

ORIGINAL ARTICLE

Human airway smooth muscle promotes eosinophil differentiation

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Summary

Introduction Human airway smooth muscle (HASM) cells in culture synthesize cytokines and chemokines that may orchestrate the tissue homing and *in situ* differentiation of haemopoietic progenitor cells from the peripheral circulation.

Objective To study the effect of a supernatant from cultured HASM cells on the differentiative and transmigration responses of haemopoietic progenitor cells.

Methods HASM cells were grown to confluence and stimulated with a cytotoxic mix of TNF- α , IL-1 β and IFN- γ . Peripheral blood-derived progenitors from atopic asthmatics ($n = 12$) and non-atopic controls ($n = 11$) were grown in a methylcellulose culture with a supernatant from stimulated HASM cells to assess clonogenic potential. The ability of HASM cells to stimulate directional migration and adhesion to fibronectin of blood progenitors was also investigated. **Results** HASM cells stimulated significant growth of eosinophil/basophil colony forming units (Eo/B CFUs) from blood progenitor cells from both groups of subjects. This activity was significantly attenuated in the presence of anti-IL-5 and anti-granulocyte macrophage-colony forming factor blocking antibodies and by pre-treatment with SB202190 [p38 mitogen-activated protein kinase (MAPK) inhibitor]. An src kinase (srcK) inhibitor (Pyrarolopyrimidine 1) was less effective at attenuating IL-5- and HASM-stimulated Eo/B CFU growth from both groups of subjects. Examination of the phosphorylation of these kinases in CD34⁺ cells following co-incubation with the major constituents of HASM showed activation of p38 MAPK but not that of the srcK pathway. The HASM supernatant had no significant effect on the migrational and adhesive responses of haemopoietic progenitor cells *in vitro*.

Conclusion We have shown that HASM cell-derived cytokines promote eosinophil differentiation that is dependent on p38 MAPK but not on the srcK pathway. This study shows that a major structural cell of the lungs, airway smooth muscle, has the capability to direct eosinophil differentiation and maturation from progenitor cells, which in turn may perpetuate an eosinophilic inflammation and consequently tissue remodelling in patients with chronic asthma.

Keywords asthma, atopy, Eo/B CFUs, eosinophilopoiesis, human airway smooth muscle, IL-5, progenitor cells

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Introduction

Chronic asthma is characterized by airflow obstruction, bronchial hyperresponsiveness and chronic inflammation with a notable eosinophilic component [1]. During exacerbations of asthma following either natural allergen exposure or controlled allergen inhalation in the laboratory [2] or due to inadequate therapy with corticosteroids [3], increased numbers of eosinophils appear in the airway

lumen and in the bronchial tissue. There is increasing evidence that, in addition to the traffic of mature eosinophils to the lung tissue from peripheral pools, haemopoietic processes are activated within the bone marrow, resulting in the mobilization and homing of progenitor cells to the lungs during an allergic inflammatory response [4]. Once within the lungs, these cells have the potential to differentiate into pro-inflammatory cells or mesenchymal tissue [5–7], their fate driven by local levels

of growth factors. There is little information about the role that lung tissue plays in the production of factors that regulate lung homing and *in situ* differentiation of bone marrow-derived haemopoietic progenitor cells.

The role of the airway smooth muscle cells in the persistence of cellular inflammation is not well known. There is increasing evidence of an interplay between smooth muscle and inflammatory cells in asthma pathophysiology [8]. Activated airway smooth muscle cells, stimulated by cytokines such as TNF- α , IL-1 β and INF- γ , are able to synthesize a variety of cytokines, chemokines and growth factors and express surface receptors that are important for cell adhesion and leucocyte activation. Cultured human airway smooth muscle (HASM) cells have been shown to synthesize IL-5 [9], RANTES [10], granulocyte macrophage-colony forming factor (GM-CSF) [11] and eotaxin [12], all of which play an important role in airway eosinophilic inflammation; however, a direct role of smooth muscle cells in the lung homing and *in situ* differentiation of immature progenitor cells has not been investigated to date.

Cytokines and chemokines that regulate the differentiation, maturation, survival and migration of eosinophils are generally derived from inflammatory cells and perhaps from mesenchymal tissue. Eosinophils are formed from CD34⁺ precursor cells in the bone marrow. The commitment of progenitor cells to the eosinophil/basophil lineage is regulated by various cytokines, in particular IL-5 and GM-CSF [13]. This process involves a number of signal transduction pathways including protein kinase C (PKC) [14], Lyn tyrosine kinase [15] and mitogen-activated protein kinases (MAPKs) [16].

