The laminin β1-competing peptide YIGSR induces a hypercontractile, hypoproliferative airway smooth muscle phenotype in chronic asthma

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Abstract

Background: Airway remodelling, including increased airway smooth muscle (ASM) mass and contractility, contributes to airway hyperresponsiveness in asthma. In vitro studies have shown that maturation of ASM cells to a (hyper)contractile phenotype is dependent on laminin, which can be inhibited by the laminin-competing peptide Tyr-Ile-Gly-Ser-Arg (YIGSR). The role of laminins in ASM remodelling in chronic asthma in vivo, however, has not yet been established.

Methods: Using an established guinea pig model of allergic asthma, we investigated the effects of topical treatment of the airways with YIGSR on features of airway remodelling induced by repeated allergen challenge, including ASM hyperplasia and hypercontractility, inflammation and fibrosis. Human ASM cells were used to investigate the effect of YIGSR on ASM proliferation in vitro.

Results: Topical administration of YIGSR attenuated allergen-induced ASM hyperplasia and pulmonary expression of the proliferative marker proliferating cell nuclear antigen (PCNA). Treatment with YIGSR also increased the expression of sm-MHC and ASM contractility, both in saline- and in allergen-challenged animals, suggesting that treatment with the laminin-competing peptide YIGSR mimics rather than inhibits laminin function in vivo. In addition, treatment with YIGSR increased allergen-induced fibrosis and submucosal eosinophilia. Culturing ASM cells on immobilized YIGSR in vitro concentration-dependently reduced PDGF-induced proliferation to a similar extent as with laminin. Remarkably, the effects of both immobilized YIGSR and laminin were antagonized by soluble YIGSR.

Conclusion: These results indicate that the laminin-competing peptide YIGSR promotes a contractile, hypoproliferative ASM phenotype in vivo, which may depend on the microenvironment of the peptide.

Background

Airway inflammation, airway obstructive reactions and development of transient airway hyperresponsiveness are primary features of acute asthma [1,2]. In addition, structural changes in the airway wall are thought to contribute to a decline of lung function and development of persistent airway hyperresponsiveness in chronic asthma [1,3]. These structural changes include goblet cell metaplasia and mucous gland hyperplasia, increased vascularity, altered deposition of the extracellular matrix (ECM) proteins and accumulation of contractile airway smooth muscle (ASM) cells [1,4-6]. ASM cells may importantly contribute to airway remodelling as they retain the ability for reversible phenotypic plasticity, enabling them to switch between contractile, proliferative, migratory and synthetic states [7,8]. In vitro, switching to a proliferative phenotype results from exposure of ASM cells to mitogenic stimuli,
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leading to increased proliferative activity and decreased contractile function [9,10](Chapter 3). Removal of growth factors, for example by serum deprivation in the presence of insulin, results in maturation of the cells to a contractile phenotype, characterized by increased expression of contractile protein markers, increased contractile function and increased expression of laminin α2, β1 and γ1 chains [7,11,12](Chapter 5).

Laminins are basement membrane ECM components composed of heterotrimers of α, β and γ chains. Five laminin α-, three β- and three γ-chains have been identified in mammals, which form at least 15 different laminin isoforms [13]. Various laminin chains are expressed in the lung and expression appears to be tissue- and developmental stage-dependent [14]. In adult asthmatics, expression of laminin α2 and β2 chains in the airways is increased [15,16]. In addition, asthmatics with compromised epithelial integrity show increased laminin γ2 chain expression in the airways [16].

Studies on the function of laminins have shown that laminins are essential for lung development and ASM function. Laminin α1 and α2 chains are required for pulmonary branching and differentiation of naive mesenchymal cells into ASM [13,17,18]. Primary ASM cells cultured on laminin-111 (laminin-1) are retained in a hypoproliferative phenotype, associated with high expression levels of contractile proteins [19]. This is of functional relevance as the induction of a hypocontractile ASM phenotype by PDGF was prevented by co-incubation with laminin-111 (Chapter 3). Increased expression of endogenous laminin-211 (laminin-2) has been found to be essential for ASM cell maturation [12], and studies from our laboratory have shown that laminin-211 is essential for the induction of a hypercontractile, hypoproliferative ASM phenotype by prolonged insulin exposure (Chapter 5).

