

The Integrin-blocking Peptide RGDS Inhibits Airway Smooth Muscle Remodeling in a Guinea Pig Model of Allergic Asthma

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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 has been 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 proliferating cell nuclear antigen (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 proliferation induced by collagen I, fibronectin, serum, and platelet-derived growth factor requires signaling via RGD-binding integrins, particularly of the $\alpha_5\beta_1$ subtype. In addition, RGDS inhibited smooth muscle α -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.

Keywords: airway remodeling; extracellular matrix; integrin; airway smooth muscle hyperplasia; airway smooth muscle contractility

Asthma is a chronic inflammatory airway disease characterized by reversible airway obstruction, persistent airway inflammation, and airway hyperresponsiveness to a variety of stimuli (1–3). Structural changes in the airway wall of individuals with asthma are thought to contribute importantly to decreased airway diameter and airway hyperresponsiveness (4). These structural changes include accumulation of airway smooth

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Integrins have been shown to modulate airway smooth muscle proliferation and contractile protein expression *in vitro*. However, the involvement of integrins in allergen-induced airway smooth muscle remodeling *in vivo* has not yet been explored.

What This Study Adds to the Field

Inhibition of RGD-binding integrins by the small peptide RGDS inhibits airway smooth muscle hyperplasia, contractile protein expression, and hypercontractility in a guinea pig model of allergic asthma.

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 individuals with asthma, the deposition of ECM 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, 12), whereas laminin, which does not affect ASM cell proliferation on its own, inhibits the induction of a proliferative phenotype by growth factors (11, 12). 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 (13). Interaction of cells with their surrounding matrix is mediated mainly through integrins, a group of heterodimeric transmembrane glycoproteins (14). Inhibition of the $\alpha_5\beta_1$ integrins attenuates serum-induced proliferation and normalizes enhanced growth factor-induced proliferation of cells cultured on collagen I or fibronectin matrices (15, 16), suggesting that $\alpha_5\beta_1$ 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 (17, 18). In culture, growth arrest in insulin-supplemented media increases the abundance of contractile proteins and contraction regulatory proteins (19–21). During this phase, ASM cells increase the expression of laminin chains α_2 , β_1 , and γ_1 , which is essential for the induction of a functionally contractile phenotype (22, 23).

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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 (24, 25), 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 (26).

METHODS

Additional detail on the methods is provided in the online supplement.

Animal Experiments

All protocols described in this study were approved by the University of Groningen (Groningen, The Netherlands) Committee for Animal Experimentation. Outbred male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, UK) weighing 150–250 g were sensitized to ovalbumin by using $\text{Al}(\text{OH})_3$ as an adjuvant, as described previously (27). Guinea pigs were challenged with aerosolized ovalbumin solutions until airway obstruction once weekly, for 12 consecutive weeks (28, 29). 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 hour before and 5.5 hours after each challenge and killed 24 hours 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 (28, 29). 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 and 40 mM KCl. After washouts and another equilibration period of 30 minutes, cumulative concentration–response curves were constructed for 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 with horseradish peroxidase-linked secondary antibodies and diaminobenzidine. In addition, hematoxylin-stained nuclei within the ASM bundle were counted. Eosinophils were identified in hematoxylin- and eosin-stained lung sections. Neutrophils were identified on the basis of morphologic criteria and tissue nonspecific alkaline phosphatase enzyme histochemistry (30). Airways within sections were digitally photographed and subclassified as cartilaginous or noncartilaginous. All immunohistochemical measurements were performed digitally, using quantification software (ImageJ; National Institutes of Health, Bethesda, MD).

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 of phosphate-buffered saline. Homogenates were incubated with 1.25 ml of 5% trichloroacetic acid on ice for 20 minutes. 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 of water by incubating them for 72 hours at room temperature, applying intermittent vortexing. In a 96-well plate, samples (5 μl) were incubated with 100 μl of chloramines-T (1.4% chloramines-T in 0.5 M sodium acetate–10%

isopropanol) for 30 minutes at room temperature. Next, 100 μl of 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 minutes. Samples were cooled to room temperature and the amount of hydroxyproline was quantified by colorimetric measurement (550 nm) (model 680 plate reader; Bio-Rad, Hercules, CA).

Cell Culture

Three human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase, were used (31, 32). For all experiments, passage 18–40 myocytes grown on uncoated plastic dishes in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL Life Technologies, Paisley, UK) supplemented with streptomycin (50 U/ml), penicillin (50 $\mu\text{g}/\text{ml}$; GIBCO), and fetal bovine serum (FBS, 10% vol/vol; 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 of University Medical Center Groningen (Groningen, The Netherlands). The tracheal smooth muscle was prepared free of mucosa and connective tissue and chopped with 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), gentamicin (45 $\mu\text{g}/\text{ml}$; GIBCO), penicillin (50 $\mu\text{g}/\text{ml}$; GIBCO), streptomycin (50 U/ml; GIBCO), amphotericin B (1.5 $\mu\text{g}/\text{ml}$; GIBCO), and 10% FBS (GIBCO); placed in 25-cm² culture flasks; and allowed to adhere. Medium was refreshed every 48–72 hours. On reaching confluency, cells were passaged by trypsinization. Cells from passages 2 and 3 were used for the present study.

Alamar Blue Proliferation Assay

ASM proliferation was assessed as described previously (32). 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 (11). Cells were then incubated with or without platelet-derived growth factor (PDGF)-AB (10 ng/ml, human; Bachem, Weil am Rhein, Germany) or FBS (10%) for 4 days. Thereafter, cells were incubated with Hanks' balanced salt solution 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- α_5 (10 $\mu\text{g}/\text{ml}$, clone P1D6; Chemicon), and/or anti- β_1 (10 $\mu\text{g}/\text{ml}$, clone 6S6; Chemicon, Chemicon, Chandler's Ford, UK) antibodies or mouse IgG control antibodies (10 $\mu\text{g}/\text{ml}$; Chemicon) 30 minutes before and during stimulation with mitogens.