In this study, we examined whether supernatant from cultured human non-asthmatic airway smooth muscle cells caused the differentiation and maturation of haemopoietic progenitor cells into eosinophils and whether it could promote migration of these cells. We also examined the relative roles of p38 MAPK, the src-kinase (srcK) Lyn and PKC in this process. Finally, we studied whether progenitor cells derived from patients with asthma respond differently from those derived from normal subjects.

Methods

Subjects

Subjects with mild asthma ($n = 12$) and healthy age- and sex-matched non-atopic volunteers ($n = 11$) were recruited after obtaining their written informed consent and with the permission of the Research Ethics Board of St Joseph's Healthcare. Atopic asthmatics were skin prick test (SPT) positive, had a PC₂₀ methacholine of < 16 mg/mL and were not on any medication other than infrequent use of short-acting bronchodilators. They were studied outside of their allergy season and did not have an upper

respiratory tract infection for at least 4 weeks before the study. The normal volunteers were SPT negative and had no history of allergic disease (non-atopic), were non-smokers and had a PC₂₀ methacholine of > 16 mg/mL.

Airway smooth muscle culture

Lung tissues were obtained from 12 non-asthmatic patients who underwent lung surgeries for lung cancer at St Joseph's Hospital, Hamilton, with the cooperation of the Divisions of Thoracic Surgery and the Department of Pathology, after approval from the Research Ethics Board and obtaining informed consent from the patients. Smooth muscle tissue was isolated from disease-free areas of bronchi, explanted, grown in culture and used between the second and fifth passages as described previously [17]. Cells were grown to confluence, trypsinized (0.05% trypsin, HyClone, Logan, UT, USA) and re-seeded at 20 000 cells/mL and maintained in growth factor-free RPMI media for 48 h. A cytokine mix comprising of TNF- α (30 ng/mL), INF- γ (100 ng/mL) and IL-1 β (5 ng/mL) (all from PeproTech Inc., Rocky Hill, NJ, USA) was added to the confluent cells 12–18 h before collecting the supernatant, referred to as an airway smooth muscle supernatant (ASMS). This cocktail of cytokines was used to cause maximal stimulation of the airway smooth muscle cells.

The concentration of cytokines in the supernatant were measured by ELISA (R&D Systems, Minneapolis, MN, USA). The lower limit of detection for the ELISA assays was GM-CSF, 3 pg/mL; RANTES, 2 pg/mL; IL-5, 3 pg/mL; IL-8, 3.5 pg/mL; Eotaxin, 5 pg/mL and SCF, 9 pg/mL.

Isolation of progenitor cells

As described previously [18], peripheral blood (60 mL) was drawn into heparinized venipuncture tubes (BD, Franklin Lakes, NJ, USA) diluted in McCoy's 5A medium (Gibco, Grand Island, NY, USA), layered on Accu-Prep (BD Falcon, Mississauga, ON, Canada) and centrifuged for 20 min at 400 g. (Megafuge 1.0, Heraeus Instruments Inc., South Plainfield, NJ, USA). The mononuclear cell interface was collected, washed and resuspended in McCoy's 3+ media (enriched with fetal bovine serum (FBS), 2 mercapto-ethanol and penicillin/streptomycin). As macrophages interfere with the growth of progenitor cells in semisolid media, the cells were incubated for 2 h (37 °C, 5% CO₂) (NuAire DH Autoflow, NuAire Corp., Plymouth, MN, USA) in 75 cm² plastic flasks (BD Falcon). Non-adherent mononuclear cells (NAMNCs) containing an enriched population of haemopoietic progenitors were collected. For the migration and adhesion assay, haemopoietic progenitors were further enriched by negative selection using magnet-activated cell sorting, i.e. depletion of lymphocytes (expressing CD3) and granulocytes (expressing CD16).

Methylcellulose culture of progenitor cells

The NAMNCs were seeded in methylcellulose (Sigma-Aldrich, St Louis, MO, USA) culture media supplemented with FBS (HyClone), Iscove's (Gibco) in modified Dulbecco's medium (plus 0.5% 2 mercapto-ethanol and 0.1% penicillin/streptomycin) [19]. Duplicate cultures were grown in the presence of a positive control, IL-5 (1 ng/mL) (BD Sciences Pharmingen, Markham, ON, Canada) or ASMS (1/1000, 1/100 and 1/10 final dilution), and cultured for 14 days (5% CO₂, 37 °C). The maximal concentration of ASMS (1/10 dilution of stock) was dictated by the methylcellulose culture set-up, whereby adding the cells, growth factor and methylcellulose results in a final 1/10 dilution of the stock concentration of the growth factor. Eosinophil/basophil colony-forming units (Eo/B CFU) were identified as tight, compact, round refractile cell aggregates staining pink with eosin under inverted light microscopy (Olympus CK 40, Olympus Co. Ltd, Tokyo Japan). The role of IL-5 and GM-CSF in ASMS stimulated Eo/B CFU formation was confirmed by adding anti-human IL-5 mAb (0.01, 0.1 and 1 µg/mL) and anti-human GM-CSF mAb (0.1, 1 and 10 µg/mL) (both from R&D Systems Inc.) to the cultures.