Recently, we have shown that in an animal model of chronic allergic asthma ASM remodelling can be inhibited by the integrin-blocking peptide Arg-Gly-Asp-Ser (RGDS) (Chapter 6), containing the RGD-binding motif present in ECM proteins like fibronectin, collagens and laminins [20,21]. The specific role of laminins in ASM remodelling in vivo, however, remains to be determined. Therefore, using a guinea pig model of chronic asthma, we explored the role of laminins in ASM remodelling in vivo, by treating the animals with the specific laminin-competing peptide Tyr-Ile-Gly-Ser-Arg (YIGSR), a binding motif present in the β1 chain of laminins [22].
Methods

Animals
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 (Sigma Chemical Co., St. Louis, MO, USA), using Al(OH)$_3$ as adjuvant, as described previously [23]. In short, 0.5 ml of an allergen solution containing 100 µg/ml ovalbumin and 100 mg/ml Al(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 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 [23]. Aerosols were produced by a DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA). 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. After an adaptation period of 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 ovalbumin (0.5, 1.0, or 3.0 mg/ml) in saline. 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
Guinea pigs were challenged with either saline or ovalbumin once weekly for 12 consecutive weeks, as described previously [24,25](Chapter 6). Animals were treated with saline or YIGSR (Calbiochem, Nottingham, UK) by intranasal instillation (2.5 mM, 200 µl), 0.5 hr prior to and 5.5 hr after each challenge with saline or ovalbumin. Treatment groups were as follows: saline-treated, saline-challenged controls (n=6); YIGSR-treated, saline-challenged animals (n=5); saline-treated, ovalbumin-challenged animals (n=7) and YIGSR-treated, ovalbumin-challenged animals (n=7). Data for the saline-treated animals (controls) have been published previously as part of a simultaneous parallel study (Chapter 6). During the 12-week challenge protocol, guinea pig weight was monitored weekly and no differences in weight gain between different treatment groups were found.
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Tissue acquisition
Guinea pigs were sacrificed by experimental concussion, followed by rapid exsanguination 24 h 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 (KH) buffer 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 into 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
Isometric contraction experiments were performed as described previously [24,25](Chapter 6). Briefly, the trachea was prepared free of connective tissue. Single open-ring, epithelium-denuded preparations were mounted for isometric recording in organ baths, containing KH buffer at 37 °C, continuously gassed with 5% CO₂ and 95% O₂, pH 7.4. During a 90-min equilibration period, resting tension was gradually adjusted to 0.5 g. Subsequently, muscle strips were precontracted with 20 mM and 40 mM KCl. Following washouts, maximal relaxation was established by the addition of 0.1 µM (-)-isoproterenol (Sigma). After washout and another 30 min equilibration period, cumulative concentration-response curves were constructed using 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 10 µM (-)-isoproterenol.

Histochemistry
Immunohistochemistry was performed as described previously [24,25](Chapter 6). Transverse cross-sections (8 µm) 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, fixed with acetone and washed in phosphate-buffered saline (PBS). 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 with PBS, after which endogenous peroxidase activity was blocked by treatment with PBS containing 0.075% H2O2 for 30 min. Sections were washed 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.

**Western analysis**
Lung homogenates were prepared as described previously [24,25](Chapter 6). Equal amounts of protein were subjected to electrophoresis and transferred onto nitrocellulose membranes, followed by immunoblotting for sm-MHC and PCNA (Neomarkers), using standard techniques. Antibodies were visualized on film using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) and analyzed by densitometry (TotallabTM, Nonlinear dynamics, Newcastle, UK). All bands were normalized to β-actin expression.

**Hydroxyproline assay**
Lungs were analyzed for hydroxyproline, an estimate of collagen content, as described previously (Chapter 6). In short, total lung homogenates were prepared by pulverizing tissue under liquid nitrogen and sonification in PBS. Homogenates were incubated with 1.25 ml 5% trichloroacetic acid on ice for 20 min, after which the samples were centrifuged. 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. To determine hydroxyproline concentrations, samples 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 hydroxyproline concentrations were 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 cells used to generate each cell line were prepared as we have described previously [26-28]. All procedures were approved by the Human Research Ethics Board of the University of Manitoba. For all experiments, passages 26–34 myocytes grown on uncoated plastic dishes in Dulbecco’s
Modified Eagle's Medium (DMEM, Gibco BRL Life Technologies, Paisley, U.K.) supplemented with 50 U/ml streptomycin, 50 μg/ml penicillin, (Gibco) and 10% vol/vol Foetal Bovine Serum (FBS, Gibco) were used.