Western Analysis

Lung homogenates were prepared as described previously (28, 29). 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 with 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 $\pm$ SEM from *n* separate experiments. Statistical significance of differences was evaluated by one-way analysis of variance, 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 hyper-responsiveness, and airway remodeling (27). 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 with saline-treated, saline-challenged controls (Figure 1A). Topical treatment of the airways with intranasally instilled RGDS 0.5 hour before and 5.5 hours after each allergen challenge reduced the ovalbumin-induced increase in *sm*-MHC-positive area by $73 \pm 7\%$ ($P < 0.01$). 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).

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 these data, the apparent cell volume of the ASM cells was also calculated. Repeated ovalbumin challenge did not change the number of nuclei per square millimeter of smooth muscle area (Figure 1B) or cell volume (see Figure E1 in the online supplement), indicating that the cell size is unchanged and that allergen-induced increases in ASM mass are caused by 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 allergen-induced ASM hyperplasia, the expression of proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA replication and repair (33), 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 with saline-treated, saline-challenged controls (Figure 1C). Treatment with RGDS attenuated the ovalbumin increase in PCNA expression by $58 \pm 9\%$ ($P < 0.01$), 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 previously. 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 (28, 29) 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 with saline-challenged controls (Figure 2A). RGDS treatment inhibited the ovalbumin-induced increase in *sm*-MHC expres-

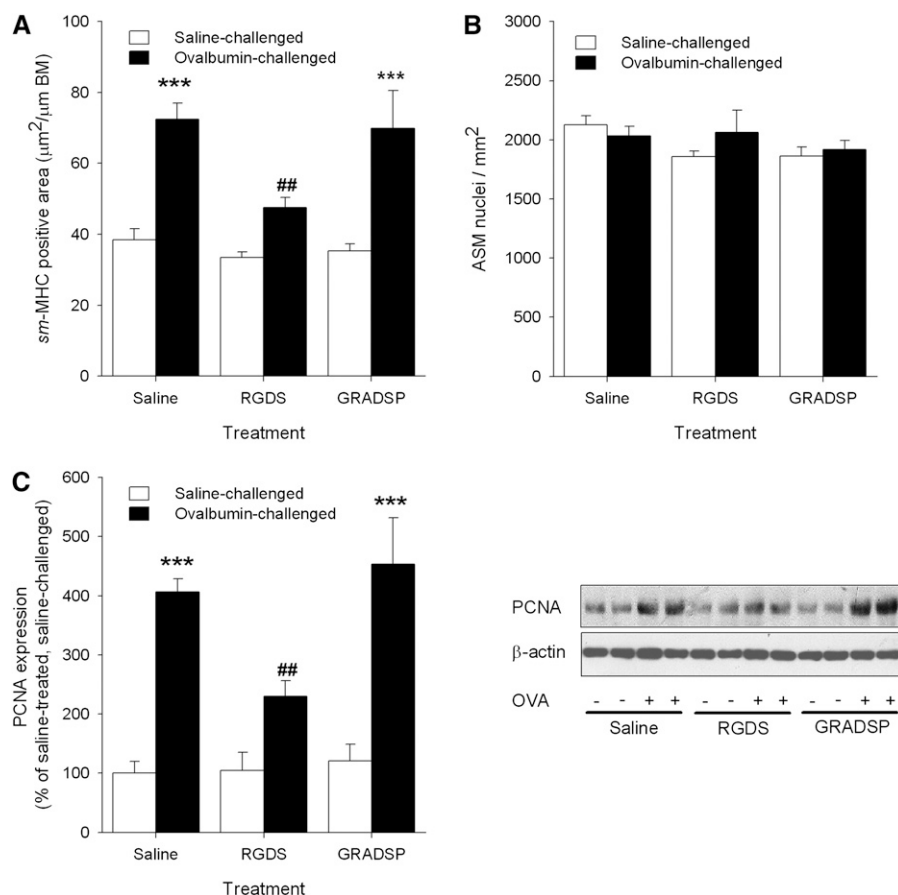


Figure 1. Airway smooth muscle (ASM) hyperplasia after repeated allergen challenge *in vivo* is inhibited by topical treatment with RGDS. To assess the role of RGD-binding integrins in ASM hyperplasia in asthma, the effects of treatment with RGDS or GRADSP were evaluated in a guinea pig model of allergic asthma. (A) Treatment with RGDS inhibited the 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 proliferating cell nuclear antigen (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 with saline-treated, saline-challenged controls; ## $P < 0.01$ compared with saline-treated, ovalbumin-challenged controls. Data represent means $\pm$ SEM of six to eight animals. BM = basement membrane.

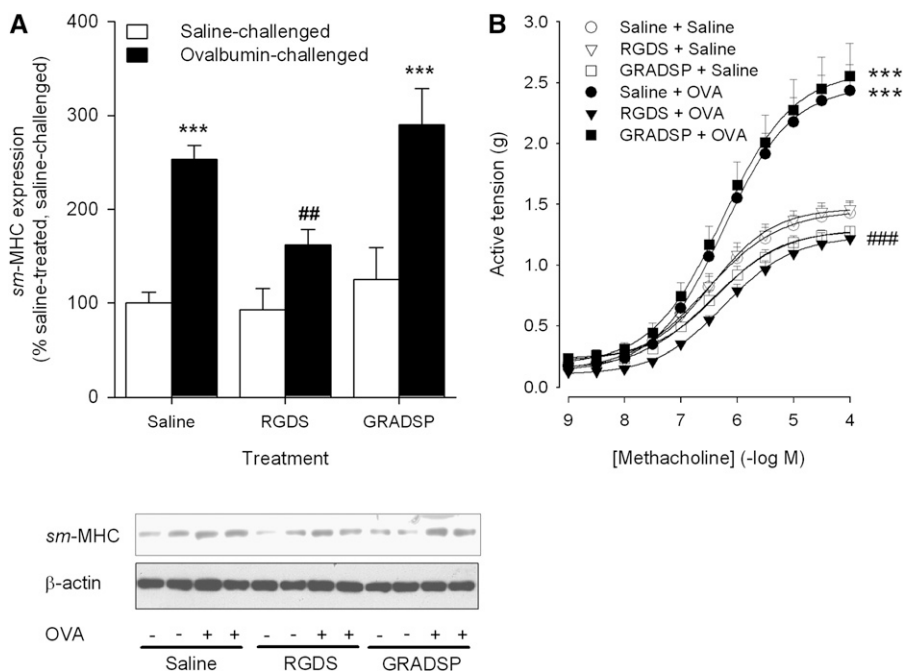


Figure 2. Increased contractile protein accumulation and airway smooth muscle (ASM) contractility after repeated allergen challenge are inhibited by topical treatment with RGDS in the airways. (A) Treatment with RGDS inhibited ovalbumin-induced accumulation of smooth muscle-specific myosin heavy chain (*sm*-MHC) in the guinea pig lung, whereas 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 with saline-treated, saline-challenged controls; ## $P < 0.01$ and ### $P < 0.001$ compared with saline-treated, ovalbumin-challenged controls. Data represent means $\pm$ SEM of six to eight animals.