Signal transduction pathways

The role of p38 MAPK, srcK Lyn and PKC in Eo/B CFU formation was investigated by adding their specific inhibitors to the methylcellulose culture media, as follows: p38 MAPK inhibitor (SB 202190, Sigma-Aldrich); srcK inhibitor [Pyrazolopyrimidine 1 (PP1)]; the conventional and novel PKC isoforms inhibitor (Calphostin C); PKC delta isoforms inhibitor (Rottlerin); and an typical PKC isoform (PKC Zeta pseudosubstrate, all purchased from Calbiochem, EMD Biosciences, La Jolla, CA, USA). The inhibitors used had the following IC₅₀ values: SB202190, 50 nM; PP1, 1.2 µM; Calphostin C, 50 nM; Rottlerin, 3–6 µM; and PKC Zeta pseudosubstrate, 10–20 µM. The concentrations used for each inhibitor, SB202190 (25 µM), PP1 (0.1–10 µM) and Calphostin C (1 µM), Rottlerin (1–100 µM) and PKC Zeta pseudosubstrate (0.1–10 µM), were evaluated in preliminary experiments to provide the optimal outcome results in methylcellulose culture. The roles of p38 MAPK and srcK pathways were further investigated by examining phosphorylation of these kinases in CD34⁺ cells when treated with IL-5 and GM-CSF. MACS-purified CD34⁺ cells from peripheral blood were co-incubated with IL-5 (1 ng/mL) and GM-CSF (10 ng/mL) for various durations of time (0, 1, 5 and 30 min) and phosphorylated, and total kinases were enumerated in the cell lysates (750 ng/tube) using Signal Transduction Assay Reaction (STAR) ELISA kits for phospho and total p38 MAPKs and phospho and total srcKs (Millipore, Temecula, CA, USA). Briefly, 96-microwell clear plates were coated with a

specific mouse monoclonal capture antibody. The detection antibody was either a specific rabbit total or phospho-specific antibody (1/1000) that was used to detect the captured target in the wells of the plate. The sensitive enzymatic detection of the sample was measured at an absorbance of 450 nm using a standard plate reader.

Adhesion assay

Enriched CD34⁺ cells were pre-incubated with diluent, positive control [stromal cell-derived factor-1α (SDF-1α) at 1, 10, 100 ng/mL] or ASMS (1/10, 1/100, 1/1000 dilution) before being added in duplicate to a 24-well plate previously coated with 20 µg/mL of human fibronectin. Plates were incubated for 30 min at 37 °C to allow cells to adhere and non-adhered cells were removed by washing with Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline. Adhered cells were resuspended with cell dissociation buffer (Invitrogen, Burlington, ON, Canada), aspirated, washed and immunostained with phycoerythrin-conjugated CD34 mAb and fluorescein isothiocyanate-conjugated CD45 mAb, fixed in 1% paraformaldehyde and enumerated by flow cytometry. Adhered cells were expressed as a percentage of the total progenitor cells added to each well.

Transwell migration assay

Using transwell chambers (24-well cell clusters with 5-µm pore size polycarbonate filters), the migrational response of peripheral blood-derived progenitors was assessed [20]. An enriched population of progenitors (5 × 10⁴ cells) as described above was loaded into each transwell insert and placed into a lower well containing 600 µL dilutions of the chemoattractants SDF-1α or SMS in serum-free medium (37 °C, 5%CO₂, 4 h). The transwell inserts were then removed and the cells in the bottom (representing migrated cells) were aspirated, washed, immuno-stained and enumerated by flow cytometry. Migrated progenitor cells in the lower wells were expressed as a percentage of the total progenitor cell numbers added to the upper wells.

Statistical analysis

Data are presented as mean ± SEM, unless otherwise stated. Counts of Eo/B CFU in methylcellulose cultures were non-normally distributed and so data were summarized using medians and inter-quartile ranges. Between-group comparisons were made using the Mann-Whitney *U*-test and within-group comparisons were made using the Kruskal-Wallis ANOVA. All other comparisons were performed using a repeated measure ANOVA. Statistical significance was assumed when *P* < 0.05. All analyses were performed using SPSS version 13.0 (Statistical Package for Social Sciences, Chicago, IL, USA).

Results

Cytokine synthesis by airway smooth muscle cells

Supernatants of ASMS cultured in the presence and absence of stimulating cytokines TNF- α , INF- γ and IL-1 β , i.e. cytomix, were assessed for the presence of various cytokine/chemokine growth factors. Stimulated ASMS produced detectable levels of GM-CSF (29.35 ± 11.9 pg/mL) and RANTES (32.5 ± 12.0 pg/mL), both of which were below the limits of detection in the unstimulated ASMS culture supernatant (Table 1). Although unstimulated ASMS spontaneously produced detectable levels of IL-5, eotaxin and IL-8 levels were increased fourfold, 50-fold

and 26-fold, respectively, in ASMS following cytomix stimulation (Table 1).

