

**Proliferation is not increased in airway myofibroblasts isolated from asthmatics**

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## **ABSTRACT:**

Airway mesenchymal cells such as myofibroblasts and airway smooth muscle cells contribute to inflammation, airway remodelling and hyperresponsiveness in asthma by excessive proliferation and inflammatory mediator production.

Using endobronchial biopsies obtained from both non-asthmatic and asthmatic subjects, *in situ* proliferation was assessed by immunostaining for cyclin D1. The number of immunoreactive cells increased with asthma severity and was restricted to the epithelium and sub-epithelial connective tissue. Despite increases in smooth muscle area, cyclin D1 was not detected in cells in intact muscle bundles.

Biopsy-derived cell cultures were characterised as predominantly myofibroblasts, and assessed to determine if proliferation and cytokine production varied with asthma status. Cell enumeration showed that basal proliferation was similar in cells from non-asthmatics and asthmatics and mitogenic responses to FGF-2, thrombin or serum were either lesser or unchanged in cells from asthmatics. Interleukin (IL)-1-dependent GM-CSF and IL-8 release was increased in cell supernatants from asthmatics.

Thus, increased rates of cellular proliferation identified *in situ* in the asthmatic airway occurred outside the expanded smooth muscle compartment. Although reduced proliferative responses were observed in cultured myofibroblasts from asthmatics, the increased cytokine production by these cells suggests that this contributes to and may perpetuate ongoing inflammation in asthma.

**Keywords:** airway smooth muscle, asthma, fluticasone propionate, granulocyte macrophage colony-stimulating factor, myofibroblast, proliferation, salmeterol

**Abbreviations:**  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; AHR, airways hyperresponsiveness; FGF-2, fibroblast growth factor-2; BAL, bronchoalveolar lavage; DMEM, Dulbecco's modified Eagle's medium; FEV, forced expiratory volume; FCS, foetal calf serum; FP, fluticasone propionate, GINA, Global Initiative for Asthma; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; MESCA, Melbourne Epidemiological Study of Childhood Asthma.

The asthmatic airway wall is characterised by tissue remodelling in which epithelial disruption, goblet cell hyperplasia, reticular basement membrane thickening, airway mesenchymal cell (comprising airway smooth muscle and airway myofibroblasts) hypertrophy and/or hyperplasia, and subepithelial collagen deposition are major features that contribute to airway hyperresponsiveness (AHR) [1] [2].

Human cultured airway smooth muscle and myofibroblasts produce multiple inflammatory mediators and proliferate in response to cytokines and polypeptide growth factors [3, 4]. Airway smooth muscle cultured from asthmatics is claimed to be hyperproliferative to 5% foetal calf serum (FCS) [5], but refractory to the antiproliferative effects of glucocorticoids [6]. Similar findings are reported with myofibroblasts using [<sup>3</sup>H]-thymidine incorporation, a marker of cells in S-phase or DNA repair [7-9]. Thrombin and fibroblast growth factor-2 (FGF-2) which are at elevated BAL concentrations in asthma are mitogenic for both myofibroblasts and airway smooth muscle [10-12]. However, it is not known whether asthma status influences the proliferative responses of airway mesenchymal cells to these specific mitogens.

Interleukin-1 (IL-1) can elicit release of granulocyte macrophage colony-stimulating factor (GM-CSF) [13, 14] and IL-8 [15] from myofibroblasts and airway smooth muscle. These cytokines play important roles in promoting recruitment, maturation and survival of eosinophils, mast cells and neutrophils, while GM-CSF may also contribute to subepithelial fibrosis by regulating collagen deposition [16]. Asthma-associated changes in the production of these inflammatory mediators have not been fully explored, although there are unconfirmed reports showing increased release of eotaxin [17] and CTGF [18] from airway smooth muscle cultured from asthmatics.

In this study, endobronchial biopsies were obtained from healthy non-asthmatic volunteers and from patients with varying asthma severity. The majority of subjects (81 of 92 studied) were recruited at age 42 from a cohort in a long-term study (Melbourne Epidemiological Study of Childhood Asthma, MESCA), in which data has been collected from participants every 7 years since age 7 [19, 20]. Initial immunohistochemical studies revealed an asthma severity-related increase in the frequency of cyclin D1 positive cells as a marker of proliferation [21]. However, despite a significant increase in smooth muscle area, we could find no evidence of ongoing proliferation of mesenchymal cells within the muscle bundles at the time of biopsy [22]. In the present study therefore, we established cell cultures from a subset of these biopsies, and after characterising these cells as predominantly myofibroblasts, assessed the relationship between proliferation or cytokine production and asthma status and the sensitivity of proliferative responses to clinically used asthma medications.

## **MATERIALS AND METHODS**

### ***Subjects***

Subjects were either non-asthmatic or asthmatic volunteers with varying asthma severity recruited from the MESCA cohort (n=82, all 42 years of age at the time of biopsy) [19], or steroid-requiring asthmatics recruited from the Royal Melbourne Hospital (n=9, mean age 43 years, range 27-67) and were stratified using Global Initiative for Asthma (GINA) guidelines [23] (Table 1). Biopsies were obtained using fiberoptic bronchoscopy [24], with approval from Human Experimental Ethics Committees of the Royal Children's and Royal Melbourne Hospitals (maximum permissible 6 biopsies per subject). Biopsies from all subjects

were fixed for immunohistochemistry, and where available, were also used to establish cell cultures (see Table 1). Further details are outlined in the on-line depository.