sion by $60 \pm 1\%$ ($P < 0.01$), 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 (28, 29), 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 - and 1.7 ± 0.1 -fold, respectively; $P < 0.001$) (Figure 2B and 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} [negative logarithm of the concentration of agonist eliciting half-maximal response]) of methacholine- or KCl-induced contraction was unaffected by all treatments (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*.

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 (34). 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 $\alpha_5\beta_1$ integrin (35). To investigate whether RGDS treatment inhibited eosinophilic airway inflammation in our guinea pig model, we assessed the number of eosinophils in various airway compartments. As we have observed previously (28), 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 RGDS or GRADSP did not significantly affect eosinophil influx into these compartments.

RGDS might also inhibit infiltration of neutrophils into the airways, by inhibition of $\alpha_5\beta_1$ integrins present on these cells (36).

TABLE 1. CONTRACTILE RESPONSES OF EPITHELIUM-DENUDED, TRACHEAL OPEN-RING PREPARATIONS AFTER REPEATED SALINE OR OVALBUMIN CHALLENGES OF GUINEA PIGS TREATED WITH SALINE, RGDS, OR GRADSP

Treatment	Challenge	Methacholine		KCl		n
		E_{max} (g)	pEC_{50} (-log M)	E_{max} (g)	EC_{50} (mM)	
Saline	Saline	1.42 ± 0.09	6.55 ± 0.18	1.02 ± 0.06	23.7 ± 0.9	6
RGDS	Saline	1.46 ± 0.07	6.54 ± 0.16	1.11 ± 0.07	24.4 ± 1.6	6
GRADSP	Saline	1.28 ± 0.04	6.46 ± 0.13	0.95 ± 0.04	24.6 ± 1.7	5
Saline	Ovalbumin	$2.43 \pm 0.22^*$	6.28 ± 0.11	$1.73 \pm 0.13^*$	23.7 ± 1.2	7
RGDS	Ovalbumin	$1.22 \pm 0.10^\dagger$	6.30 ± 0.08	$0.82 \pm 0.07^\dagger$	23.6 ± 1.1	8
GRADSP	Ovalbumin	$2.55 \pm 0.27^*$	6.29 ± 0.07	$1.88 \pm 0.25^*$	25.7 ± 1.0	6

Definition of abbreviations: EC_{50} = concentration of agonist eliciting half-maximal response; E_{max} = maximal contraction; pEC_{50} = negative logarithm of the EC_{50} value.

Data represent means $\pm$ SEM.

* $P < 0.001$ compared with saline-treated, saline-challenged guinea pigs.

† $P < 0.001$ compared with saline-treated, ovalbumin-challenged guinea pigs.

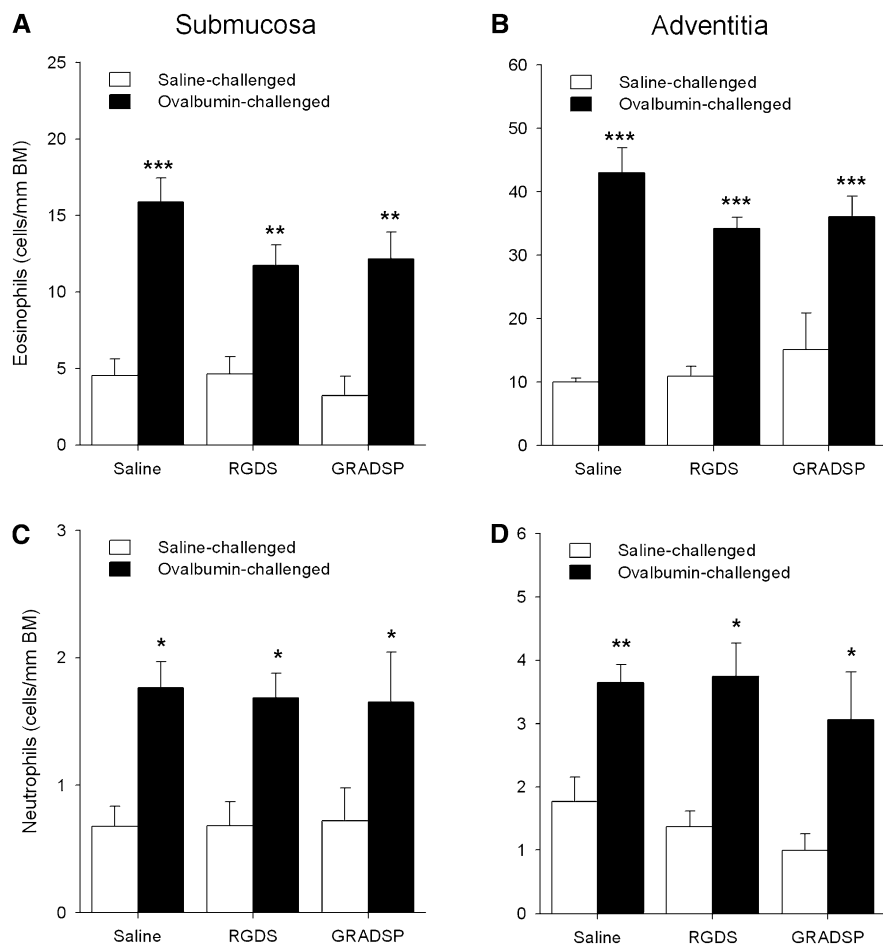


Figure 3. RGDS does not affect allergen-induced airway inflammation. Eosinophil numbers after saline or ovalbumin challenges in the (A) submucosal and (B) adventitial compartments of the cartilaginous airways were unaffected by RGDS or GRADSP treatment. Similarly, neutrophilic inflammation in the (C)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

Repeated ovalbumin challenges increased the infiltration of neutrophils into the submucosa and adventitia of the cartilaginous airways ($P < 0.05$; Figures 3C and 3D). However, as observed for eosinophils, no effects of RGDS or GRADSP treatment were found on 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.