Airway smooth muscle supernatant and Eo/B colony formation

A concentration-dependent clonogenic response by blood progenitor cells was observed following co-culture with stimulated ASMS, optimal at a 1/10 dilution (Fig. 1). In contrast, no clonogenic response was detected in cultures with supernatants from unstimulated ASMS. In addition, constituents of the cytomix cocktail, i.e. TNF- α , INF- γ and IL-1 β , used either alone or in combination, did not stimulate the growth of eosinophil colonies in methylcellulose culture from either group of subjects (data not shown). This eosinophilopoietic activity, although significantly greater than diluent levels, was lower than the clonogenic response elicited by the positive control, IL-5 (1 ng/mL). When the eosinophilopoietic response of blood progenitor cells from asthmatics and non-asthmatic donors to stimulated ASMS was compared, there was no significant group difference (Figs 1a and b, respectively).

A concentration-dependent inhibition of eosinophilopoietic activity in stimulated ASMS was observed in the presence of optimal concentrations of IL-5 and GM-CSF-neutralizing antibodies (assessed in control experiments), implicating both IL-5 and GM-CSF as the possible eosinophilopoietic factors in ASMS (Fig. 2). In addition, in the control experiments, we found that the clonogenic activity of ASMS was ablated by heat treatment (56 °C, 30 min), indicating the protein nature of the growth factor (data not shown).

Table 1. Cytokine production by human airway smooth muscle

Cytokines	Unstimulated ASMS (pg/mL)	Stimulated ASMS (pg/mL)
GM-CSF	ND	29.4 ± 12
RANTES	ND	32.5 ± 12
IL-5	20 ± 4.2	80 ± 14
IL-8	265 ± 235	6942.5 ± 298.4
Eotaxin	8.7 ± 5	438 ± 192
Stem cell factor	15.7 ± 8.7	19.95 ± 8.9

ELISA was performed on 20 000 cells, pre- and post-stimulation with cytomix (TNF- α , IL-1 β and INF- γ). Secretion of cytokines and growth factors was detectable at baseline and enhanced by stimulation with cytomix, in particular IL-8. Data are presented as the mean $\pm$ SEM of $n = 5$ separate ASMS cultures. The lower limit of detection for the ELISA assays was GM-CSF, 3 pg/mL; RANTES, 2 pg/mL; IL-5, 3 pg/mL; IL-8, 3.5 pg/mL; Eotaxin, 5 pg/mL; and SCF, 9 pg/mL.

ASMS, airway smooth muscle supernatant, GM-CSF, granulocyte macrophage-colony forming factor.

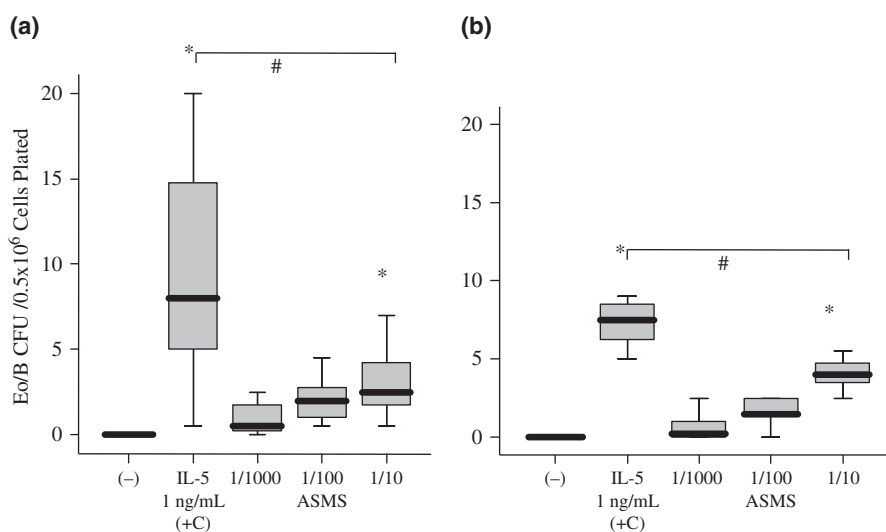


Fig. 1. Stimulated airway smooth muscle supernatant (ASMS) elicited an eosinophilopoietic response by blood progenitors from (a) normals ($n = 11$) and (b) atopic asthmatics ($n = 12$), optimal at 1/10 dilution. No significant difference in the eosinophil/basophil colony forming unit (Eo/B CFU) numbers was detected between the subject groups. * $P < 0.05$ for diluent vs. IL-5- or ASMS-stimulated colonies; # $P < 0.05$ for IL-5- vs. ASMS-stimulated colonies.