Coating of culture plates with laminin and integrin-blocking peptides
Dilutions of mouse Engelberth-Holm-Swarm (EHS) laminin-111 (10 μg/ml, Invitrogen, Grand Island, NY, USA), YIGSR (1-100 μM), Arg-Gly-Asp-Ser (RGDS, 100 μM, Calbiochem) and Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 100 μM, Calbiochem) were prepared in PBS and absorbed to 24-well culture plates overnight. Unoccupied protein-binding sites were blocked by a 30-min incubation with 0.1% BSA in PBS. Subsequently, plates were washed twice with plain DMEM and dried before further use.

[^]-Thymidine incorporation
Cells in DMEM supplemented with streptomycin, penicillin and 10% FBS were plated on uncoated or coated 24-well culture plates at a density of 20,000 cells per well and allowed to attach overnight. Subsequently, cells were maintained in serum-free DMEM supplemented with antibiotics and 1% ITS (Insulin, Transferrin and Selenium, Gibco) for 3 days. Cells were then incubated with or without PDGF-AB (10 ng/ml, human, Bachem, Weil am Rhein, Germany) for 28 h, the last 24 h in the presence of [methyl-3H]-thymidine (0.25 μCi/ml) in DMEM supplemented with antibiotics. After incubation, the cells were washed twice with 0.5 ml PBS at room temperature. Subsequently, the cells were treated with 0.5 ml ice-cold 5% trichloroacetic acid on ice for 30 min, and the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [3H]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

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

The laminin $\beta_1$-competing peptide YIGSR inhibits allergen-induced ASM accumulation in a guinea pig model of chronic allergic asthma.

In our guinea pig model, 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 YIGSR 0.5 h prior to and 5.5 h after each ovalbumin-challenge almost fully inhibited the ovalbumin-induced increase in ASM area (by 96±3%, P<0.001). No significant effect of YIGSR treatment was observed in saline-challenged animals.

To determine whether the changes in ASM content were associated with changes in cell number and/or cell size, the number nuclei within the ASM layer were counted and expressed relative to total ASM area. Repeated ovalbumin challenge did not change the number of nuclei per mm$^2$ of smooth muscle area (Figure 1B), indicating that the cell size is unchanged and ovalbumin-induced increases in ASM mass were caused by an increased cell number. YIGSR treatment did not change ASM cell size in saline-challenged animals; however, a small, but significant (P<0.05) decrease in the number of nuclei/mm$^2$ was observed in ovalbumin-challenged animals (Figure 1B), suggesting a slight increase in cell size.

To assess whether the changes in ASM area were associated with changes in proliferative responses, Western analysis was used to determine expression of the proliferative marker PCNA in whole lung homogenates. After repeated ovalbumin-challenge, a considerable increase (4.2±0.2-fold, P<0.001) in PCNA was observed compared to saline-treated, saline-challenged controls (Figure 1C). Treatment with YIGSR fully normalized the ovalbumin-induced increase in PCNA, when compared to saline-challenged controls (P<0.001). In the saline-challenged animals, no significant effect of YIGSR treatment on PCNA expression was observed. Unfortunately, specific characterization of the proliferating cells in guinea pig lung sections by immunohistochemistry was not possible with the antibody used. Collectively, these in vivo data indicate that YIGSR treatment inhibits allergen-induced ASM hyperplasia as well as proliferative responses that may underlie ASM hyperplasia.
Figure 1: Increased ASM mass after repeated allergen challenge in vivo is inhibited by topical treatment with YIGSR. To assess the role of laminins in increased ASM mass in asthma, the effects of treatment with YIGSR were evaluated in a guinea pig model of chronic allergic asthma. (A) Treatment with YIGSR fully inhibited ovalbumin-induced increase in sm-MHC positive area in cartilaginous airways. (B) Changes in ASM mass were mainly dependent on changes in ASM cell number, only a small increase in cell size was observed for the YIGSR-treated, ovalbumin-challenged animals. (C) Increased pulmonary expression of the proliferative marker PCNA after repeated ovalbumin-challenges, was almost fully reversed by YIGSR. Representative blots of PCNA and β-actin are shown. No effects of YIGSR were shown in saline-challenged animals for any of the parameters. *P<0.05, ***P<0.001 compared to saline-treated, saline-challenged controls. Data represent means ± SEM of 5-7 animals.