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 (37, 38). 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 with 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

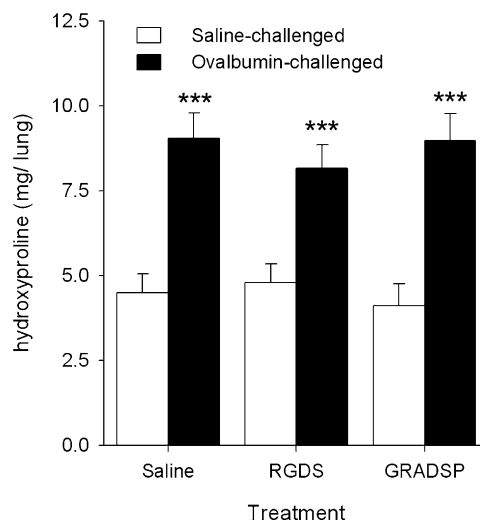


Figure 4. RGDS treatment does not affect allergen-induced fibrosis in guinea pig lung. Shown is the hydroxyproline content in guinea pig lung after repeated saline or ovalbumin challenges in saline-, RGDS-, and GRADSP-treated animals. *** $P < 0.001$ compared with saline-treated, saline-challenged controls. Data represent means $\pm$ SEM of six to eight animals.

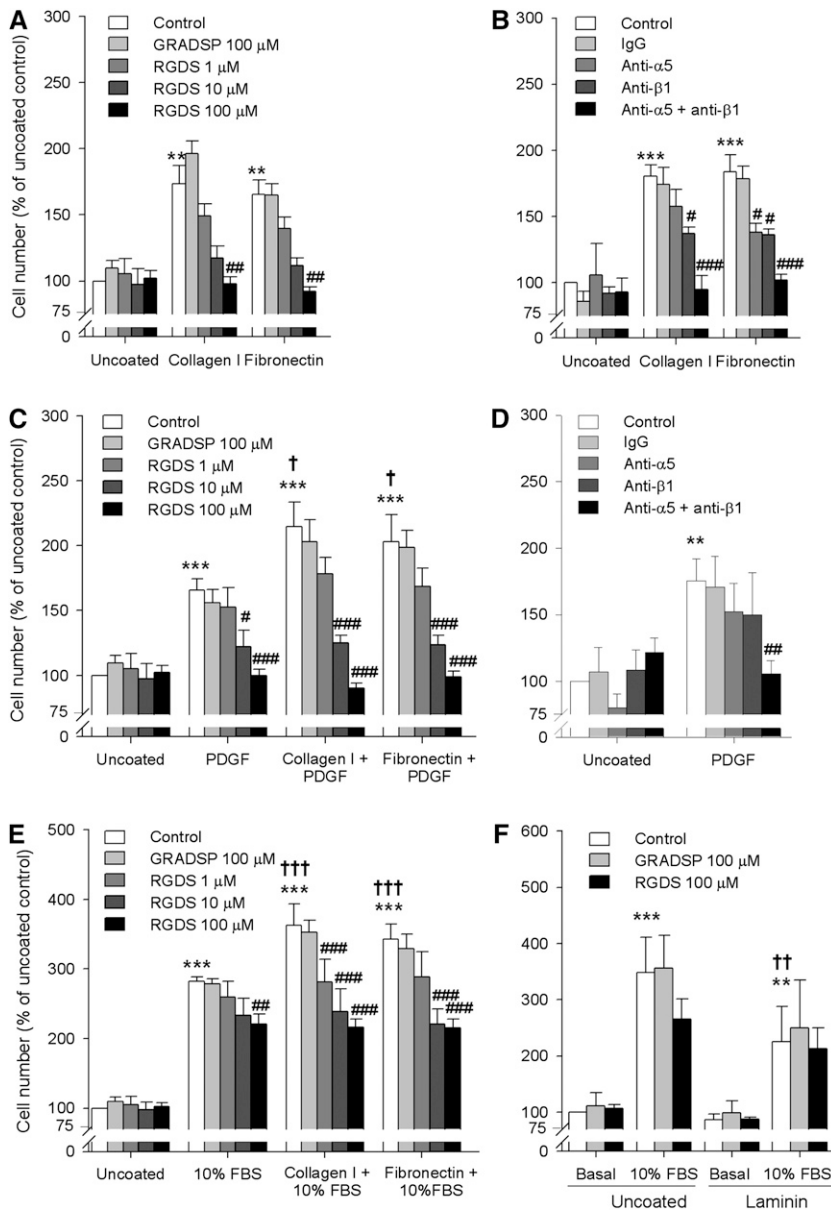


Figure 5. Extracellular matrix (ECM)- and growth factor-induced human airway smooth muscle (ASM) cell proliferation are dependent on RGD-binding integrins. (A) Increased ASM cell numbers were observed after culturing on collagen I or fibronectin matrices compared with an uncoated surface. Increases in proliferation were concentration-dependently inhibited by RGDS. (B) ECM-induced proliferation required interaction with the RGD-binding integrin $\alpha_5\beta_1$. (C) Platelet-derived growth factor (PDGF)- and (E) fetal bovine serum (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 $\alpha_5\beta_1$. (F) Growth-attenuating effects of laminin-111 were not affected by RGDS treatment. ** $P < 0.01$, *** $P < 0.001$ compared with cells (control) on uncoated surface. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with cells grown on ECM matrices and/or stimulated with mitogens in the absence of inhibitors. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared with mitogen-stimulated cells grown on uncoated surface. Data represent means $\pm$ SEM of six independent experiments of three donors, performed in duplicate.