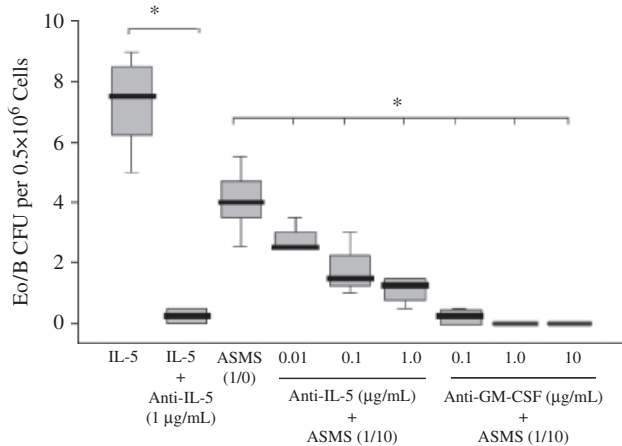


Fig. 2. The eosinophilopoietic response stimulated by airway smooth muscle supernatant (ASMS) co-cultured with progenitors from atopic asthmatics was attenuated by IL-5 or granulocyte macrophage-colony forming factor (GM-CSF) monoclonal antibodies. There was a trend for anti-GM-CSF to be more effective than IL-5, although this effect was not significantly different. IL-5 and GM-CSF may contribute to the clonogenic activity detected in ASMS. * $P < 0.05$ for ASMS-stimulated colonies plus anti-cytokine antibody.

Signalling mechanisms

Pharmacological antagonists of p38 MAPK (SB202190), PKC-conventional (Calphostin) tested at sub-optimal concentrations completely ablated the growth of Eo/B CFUs from asthmatic and normal healthy subjects in cultures with either IL-5 or ASMS (Table 2). The inhibitory effect of srcK (PP1), however, was less effective than that observed with SB202190 and Calphostin.

In a subsequent set of experiments, we investigated the effect of (a) Rottlerin (1, 10, 50 and 100 μM), an inhibitor of PKC delta, and (b) PKC Zeta pseudosubstrate (0.1, 1 and 10 μM), the specific inhibitor of the atypical PKC Zeta isoform, on stimulated eosinophilopoiesis. As shown in Fig. 3, all concentrations of both inhibitors caused a statistically significant concentration-dependent attenuation of IL-5-induced Eo/B CFU grown from progenitor cells isolated from asthmatics. In addition, in co-cultures of ASMS with blood-derived progenitor cells from asthmatics, we found that Rottlerin (50 μM) and PKC Zeta pseudosubstrate (1 μM) caused a significant attenuation of the Eo/B CFU (Fig. 4). Both signalling inhibitors were equi-effective on the stimulated growth and differentiation of progenitor cells from asthmatics and normal subjects (data not shown).

Using STAR ELISA kits for phospho and total p38 MAPKs and phospho and total srcKs, we examined the activation of p38 MAPK and srcK in CD34⁺ cells during eosinophilopoiesis. Table 3 shows that for four separate experiments, compared with baseline, a significant increase in the ratio of phospho/total p38 was detected at 1 min following co-incubation of CD34⁺ cells with IL-5 and

Table 2. Effect of signalling inhibitors on clonogenic responses of blood progenitor cells

Conditions	Eo/B CFU (mean $\pm$ SEM) and (median)	
	Normal healthy controls ($n = 11$)	Asthmatic subjects ($n = 12$)
Diluent	0.6 $\pm$ 0.5 (0)	0 (0)
IL-5 (1 ng/mL)	9.4 $\pm$ 1.8 (8)	7.8 $\pm$ 0.7 (7.5)
IL-5 (1 ng/mL) + SB202190 (25 μM)	1.5 $\pm$ 0.4 (1)*	1.3 $\pm$ 0.4 (0.8)*
IL-5 (1 ng/mL) + CP (1 μM)	0 (0)*	0 (0)*
IL-5 (1 ng/mL) + PP1 (0.1 μM)	6.5 $\pm$ 2.4 (3.3)*	3.4 $\pm$ 0.7 (2.8)*
IL-5 (1 ng/mL) + PP1 (10 μM)	8.4 $\pm$ 1.6 (5.5)	4.3 $\pm$ 0.7 (4.3)*
ASMS (1/10)	3.3 $\pm$ 0.7 (2.5)	4.1 $\pm$ 0.4 (4)
ASMS (1/10) + SB202190 (25 μM)	1.2 $\pm$ 0.4 (0.5)*	0.8 $\pm$ 0.4 (0)*
ASMS (1/10) + CP (1 μM)	0 (0)*	0 (0)*
ASMS (1/10) + PP1 (0.1 μM)	1 $\pm$ 0.5 (0.3)*	1.1 $\pm$ 0.3 (0.5)*
ASMS (1/10) + PP1 (10 μM)	2.5 $\pm$ 0.7 (1.5)	1.6 $\pm$ 0.4 (1.5)*