**YIGSR treatment increases contractile protein accumulation and ASM contractility.**

Previously, we have shown that repeated ovalbumin-exposure increased maximal methacholine- and KCl-induced isometric contractions of epithelium-denuded, tracheal smooth muscle preparations ex vivo [24,25](Chapter 6). Interestingly, treatment with the YIGSR peptide augmented the ovalbumin-induced increases in maximal methacholine- and KCl-induced contractions further (1.33±0.08-fold (P<0.001) and 1.28±0.11-fold (P<0.05), respectively, compared to saline-treated, ovalbumin-challenged controls, Figure 2A, Table 1). Similarly, in saline-challenged animals YIGSR treatment increased methacholine- and KCl-induced contractions (1.29±0.03-fold and 1.39±0.04-fold (P<0.05), respectively, compared to saline-treated, saline-challenged animals).
The sensitivity to either contractile stimulus was unaffected by all treatments (Table 1).

Previously, we have found that increased ASM contractility induced by repeated ovalbumin challenge is associated with increased pulmonary sm-MHC expression [24,25](Chapter 6). In saline-treated animals, repeated ovalbumin-challenge increased sm-MHC by 2.5±0.1-fold compared to saline-challenged controls (P<0.001, Figure 2B). In line with the increased methacholine- and KCl-induced contractions, treatment with YIGSR increased pulmonary sm-MHC expression in saline-challenged animals (2.40±0.28-fold, P<0.001), whereas in ovalbumin-challenged animals the increase in sm-MHC was increased further (1.37±0.08-fold compared to ovalbumin-challenged controls, P<0.01). Collectively, these data indicate that in vivo treatment with the laminin-competing peptide YIGSR increases ASM contractility and contractile protein expression both in saline- and allergen-challenged animals.

**Figure 2:** Topical treatment of the airways with YIGSR increases ASM contractility and contractile protein accumulation. (A) Treatment with YIGSR enhanced the maximal methacholine-induced isometric contraction of epithelium-denuded tracheal smooth muscle preparations both in saline- and in ovalbumin-challenged animals. (B) Treatment with YIGSR increased pulmonary expression of sm-MHC, both in saline- and in ovalbumin-challenged animals. Representative blots of sm-MHC and β-actin are shown. ***P<0.001 compared to saline-treated, saline-challenged controls. ###P<0.01 compared to saline-treated, ovalbumin-challenged controls. Data represent means ± SEM of 5-7 animals.
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Table 1: Contractile responses of epithelium-denuded, tracheal smooth muscle preparations after repeated saline or ovalbumin challenge of saline- or YIGSR-treated guinea pigs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Challenge</th>
<th>E_max (g)</th>
<th>pEC_{50} (- log M)</th>
<th>E_max (g)</th>
<th>EC_{50} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>1.42±0.09</td>
<td>6.55±0.18</td>
<td>1.02±0.06</td>
<td>23.7±0.9</td>
</tr>
<tr>
<td>YIGSR</td>
<td>Saline</td>
<td>1.84±0.04</td>
<td>6.82±0.13</td>
<td>1.41±0.04*</td>
<td>20.4±2.2</td>
</tr>
<tr>
<td>Saline</td>
<td>Ovalbumin</td>
<td>2.33±0.22***</td>
<td>6.28±0.11</td>
<td>1.73±0.13**</td>
<td>23.7±1.2</td>
</tr>
<tr>
<td>YIGSR</td>
<td>Ovalbumin</td>
<td>3.11±0.18***</td>
<td>6.61±0.08</td>
<td>2.12±0.19***###</td>
<td>24.5±1.1</td>
</tr>
</tbody>
</table>

Data represent means ± SEM. Abbreviations: E_max: maximal contractile effect; EC_{50}: concentration of the stimulus eliciting half-maximal response; pEC_{50}: negative logarithm of the EC_{50} value. *P<0.05, **P<0.01, ***P<0.001 compared to saline-treated, saline-challenged animals. ###P<0.001 compared to saline-treated, ovalbumin-challenged animals.

Effects of YIGSR treatment on allergen-induced airway inflammation.