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_{50}) of ECM-induced proliferation was not significantly different for cells cultured on monomeric collagen I or fibronectin (5.2 ± 2.4 and 6.7 ± 2.0 μ M, respectively). No effects were observed for GRADSP. In addition, no effects of RGDS or GRADSP were observed in cells cultured on uncoated surface.

To identify the integrin inhibited by RGDS, monoclonal anti- α_5 and anti- β_1 function-blocking antibodies were used. Treatment with the anti- β_1 -blocking antibody reduced both monomeric collagen type I- and fibronectin-induced proliferation by 54 ± 4 and $55 \pm 4\%$, respectively (both $P < 0.05$; Figure 5B), whereas the anti- α_5 -blocking antibody inhibited only the fibronectin-induced increase in cell number ($51 \pm 9\%$; $P < 0.05$). Notably, combining the anti- α_5 - and anti- β_1 -blocking antibodies fully abrogated the proliferation induced when cells

were grown on either matrix ($P < 0.001$). Treatment with nonimmune control IgG antibodies did not affect ASM cell number and no effects of the blocking antibodies were observed in cells cultured on an uncoated surface (Figure 5B).

The effects of RGDS on FBS (10%)-induced 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 ($IC_{50} = 4.4 \pm 1.6$ and 4.0 ± 1.6 μ M, 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 $37 \pm 7\%$ ($P < 0.01$; 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 and 5.0 ± 1.4 μ M, respectively, for PDGF-induced proliferation, and 5.1 ± 3.5 and 2.6 ± 1.1 μ M, respectively, for

FBS-induced proliferation). As with RGDS, PDGF-induced proliferation was fully normalized by culturing in the presence of α_5 and β_1 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 promitogenic responses of ASM are dependent on signaling by RGDS-binding integrins, in particular $\alpha_5\beta_1$ 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 medium, in the presence of various 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 μ M and a maximal inhibition of $72 \pm 14\%$ ($P < 0.05$). 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 an RGD-containing peptide.

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 and hyperplasia, and 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 (24, 25), could be intrinsic regulators of these processes.

Accumulation of ASM and increased deposition ECM proteins, including collagen type I, fibronectin, and laminin $\alpha_2(\beta_2)$, in the airway wall are characteristic features of remodeling in asthma (7, 39). Previous reports have indicated that monomeric collagen type I and fibronectin increase basal ASM proliferation in cells of bovine origin (11, 40). 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 (24), of which the $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$ integrins are expressed by ASM cells in culture (15, 41). Previous studies using human ASM indicated that enhanced PDGF-induced proliferation on monomeric collagen I and fibronectin matrices is inhibited by function-blocking antibodies directed against $\alpha_5\beta_1$ integrin, but not $\alpha_3\beta_1$, $\alpha_v\beta_1$, or $\alpha_v\beta_3$ integrin (15). Using function-blocking antibodies we have now established that increased basal ASM proliferation on both collagen and fibronectin matrices also requires primarily interaction with the RGD-sensitive, fibronectin-selective integrin $\alpha_5\beta_1$. Involvement of the $\alpha_5\beta_1$ 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 (15), suggesting that the proliferative responses share a common mechanism. Indeed, studies in vascular

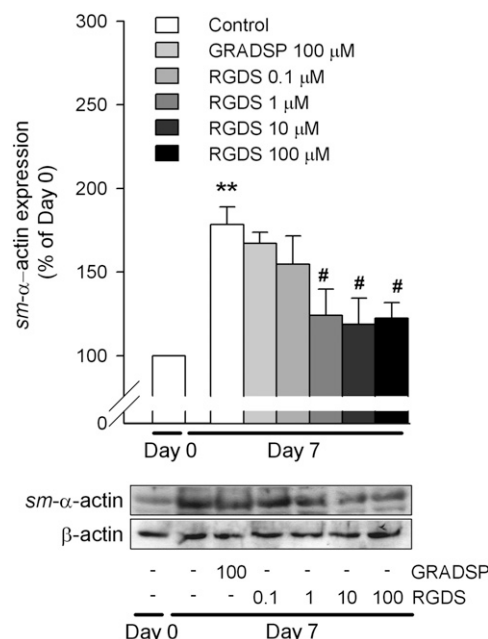


Figure 6. RGD-binding integrins are involved in contractile protein accumulation in serum-deprived human airway smooth muscle (ASM) cells. Human ASM cells were grown to confluence in the presence of 10% fetal bovine serum (FBS). Subsequently, ASM cells were kept in serum-free medium for 7 days in the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

smooth muscle cells have indicated that culturing on monomeric collagen type I markedly increased the expression of other ECM proteins (42), including fibronectin, suggesting that the proliferative responses on monomeric collagen type I could be influenced by autocrine fibronectin deposition and integrin $\alpha_5\beta_1$ 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 and colleagues (43), who showed that FBS-induced proliferation of ASM cells obtained from both patients without asthma and patients with asthma was reduced by anti- α_5 and anti- β_1 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 (15), 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 previously 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 various stimuli were observed, strongly suggesting the involvement of a single integrin in these processes. The inhibitory effects of RGDS were specific for proliferative processes, as the peptide did not normalize the inhibitory effects of laminin-111, previously shown to decrease proliferative responses in ASM cells (11, 12). Collectively, these findings identify the $\alpha_5\beta_1$ integrin, which is universally expressed on