Effect of signalling inhibitors on clonogenic responses of blood progenitor cells from normal subjects and atopic asthmatics in methylcellulose culture. In co-cultures with either IL-5 or cytomix-stimulated airway smooth muscle supernatant (ASMS), there was a significant inhibition of eosinophil/basophil colony forming unit (Eo/B CFU) in the presence of SB 202190 (p38 mitogen-activated protein kinase inhibitor) and Calphostin (CP; protein kinase C inhibitor) and to a lesser extent PP1 (Src kinase inhibitor).

* $P < 0.05$ for comparisons between either IL-5 or ASMS-stimulated cultures and respective cultures in the presence of various signalling inhibitors.

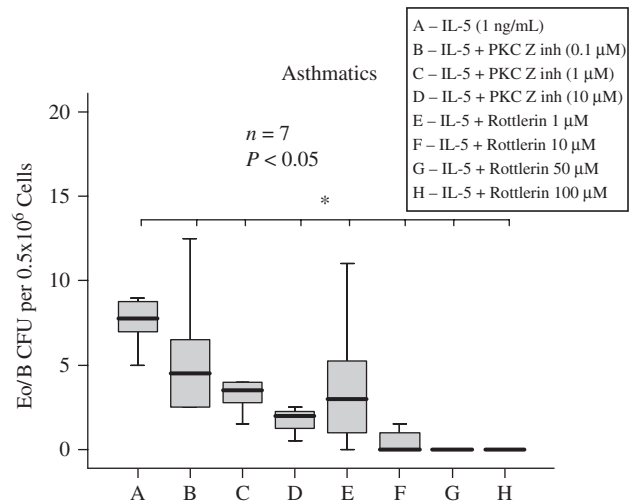


Fig. 3. Inhibitory effect of protein kinase C (PKC) Zeta pseudosubstrate (atypical PKC isoform inhibitor) and Rottlerin (novel PKC isoforms inhibitor) on IL-5-stimulated eosinophil/basophil colony forming unit (Eo/B CFU) using progenitor cells from asthmatics ($n = 7$). A concentration-dependent attenuation of the clonogenic response was observed in the presence of each signalling inhibitor. A similar response was observed with cells from normal subjects ($n = 7$; data not shown). * $P < 0.05$ for comparisons between IL-5-stimulated colonies vs. conditions with IL-5 plus signalling inhibitor.

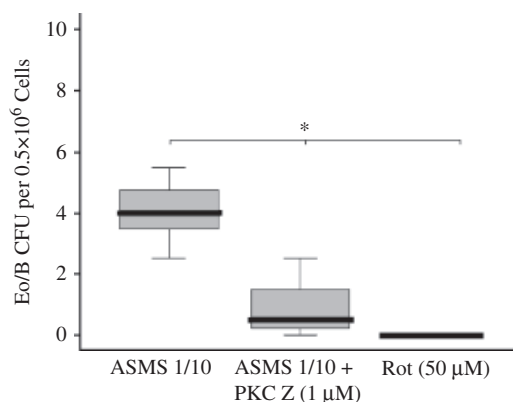


Fig. 4. Inhibitory effect of protein kinase C (PKC) Zeta pseudosubstrate (atypical PKC isoform inhibitor) and Rotterlin (novel PKC isoform inhibitor) on airway smooth muscle supernatant (ASMS)- (1/10 dilution) stimulated eosinophil/basophil colony forming unit (Eo/B CFU) using progenitor cell populations from asthmatic subjects ($n = 5$). A similar response was observed with cells from normal subjects ($n = 5$; data not shown). * $P < 0.05$ for comparisons between ASMS-stimulated colonies vs. conditions with ASMS in the presence of signal inhibitors.

Table 3. Detection of phosphorylated and total signalling moieties in CD34⁺ cell

Phospho/total ratio	0 min	1 min	5 min	30 min
p38 MAP kinase	0.18±0.07	0.61±0.23*	0.59±0.36	0.44±0.34
Src kinase	0.05±0.04	0.040±0.02	ND	ND

Detection of Phosphorylated and total signalling moieties were enumerated in the CD34⁺ cell lysates (750 ng/tube) using Signal Transduction Assay Reaction ELISA kits for phospho and total p38 mitogen-activated protein (MAP) kinases and phospho and total src kinases (Millipore, CA, USA). The data (mean±SD) show that for four separate experiments, compared with baseline, a significant increase in the ratio of phospho/total p38 was detected at 1 min following co-incubation of CD34⁺ cells with IL-5 (1 ng/mL) and granulocyte macrophage-colony forming factor (10 ng/mL).