Infiltration of eosinophils into the airways is a characteristic feature of allergic asthma and is generally considered to contribute to airway remodelling [2]. As observed previously [25](Chapter 6), repeated ovalbumin challenge increased the number of eosinophils in the submucosal and adventitial compartments of the airways (P<0.001 both, Figures 3A and 3B). No significant effect of YIGSR on the increased adventitial eosinophil number after ovalbumin-challenge was observed (Figure 3B). Treatment with YIGSR did not affect eosinophil numbers in the adventitial compartment of saline-challenged animals. Remarkably, however, treatment with YIGSR significantly increased the number of eosinophils in the submucosal airway compartment after repeated allergen challenge (P<0.05, Figure 3A). Similarly, although not statistically significant, a increase in eosinophil numbers in saline-challenged animals was observed.
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Figure 3: YIGSR treatment increases allergen-induced eosinophilic inflammation in the submucosal airway compartment. (A) Ovalbumin-induced eosinophil numbers in the submucosal compartment are increased by YIGSR treatment. (B) YIGSR treatment does not affect eosinophilic cell number in the adventitial compartment. No effects of YIGSR were found in saline-challenged animals for any of the conditions. ***P<0.001 compared to saline-treated, saline-challenged controls. #P<0.05 compared to saline-treated, ovalbumin-challenged animals. Data represent means ± SEM of 5-7 animals.

Effects of YIGSR treatment on allergen-induced fibrosis.
Aberrant deposition of ECM proteins, including collagens, in the airway wall is another characteristic feature of chronic asthma [29,30]. In line with previous studies (Chapter 6), we demonstrated that lung hydroxyproline content, as an estimate of collagen, is increased after repeated ovalbumin challenge (P<0.001, Figure 4). Treatment with YIGSR of the ovalbumin-challenged animals further augmented the hydroxyproline content (P<0.01). YIGSR did not change the hydroxyproline content in saline-challenged animals. Collectively, these findings indicate that YIGSR treatment increases allergen-induced submucosal airway eosinophilia as well as collagen deposition in the lung.
Immobilized YIGSR inhibits ASM cell proliferation in vitro.

Paradoxically, previous in vitro studies have indicated that soluble YIGSR inhibits ASM cell maturation and the development of a hypercontractile, hypoproliferative ASM phenotype induced by insulin [12](Chapter 5). To further investigate this apparent paradox, the effects of immobilized and soluble YIGSR on basal and growth factor-induced ASM cell proliferation in vitro were compared. First, human ASM cells were cultured on 24 well plates coated with increasing concentrations of YIGSR (1-100 μM) and stimulated with PDGF (10 ng/ml). Surprisingly, culturing the cells on immobilized YIGSR concentration-dependently inhibited PDGF-induced DNA synthesis compared to culturing on uncoated plates (Figure 5A). No effect of YIGSR was observed on basal DNA synthesis. Similar effects were observed for cell number (data not shown). By contrast, culturing on immobilized RGDS (100 μg/ml) or its negative control Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 100 μg/ml) did not affect basal or PDGF-induced proliferation (Figure 5B).

To assess the effects of soluble YIGSR on proliferative responses of human ASM, cells were cultured on immobilized laminin-111 (10 μg/ml) or YIGSR (100 μM). Subsequently, the cells were stimulated with vehicle or PDGF in the absence or presence of soluble YIGSR. As observed previously (Chapter 3 & 5), we found that culturing on laminin-111 inhibited PDGF-induced DNA-synthesis (by 56±11%, P<0.05, Figure 5C). This inhibitory effect was fully normalized by soluble YIGSR. Surprisingly, the inhibitory effect of coated YIGSR on PDGF-induced proliferation was also fully normalized by soluble YIGSR. Similar results were obtained for cell number (data not shown). Collectively, these results indicate that the effects of the laminin-competing peptide YIGSR on ASM proliferative responses may depend on the microenvironment of the peptide.
Figure 5: Effects of immobilized and soluble YIGSR on basal and PDGF-induced human ASM cell proliferation. (A) Culturing of human ASM cells on immobilized YIGSR matrices inhibits PDGF-induced thymidine-incorporation in a YIGSR concentration-dependent fashion. Under unstimulated (Basal) conditions, no effects of immobilized YIGSR were observed. (B) Immobilized RGDS or its negative control GRADSP did not affect basal or PDGF-induced thymidine-incorporation. (C) The inhibitory effects of immobilized laminin-111 and YIGSR matrices on PDGF-induced thymidine-incorporation were normalized by soluble YIGSR. ***P<0.001 compared to thymidine-incorporation of unstimulated cells (basal) cultured on uncoated matrices (plastic). #P<0.05 and ##P<0.01 compared to PDGF-induced thymidine-incorporation of cells cultured on uncoated matrices. Data represent means ± SEM of 4-5 independent experiments of 3 different donors, performed in duplicate.
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Discussion