ASM cells (15, 41), 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, 44). Thus far, increased ASM mass has been attributed primarily to the action of growth factors, inflammatory mediators, and neurotransmitters (45). From animal models of allergic asthma it has been shown that transforming growth factor- β , cysteinyl leukotrienes, and acetylcholine are important endogenous mediators of ASM remodeling (46). 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 increased expression of the proliferative marker PCNA in the lung. Although the specific contribution of the various 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 the *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 (47, 48). Indeed, previous studies by Nguyen and colleagues (15) 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 (49). In asthmatic airways, however, accumulation of ASM cells with increased contractile properties and high expression levels of contractile proteins has been described (17, 18). 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 (21). 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 (22, 23). 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 IC_{50} 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 α_7 integrin, may be involved. Previous studies

have indicated that the α_7 integrin is an important regulator of ASM maturation (50). Collectively, our *in vitro* findings indicate that RGD-binding integrins are not only importantly involved in ASM proliferation via the $\alpha_5\beta_1$ integrin, but also in ASM maturation to a (hyper)contractile phenotype, presumably via a mechanism involving the $\alpha_7\beta_1$ 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 (44). Eotaxin is a key chemokine for the recruitment of eosinophils (51) and ASM cells derived from patients with asthma release enhanced levels of this chemokine, presumably as a result of enhanced fibronectin deposition and subsequent $\alpha_5\beta_1$ integrin activation (35). In addition, integrin-blocking peptides containing the RGD sequence inhibit the enhanced eotaxin release from healthy ASM cells cultured on fibronectin matrices (34). 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 $\alpha_5\beta_1$ integrin on their cell membrane (36), was observed either. 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 of 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-transforming growth factor- β antibodies during allergen challenge, which showed that inhibition of peribronchiolar ECM deposition is associated with normalization of ASM cell proliferation, without affecting airway inflammation in bronchoalveolar lavage fluid or in tissue sections (52). 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 $\alpha_4\beta_1$ subtype, are involved in the recruitment of inflammatory cells to the airways (for a detailed review see Reference 36). However, thus far phase II clinical trials with small integrin $\alpha_4\beta_1$ antagonists have met with limited success in affecting lung function and asthma exacerbations (53), 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 (54, 55). 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 into 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 airway disease. On the basis of these findings, RGD-binding integrins may represent a novel target in the treatment of airway remodeling in asthma.

Conflict of Interest Statement: B.G.J.D. received more than \$100,001 from Boehringer Ingelheim in industry-sponsored grants. I.S.T.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.G. received up to \$1,000 from Boehringer Ingelheim in lecture fees and more than \$100,001 from Boehringer Ingelheim in industry-sponsored grants. A.J.H. received a \$50,001–\$500,001 research grant (leukotriene receptor function) from Merck Frosst, a more than \$100,001 research grant (statins in airway inflammation/remodeling) from GlaxoSmithKline, and up to \$1,000 from Merck Frosst for support for meeting proceedings publication. J.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.M. received more than \$100,001 from Schering Plough and more than \$100,001 from Boehringer Ingelheim in industry-sponsored grants.

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ONLINE DATA SUPPLEMENT

THE INTEGRIN-BLOCKING PEPTIDE RGDS INHIBITS AIRWAY SMOOTH MUSCLE REMODELING IN A GUINEA PIG MODEL OF ALLERGIC ASTHMA

Bart G.J. Dekkers, I. Sophie T. Bos, Reinoud Gosens, Andrew J. Halayko, Johan
Zaagsma and Herman Meurs.

Methods (Detailed description)

Animal Experiments

All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation (University of Groningen, Groningen, The Netherlands). Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, UK) weighing 150–250 g were sensitized to ovalbumin (Sigma Chemical Co., St. Louis, MO, U.S.A) using $\text{Al}(\text{OH})_3$ as adjuvant as described previously (E1). In short, 0.5 ml of an allergen solution containing 100 $\mu\text{g}/\text{ml}$ ovalbumin and 100 mg/ml $\text{Al}(\text{OH})_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck. The animals were used experimentally 4 weeks later. The animals were group-housed in cages in climate controlled animal quarters and given water and food *ad libitum*, while a 12-hour on/12-hour off light cycle was maintained.

Provocation Procedures

Four weeks after sensitization, allergen-provocations were performed by inhalation of aerosolized solutions of saline (control) or ovalbumin as described previously (E1). Aerosols were produced by a DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA) with an airflow of 8 l/min resulting in an output of 0.33 ml/min. Provocations were carried out in a specially designed Perspex cage (internal volume 9 L), in which the guinea pigs could move freely. Before the start of the experimental

protocol, the animals were habituated to the provocation procedures on two sequential days. After an adaptation period of at least 30 min, three consecutive provocations with saline were performed, each provocation lasting 3 min, separated by 7 min intervals. Ovalbumin challenges were performed by inhalation of increasing concentrations of 0.05%, 0.1% and 0.3% w/v ovalbumin in saline for 3 min each, with 7 min intervals. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. No anti-histaminic was needed to prevent the development of anaphylactic shock.

Study Design

Treatment groups were as follows: (1) ovalbumin-sensitized, saline-treated, saline-challenged controls (n=6), (2) ovalbumin-sensitized, RGDS-treated saline-challenged animals (n=6), (3) ovalbumin-sensitized, GRADSP-treated, saline-challenged animals (n=5), (4) ovalbumin-sensitized, saline-treated, ovalbumin-challenged animals (n=7), (5) ovalbumin-sensitized, RGDS-treated, ovalbumin-challenged animals (n=8) and (6) ovalbumin-sensitized, GRADSP-treated, ovalbumin-challenged animals (n=6). Guinea pigs were challenged with either saline or ovalbumin once weekly for 12 consecutive weeks, to induce airway remodeling as described previously (E2, E3). Animals were treated with saline, RGDS or GRADSP (2.5 mM, both) by intranasal instillation (200 μ l), 0.5 hr prior to and 5.5 hr after each challenge with saline or ovalbumin. During the 12-week challenge protocol, guinea pig weight was monitored weekly and no differences in weight gain between the different treatment groups were found.

Tissue Acquisition

Guinea pigs were sacrificed by experimental concussion followed by rapid exsanguination 24 hr after the last challenge. The lungs were immediately resected and kept on ice for further processing. The trachea was removed and transferred to a Krebs–Henseleit solution of the following composition (mM): 117.5 NaCl, 5.60 KCl, 1.18 MgSO₄, 2.50 CaCl₂, 1.28 NaH₂PO₄, 25.00 NaHCO₃, and 5.50 glucose, pregassed with 5% CO₂ and 95% O₂, pH 7.4 at 37°C. Lungs were divided in three parts and weighed. One part was snap frozen in liquid nitrogen for the measurement of hydroxyproline content. One part was frozen at -80 °C in isopentane and stored at -80 °C for histological purposes. The remaining part was snap frozen in liquid nitrogen and stored at -80 °C to be used for Western analysis.