* $P < 0.05$. In contrast, no changes were detectable in the stimulated cell lysates for phospho src kinases.

GM-CSF (* $P < 0.05$). In contrast, no changes were detectable in the stimulated cell lysates for phospho srcKs.

Adhesion and transmigration assay

Because various chemokines including eotaxin and RANTES were detected in ASMS (Table 1), we investigated whether airway smooth muscles could stimulate lung homing of blood-derived progenitor cells. The adhesive and migrational properties of ASMS compared with a potent progenitor cell chemoattractant, SDF-1, were assessed *in vitro*. When tested over a wide dilution range, stimulated ASMS did not elicit the adhesion of blood-derived progenitor cells from atopic asthmatics to fibronectin. In addition, stimulated ASMS had no effect on the directional migration of blood progenitor cells from atopic asthmatics (Table 4).

Table 4. Adhesive and migrational properties of human airway smooth muscle supernatants

Conditions	Adhered cells (% of total CD34 ⁺ cells)	Migrated cells (% of total CD34 ⁺ cells)
Diluent	11±5	13±5
SDF-1 (10 ng/mL)	24±3*	26±7*
ASMS (1/1000)	19±5	15±8
ASMS (1/100)	20±7	18±4
ASMS (1/10)	18±5	13±8

Adhesive and migrational properties of human airway smooth muscle supernatants (ASMS) on cytomix-stimulated peripheral blood-derived progenitor cells were investigated. Data represent the mean±SEM of seven separate experiments on progenitor cells from atopic asthmatics. Stimulated ASMS had no effect on the adhesion and migrational responses of CD34⁺ progenitor cells.

* $P < 0.05$ compared with diluent control.

Discussion

In the current study, we report for the first time that HASM cells have the capacity to stimulate the growth and differentiation of progenitor cells into mature eosinophils *in vitro*. This eosinophilopoietic response was dependent on p38 MAPK pathways but not on activation of the srcK pathway. Smooth muscle supernatant, however, did not exert chemotactic effects on these progenitor cells. The findings of this study indicate that the synthetic products of activated airway smooth muscle cells can promote the local differentiation of haemopoietic progenitor cells into pro-inflammatory cells once they have been recruited to the lungs in the allergic asthmatic process and thus contribute to the persistence of eosinophilic airway inflammation.

It has previously been shown that increased numbers of CD34⁺ progenitor cells are present in the blood and bone marrow of atopic subjects compared with non-atopic controls [19]. Furthermore, it was shown that progenitors from atopic subjects displayed greater clonogenic responsiveness to IL-5 compared with blood progenitors from normal subjects. Depletion of CD34⁺ cells from cell cultures resulted in the ablation of the IL-5-induced eosinophilopoietic response, indicating that the responding cells in the NAMNC populations plated in methylcellulose culture were indeed CD34⁺ cells [19]. It was therefore proposed that in the present study if ASMS stimulated eosinophilopoiesis, then a greater number of colonies would be grown from atopics compared with normal subjects. That this was not observed may indicate that in the current group of subjects there were no significant differences in the numbers of CD34⁺ cells between the normal and atopic asthmatic subjects, the latter being a very mild group. An alternative explanation may be that the magnitude of the eosinophilopoietic response is determined by the local airway milieu and airway smooth muscle synthetic phenotype rather than

being a property of circulating progenitor cells. Because the atopic status of the lung biopsy donors was not ascertained in this study, we are unable to investigate this variable. However, we propose that airway smooth muscle is likely to contribute to *in situ* eosinophil differentiation, thus perpetuating an eosinophilic inflammation and consequently tissue remodelling in patients with chronic asthma. It is likely that airway smooth muscle cells from asthmatic airways produce greater amounts of eosinophilopoietic factors than cells from normal airways, and that this may therefore contribute to the magnitude of the airway eosinophilia.