In the current study, we demonstrate that treatment with the laminin β1 chain-competing peptide YIGSR promotes the formation of a hypercontractile, hypoproliferative ASM phenotype in an animal model of chronic asthma. Thus, it was shown that topical application of YIGSR to the airways inhibited ASM hyperplasia induced by repeated allergen challenge, whereas ASM contractility and contractile protein expression were increased, both under basal and under allergen-challenged conditions. Apparently, these results are in contrast to previous in vitro studies, demonstrating that YIGSR inhibited maturation of human ASM cells to a hypercontractile, hypoproliferative ASM phenotype [12](Chapter 5).

Accumulation of ASM in the airway wall is a characteristic feature of asthma, which may be due to an increase in cell number (hyperplasia) [31,32] as well as an increase in cell size (hypertrophy) [31,33]. Switching of the ASM phenotype from a contractile to a proliferative state is thought to contribute to the increased ASM mass in asthma [8]. In support, various mitogenic stimuli, including growth factors and ECM proteins, have been shown to induce a proliferative ASM phenotype in vitro [9,10](Chapter 3), which may be inhibited by culturing the cells on immobilized laminin-111 [19](Chapter 3 & 6) or by endogenously produced laminin-211 (Chapter 5). These inhibitory effects are reversed, when cells are cultured in the presence of soluble YIGSR (Chapter 5), a binding motif present in the laminin β1 chain [22]. In accordance with these findings, we found that culturing human ASM cells on laminin-111 reduced proliferation of these cells induced by PDGF, which effect was fully normalized in the presence of soluble YIGSR. Surprisingly, although culturing of human ASM cells on immobilized YIGSR had no effect on basal proliferation, growth factor-induced proliferation of these cells was concentration-dependently inhibited on this matrix, to a similar extent as with laminin-111. These effects were specific for the YIGSR peptide, since culturing on RGDS, containing the RGD binding motif found in fibronectin, collagens as well as laminin [20,21], or its negative control GRADSP did not affect basal or PDGF-induced proliferation. Our observations are in agreement with previous findings, showing that immobilized YIGSR promoted attachment of various cells to a similar extent as laminin-111 [22,34,35], whereas addition of soluble YIGSR to the culture medium blocked the attachment to laminin-111 [34] or matrigel [35]. In our study, addition of soluble YIGSR to the culture medium also normalized the effects of immobilized YIGSR, which corresponds to previous findings in alveolar cells, using the Ser-Ile-Asn-Asn-Asn-Arg (SINNNR) sequence derived from the laminin α chain [36]. Collectively, these findings suggest that the laminin-competing peptide YIGSR may either promote or inhibit ASM proliferative responses, depending on the microenvironment of the peptide. The mechanisms underlying these differential effects are currently unknown. However, our results, as that of others [39], may suggest that bridging of the 67 kDa laminin receptor LAMR1, that has high
affinity to the YIGSR binding epitope [37], could be important. Previous studies have found similar effects for fibronectin and the α5β1 integrin, showing that monovalent ligand binding to this integrin resulted in the targeting of the receptor to the focal contacts, whereas multivalent ligand binding resulted in reorganization of the cytoskeleton and activation of protein tyrosine phosphorylation [38].