Isometric Tension Measurements

The trachea was prepared free of connective tissue. Single open-ring, epithelium-denuded preparations were mounted for isometric recording in 20 ml water-jacketed organ baths, containing Krebs–Henseleit solution at 37 °C, continuously gassed with 5% CO₂ and 95% O₂, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 0.5 g. Subsequently, muscle strips were precontracted with 20 mM and 40 mM KCl. Following two washouts, maximal relaxation was established by the addition of 0.1 µM (-)-isoproterenol. Tension was readjusted to 0.5 g, immediately followed by two changes with fresh KH-buffer. After another equilibration period of 30 min, cumulative concentration response curves were constructed to stepwise increasing concentrations of KCl

(5.6–50 mM) or methacholine (1 nM – 0.1 mM). When maximal tension was reached, the strips were washed several times and maximal relaxation was established using (-)-isoproterenol.

Histochemistry

Smooth muscle area was determined in 8 µm thick cryostat lung sections. Transverse cross-sections of the main bronchi from both right and left lung lobes were used for morphometric analyses. To identify smooth muscle, the sections were stained for smooth-muscle-specific myosin heavy chain (*sm*-MHC). Sections were dried for 30 min, fixed with acetone for 10 min and then washed three times in phosphate-buffered saline (PBS, composition in mM: 140.0 NaCl, 2.6 KCl, 1.4 KH₂PO₄, 8.1 Na₂HPO₄·2H₂O, pH 7.4). Subsequently, sections were incubated for 1 h in PBS supplemented with 1% bovine serum albumin (BSA, Sigma) and anti-*sm*-MHC (diluted 1:100, Neomarkers, Fremont, CA, USA) at room temperature. Sections were then washed three times with PBS after which endogenous peroxidase activity was blocked by treatment with PBS containing 0.075% H₂O₂ for 30 min. Sections were washed for another three times with PBS, after which the horseradish peroxidase (HRP)-linked secondary antibody (rabbit anti-mouse IgG, Sigma, diluted 1:200) was applied for 30 min at room temperature. After another three washes, sections were incubated with diaminobenzidine (1 mg/ml) for 5 min in the dark, after which sections were washed and stained with haematoxylin. After rinsing with water the sections were embedded in Kaisers glycerol gelatin. Airways within sections were digitally photographed and subclassified as cartilaginous or non-cartilaginous.

All immunohistochemical measurements were carried out digitally using quantification software (ImageJ). For this purpose, digital photographs of lung sections were analyzed at a magnification of 40-100x. For both types of airways, *sm*-MHC positive areas were measured by a single observer in a blinded fashion. In addition, haematoxylin-stained nuclei within the ASM bundle were counted. Of each animal 4 lung sections were prepared per immunohistochemical staining, in which a total of 4 to 5 airways of each classification were analyzed. Eosinophils were identified in haematoxylin-and-eosin-stained lung sections. Neutrophils were identified by a combination of morphologic criteria and tissue non-specific alkaline phosphatase enzyme histochemistry (E4).

Hydroxyproline Assay

Lungs were analyzed for hydroxyproline as an estimate of collagen content. Lung homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent 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 baked overnight at 110 °C. The samples were dissolved in 2 ml water by incubating for 72 hr 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 for all experiments. The primary cultured human bronchial smooth muscle cells used to generate each cell line were prepared from macroscopically healthy segments of second to fourth generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (H. Unruh, Section of Thoracic Surgery, University of Manitoba, Canada), as we have described previously (E2,E5). All procedures were approved by the Human Research Ethics Board of the University of Manitoba.

As detailed previously (E2,E5), each cell line was thoroughly characterized to passage 10 and higher and was shown to express a number of smooth muscle contractile phenotype marker proteins (e.g., *sm*-MHC, *sm*- α -actin and desmin). For all experiments, passages 18–41 myocytes grown on uncoated plastic dishes in DMEM (Gibco BRL Life Technologies, Paisley, U.K.) supplemented with 50 U/ml streptomycin, 50 μ g/ml penicillin, (Gibco) and 10% vol/vol FBS (Gibco) were used.

Coating of Culture Plates with Extracellular Matrix Proteins.

Calf skin monomeric collagen I (Fluka, Buchs, Switzerland), was reconstituted in 10 mM hydrochloric acid at 5 mg/ml before diluting. Human plasma fibronectin (Sigma) was reconstituted in sterile PBS. Dilutions of collagen I (50 μ g/ml), fibronectin (10

µg/ml) and mouse Engelberth-Holm-Swarm (EHS) laminin (10 µg/ml, Invitrogen, Grand Island, NY, USA) were prepared in PBS. ECM proteins were absorbed to culture plates overnight and air-dried at room temperature. Unoccupied protein-binding sites were blocked by a 30-min incubation with a sterile 0.1% BSA in PBS. Subsequently, plates were washed twice with plain DMEM and dried before further use.

Alamar Blue Proliferation Assay

Cells were plated on uncoated or ECM-coated culture plates and were allowed to attach overnight in DMEM supplemented with 50 U/ml streptomycin, 50 µg/ml penicillin and 10% vol/vol FBS. The next day, cells were washed twice with sterile PBS and made quiescent in DMEM supplemented with antibiotics and 1% ITS (Insulin 5 µg/ml, transferrin 5 µg/ml and selenium 5 ng/ml, Gibco) for 3 days. Cells were then incubated with or without PDGF-AB (10 ng/ml, human, Bachem, Weil am Rhein, Germany) or FBS (10%) for 4 days in DMEM supplemented with antibiotics. Thereafter, cells were washed two times with PBS and incubated with Hank's balanced salt solution (HBSS) containing 5% vol/vol Alamar blue solution (Biosource, Camarillo, CA). Conversion of Alamar blue into its reduced form by mitochondrial cytochromes was then quantified by fluorimetric analysis, as indicated by the manufacturer. In some experiments, cells were pretreated for 30 minutes 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) before and during stimulation with mitogens.