The roles of three signalling pathways previously reported to play a part in eosinophil differentiation, namely PKC [14], src tyrosine kinase [15] and p38 MAPK [16] pathways, were investigated in ASMS-stimulated eosinophil differentiation from blood progenitor cells. Using a specific pharmacological antagonist of the typical isoform of PKC, known to promote myeloid lineage commitment and eosinophil differentiation in various progenitor cell lines, we found that Calphostin (1 μ M) completely ablated IL-5 and ASMS stimulated eosinophil differentiation (Table 2). The concentration of Calphostin that was used was not cytotoxic to the progenitor cells as determined by Trypan blue exclusion after 24 h in culture. In addition, antagonists of the atypical isoform of PKC (PKZ pseudosubstrate) [14], identified as integral to *in vitro* transmigrational responses of CD34⁺ cells elicited by SDF-1, and Rottlerin, inhibitor of the novel isoforms of PKC, both attenuated IL-5- and ASMS-stimulated eosinophil differentiation (Figs 3 and 4). Similarly, modulation of p38 MAPK, known to transduce IL-5-mediated eosinophil differentiative signals in mice [16], by SB202190 significantly attenuated IL-5- and ASMS-stimulated eosinophil differentiation. The role of the src tyrosine kinase pathway was investigated using PP1. This inhibitor, in contrast to the various PKC isoform inhibitors and p38 MAPK inhibitor, was least effective at attenuating eosinophil differentiation (Table 2). We confirmed the role of p38 MAPK but not srcK in this process by demonstrating activation of the kinase in CD34⁺ cells following co-culture with the major constituents of HASM, i.e. IL-5 and GM-CSF (Table 3). Although our data with soluble inhibitors suggest the involvement of PKC delta and PKC Zeta, the lack of analyses of the activation of these kinases in CD34⁺ cells does not make it possible to be conclusive about the involvement of these two PKC isoforms in HASM-stimulated eosinophilopoiesis. Overall, the data indicate that HASM cell-derived growth factors can promote eosinophil growth and differentiation that is dependent on p38 MAPK, but not on src tyrosine kinase pathways. These data show that ASMS activity uses a similar signalling pathway as IL-5 to stimulate eosinophilopoiesis.

There is considerable evidence that haemopoietic events are activated within the bone marrow during the

course of the allergic asthmatic response [21–23]. Studies have shown that the phenotype and responsiveness of these cells are altered so as to allow the cells to mobilize to the peripheral circulation and ultimately home to the site of airway inflammation [7, 20, 24]. Factors that orchestrate the lung homing of progenitor cells still remain to be investigated although eotaxin and SDF-1 have been implicated in the context of allergic inflammatory diseases [4, 25, 26]. The current study shows that although low levels of eotaxin (0.7–0.9 ng/mL) were detected in ASMS (Table 1), this supernatant was unable to elicit significant adhesive or migrational responses by blood progenitor cells (Table 4). In a previous study, we have shown that at high concentrations, eotaxin (200–500 ng/mL) stimulated a weak migrational response by bone marrow-derived progenitor cells [20, 27]. These levels were much higher than those detected in the ASMS and may account for the lack of pro-migrational or adhesive responses by blood progenitor cells in the current study. In addition, although eotaxin has been shown to promote eosinophilopoiesis from cord blood-derived progenitors [28], we have been unable to demonstrate any clonogenic activity of this chemokine on blood or bone marrow-derived progenitors when used either alone or in combination with various cytokines [27]. Thus it is unlikely that eotaxin levels in the ASMS promoted eosinophil colony formation in this study.

Evidence exists to support the view that once within the lung, progenitor cells can differentiate to form mature pro-inflammatory cells [5, 6] that may be primarily IL-5 mediated. Other studies have indicated that GM-CSF may play a role in *in situ* differentiation of eosinophil progenitor cells in allergic inflammatory responses [29–31]. In agreement with this, our data showed that, in the presence of neutralizing antibodies to IL-5 and GM-CSF, the eosinophilopoietic potential of ASMS was ablated, suggesting that these may be the prime factors produced by airway smooth muscle cells to promote *in situ* eosinophilopoiesis in the asthmatic lung.

While there are no reports of eosinophilic infiltration of airway smooth muscle bundles with eosinophils in patients with asthma, our findings are not in contradiction to this observation. We have demonstrated that smooth muscle supernatant is not chemotactic to eosinophil progenitor cells. This is consistent with the lack of eosinophil or progenitor infiltration into the smooth muscle bundle in patients with asthma. In addition, we have demonstrated that products of smooth muscle cells are capable of promoting eosinophil differentiation. This does not imply that this is the only source of such factors. Clearly, other inflammatory cells, in particular, lymphocytes and macrophages, produce these cytokines and chemokines. Other structural cells including fibroblasts may also elaborate these products although this was not the focus of the current study.

It is known that various proteases such as mast cell-derived tryptase are capable of inactivating a number of cytokines and chemokines including eosinophil chemoattractants such as eotaxin. This does not invalidate our observations as in asthma, it is very likely that there is an imbalance between proteases and anti-proteases in the milieu of growth factors, enzymes, mediators, chemokines and cytokines in the airway wall and lumen, thus perpetuating the effects of various factors including smooth muscle-derived eosinophilopoietic factors.

In summary, the current study shows that a major structural cell of the lungs, airway smooth muscle, which undergoes significant changes within the asthmatic lung, has the capability to direct eosinophilic differentiation. These data not only expand our understanding of the role of airway smooth muscle cells, but may provide a therapeutic target for the optimal control of asthma symptoms.

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