In addition to ASM accumulation, increased expression of expression of contractile proteins and ASM contractility, as well as increased ECM deposition, including laminins, are also characteristic features of airway remodelling in asthma (Chapter 2). Thus, in the airways of asthmatics, increased expression of laminin α2 and β2 chains has been observed [15,16], whereas expression of laminin γ2 chains inversely correlated with epithelial integrity [16]. Laminins have not only been shown to inhibit ASM proliferation, but also to be critically involved in both the maintenance and induction of a (hyper)contractile ASM phenotype. Thus, culturing of ASM cells on a laminin-111 matrix inhibits proliferation and maintains contractile protein expression in the presence of various growth factors [19], and prevents the induction of a functionally hypocontractile ASM phenotype by PDGF (Chapter 3). Induction of a contractile ASM phenotype by serum deprivation is associated by increased expression of laminin α2, β1 and γ1 chains, all found in the laminin-211 isoform [12]. Similarly, the induction of a hypercontractile, hypoproliferative ASM phenotype by insulin is associated with increased laminin α2, β1 and γ1 chain expression (Chapter 5). The increased expression of endogenous laminin is required for the observed ASM phenotype maturation, as the laminin competing peptides YIGSR, GRGDSP and RGDS prevented the induction of contractile protein expression and hypercontractility [12](Chapter 5 & 6). Recently, using the same guinea pig model of chronic asthma, we have shown that in vivo treatment with the RGD-containing peptide RGDS largely inhibits allergen-induced ASM hyperplasia and hypercontractility (Chapter 6). The RGD sequence, however, can be found in several ECM proteins [20,21], and the specific contribution of laminins to ASM remodelling in chronic asthma, is unknown thus far. In present study we found that in vivo treatment with YIGSR inhibited allergen-induced ASM hyperplasia, but increased both the expression of sm-MHC and ASM contractility in allergen-challenged as well as in control animals. In addition, a small increase in cell size in the allergen-challenged YIGSR treated animals was observed suggesting that hypertrophy may also have played a role in the observed effects. Collectively, our results indicate that treatment with YIGSR inhibits allergen-induced ASM hyperplasia and increases ASM contractility in vivo, suggesting that YIGSR mimics rather than inhibits laminin function under this condition.

Previous studies have shown that treatment with YIGSR inhibits tumour growth [39,40], which may involve blockade of the laminin receptor on endothelial cells, leading to inhibition of angiogenesis and thereby limiting the blood supply to the tumour [35]. This mechanism may also have contributed to the effects of YIGSR described in the present study, as the allergen-induced
YIGSR induces a hypercontractile ASM phenotype in vivo

increase in sm-MHC positive microvasculature in the airway wall (1.33±0.17-fold increase, P<0.05 compared saline-treated, saline-challenged controls, data not shown), an indication for the accumulation of stabilized blood vessels [41], was significantly inhibited by YIGSR treatment (90±27% reduction, P<0.05, data not shown). The potential role of angiogenesis in increased ASM growth and contractility in asthma, however, is completely unknown and would warrant further investigation.

Eosinophils express a number of integrins, of which the α6β1 integrin mediates adhesion to laminin, but not to collagen type I or type IV [42,43]. Eosinophils isolated from allergic donors also showed a higher adhesion to laminin than those isolated from healthy subjects [43]. In addition, migration of eosinophils through matrigel, a basement membrane extract containing laminin-111, required interaction with β1-integrins [43]. These findings suggest that laminin-competing peptides could affect allergen-induced infiltration of inflammatory cells to the airways. Thus far, no reports on the effects of YIGSR on eosinophil migration are available. In the current study, we observed that YIGSR increased allergen-induced eosinophil cell numbers in the submucosal compartment, without affecting eosinophil numbers in the adventitial compartment. The increased number of eosinophils in the submucosal compartment suggests that, rather than, infiltration, retention time of the eosinophils in the compartment is increased.

Increased and altered deposition of ECM proteins, including laminins and collagens, in the airway wall is another characteristic feature of remodelling in chronic asthma [29,30]. The effects of laminins on the deposition of collagens and other ECM components by fibroblasts and other structural cells is currently unknown. However, increased ECM deposition may be secondary to prolonged airway inflammation [2] and therefore increased allergen-induced airway fibrosis in YIGSR-treated animals could also indirectly result from increased eosinophilia.

Conclusions

Our results indicate that the laminin-competing peptide YIGSR promotes a contractile, hypoproliferative ASM phenotype in vivo, which may depend on the microenvironment of the peptide. In addition, treatment with YIGSR increased allergen-induced fibrosis and submucosal eosinophilia. Collectively, our data indicate that YIGSR mimics rather than competes with laminin function in vivo.

Competing interests

The authors declare that they have no competing interests
Chapter 7

Authors’ contributions

BGJD: design of the study, acquisition of data, data analysis and interpretation, manuscript writing; ISTB: design of the study, acquisition of data, data analysis and interpretation; AJH: preparation of ASM cell lines and critical revision of the MS; JZ: design of the study, data interpretation and critical revision of the MS; HM: design of the study, data interpretation and critical revision of the MS. All authors have read and approved the manuscript.

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