Preparation of Cell and Lung Homogenates

Cells were plated on uncoated culture plates and grown to confluence in DMEM supplemented with 50 U/ml streptomycin, 50 µg/ml penicillin and 10% vol/vol FBS. At confluence, cells were serum deprived for up to 7 days in DMEM supplemented with antibiotics and 1% ITS, to induce a contractile phenotype. In some experiment cells were serum deprived in the presence of RGDS (1-100 µM) or GRADSP (100 µM). Media was replaced every third day. For preparation of cell lysates, cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA 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, 10 µg/ml aprotinin, 10 µg/ml pepstatin, Na-deoxycholate 0.25 % and 1% Igepal (NP-40)), before serum deprivation (Day 0) and following 7 days of serum-deprivation (Day 7). Homogenates were stored at –80 °C until further use. Guinea pig lung homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent sonification in RIPA buffer. Homogenates were stored at –80 °C until further use.

Western Analysis

Protein content was determined according to Bradford (E6). Equal amounts of protein were subjected to electrophoresis and transferred onto nitrocellulose membranes. Membranes were subsequently blocked in blocking buffer

(composition: Tris-HCl 50.0 mM; NaCl 150.0 mM; Tween-20 0.1%, dried milk powder 5%) for 60 minutes at room temperature. Next, membranes were incubated overnight at 4 °C with primary antibodies (anti-*sm*-MHC and anti-PCNA (clone PC10, Neomarkers)), both diluted 1:200 in blocking buffer. After three washes with TBS-Tween 20 (0,1% TBST, containing 50.0 mM Tris-HCl, 150.0 mM NaCl and 0,1% Tween 20) of 10 min each, membranes were incubated with horseradish peroxidase-labelled secondary antibodies (dilution 1:2000 in blocking buffer) at room temperature for 90 min, followed by another three washes with 0,1% TBST. Antibodies were then visualized on film using enhanced chemiluminescence reagents and analyzed by densitometry (TotallabTM). All bands were normalized to β -actin expression.

Statistics

All data represent means $\pm$ 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 $P < 0.05$

Figure legends

Figure E1:

An increase in cell volume does not account for changes in allergen-induced ASM mass. The number of haematoxylin-positive nuclei within the ASM layer of the different animal groups were counted. From this data, the ASM area and the thickness of the sections, the apparent cell volume was calculated. Data represent means $\pm$ S.E.M of 6-8 animals.

Figure E2:

Fibronectin and growth factor-induced proliferation of primary human tracheal smooth muscle cells are inhibited by RGDS. Increased tracheal smooth muscle cell numbers were observed after culturing on fibronectin matrices (50 μ g/ml) compared to uncoated surface, which was fully inhibited by RGDS (100 μ M). The effect of PDGF and the additive effect of fibronectin on PDGF-induced proliferation were also fully normalized by RGDS. *P< 0.05, **P<0.01 compared to cells (basal) on uncoated surface. #P<0.05, ###P<0.01 compared to cells grown on ECM matrices and/or stimulated with mitogens in the absence of inhibitors. ^{††}P<0.01 compared to PDGF-stimulated cells grown on uncoated surface. Data represent means $\pm$ SEM of 5 independent experiments with cells of 5 different donors, performed in duplicate.

Figure E1

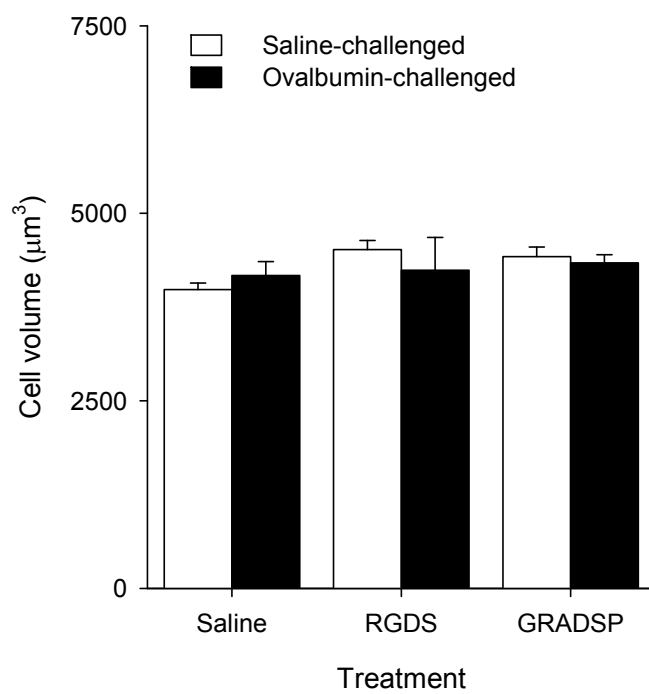
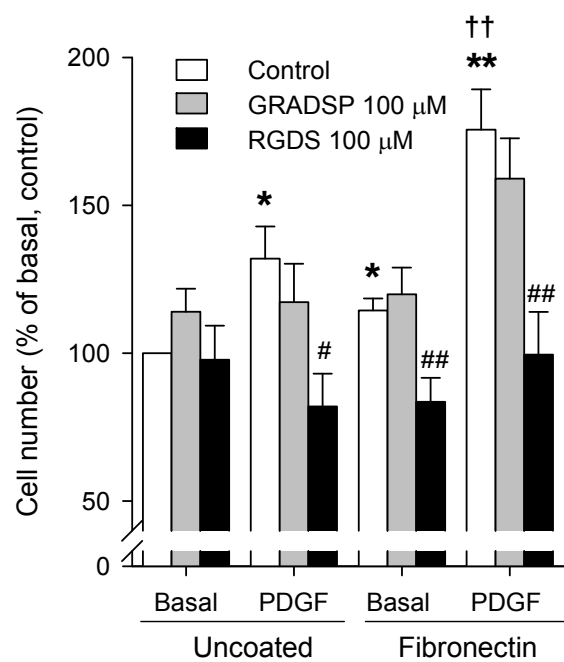


Figure E2



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