### ***Immunohistochemistry of biopsies***

The number of cyclin D1 positive cells was counted on 5  $\mu\text{m}$  thick biopsy sections as a measure of cell proliferation, and expressed per  $\text{mm}^2$  of quantifiable biopsy area as described elsewhere [24] and in the on-line depository.

### ***Cell culture***

Cell cultures were established from endobronchial biopsies from a subset of the MESCA cohort (Table 1). After removal of the epithelium, the remaining tissue was teased apart, attached to a 6-well culture plate and maintained in Dulbecco's modified Eagle's medium (DMEM) with 20% v/v foetal calf serum (FCS), 2 mM L-glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin, 100 U/ml penicillin-G and 2  $\mu\text{g}/\text{ml}$  amphotericin. In addition to these cultures, separate airway myofibroblast and smooth muscle cultures were also established from macroscopically normal lung resection tissues as previously described [10]. For all biopsy- and resection-derived cultures, cells were used between passages 4-15.

### ***Flow cytometry, immunocytochemistry for phenotyping biopsy-derived cells***

In three experiments, properties of airway myofibroblasts and smooth muscle derived from within the same resection specimen were compared, and subsequently used to phenotype biopsy-derived cells. Morphometric properties were analysed by flow cytometry to determine differences in cell size (forward scatter, FACS Calibur II). Expression of mesenchymal cell phenotypic markers was assessed using antibodies to proline-4-hydroxylase,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and myosin

and vimentin as previously described [25]. Airway epithelial cell contamination of cultures was assessed by cytokeratin immunoreactivity. Immunocytochemistry for these phenotypic markers is described in an on-line supplement. On the basis of these studies, the biopsy-derived cells were characterised as predominantly myofibroblasts [26].

### ***Proliferation and cytokine assays***

For proliferation studies, biopsy-derived cells were cultured on 24-well or 6-well plates, at a density of  $1.5-2 \times 10^4$  cells per  $\text{cm}^2$  in DMEM with 10% FCS, and rendered quiescent by 24h serum deprivation. Cells were treated with thrombin (Thr, 0.3U/ml), FGF-2 (300pM) or FCS (5% v/v) with Monomed A (1% v/v) to provide progression factors essential for mitogen activity. In some experiments, fluticasone propionate (FP, 0.1 or 1nM) and/or salmeterol (10 or 30nM) were added 30min before thrombin. Briefly, cells were stimulated for 24h before being pulsed with [ $^3\text{H}$ ]-thymidine (1 $\mu\text{Ci/ml}$ ) for 4h, or stimulated for 48h before enumeration by haemocytometry. Cell viability (Trypan Blue exclusion) was greater than 98% in all experiments. IL-1 $\alpha$  (1ng/ml) was used as the stimulus for cytokine production. Supernatants were assayed for GM-CSF and IL-8 by ELISA (Endogen), and PGE $_2$  by radioimmunoassay [27]. Justification of concentrations used and details on proliferation and cytokine assays are provided in an online depository.

### ***Statistical analyses***

For non-parametric data (subject characteristics, total biopsy area, smooth muscle area and cyclin D1 data), comparisons were made using Kruskal-Wallis test followed by Dunn's *post-hoc* test for inter-group comparisons across groups with increasing asthma severity. For all other analyses, comparisons were made with all

asthma subjects grouped together, as relatively few cell cultures were obtained from moderate and severe asthmatics. Mitogen responses were normally distributed, so one-tailed t-tests and unpaired t-tests were performed to test for responses within groups and differences between non-asthmatic and asthmatic groups, respectively. The effects of FP and/or salmeterol and of asthma status on thrombin responses were analysed using a fixed-effects model, two-way ANOVA. Cytokine levels were log-transformed to allow unpaired t-tests to be performed on normally distributed data. Differences were considered to be statistically significant when  $p < 0.05$ , using Graph Pad Prism<sup>TM</sup> software version 4.0.



**TABLE 1** Demographic data for subjects from the MESCA cohort from whom biopsies were obtained for immunohistochemistry and cell culture

	Immunohistochemistry Studies				Culture Studies	
	non-asthmatic	mild asthma	moderate asthma	severe asthma	non-asthmatic	asthmatic <sup>a</sup> mild/mod/sev
<b>Subjects (n)</b>	34	38	5	14	22	25 20 / 4 / 1
<b>Gender (M/F)</b>	21 / 13	24 / 14	4 / 1	6 / 8	15 / 7	16 / 9
<b>FEV1 % predicted (range)</b>	104 (96, 115)	102 (93, 108)	67* (66, 71)	60** (57, 74)	101 (98, 110)	97 (85, 115)
<b>Atopic (%)</b>	65	85	100	71	68	77
<b>Current smokers (%)</b>	71	49 <sup>#</sup>	67	0 <sup>##</sup>	82	52
<b>Current treatment (%)</b>						
• $\beta_2$ -adrenoceptor agonists	0	74	100	100	0	76
• Inhaled steroids	0	37	80	100	0	40
• Oral steroids	0	0	0	64	0	0

Subjects were classified using GINA guidelines. COPD was excluded in all these subjects. Cyclin D1 immunohistochemistry data (n=91) is stratified by asthma status. <sup>a</sup>For the smaller cohort from whom myofibroblast cultures were also derived (n=47), all asthmatics are combined for analysis since few biopsies from moderate or severe asthmatics were available for culture. FEV1 % predicted data are median (interquartile range). \*p<0.01, \*\*p<0.001 (Kruskal-Wallis test followed by Dunn's post-hoc test). Smoking status is unknown for <sup>#</sup>3% mild and <sup>##</sup>14% severe asthmatics.



## RESULTS

### *Detection of proliferation in biopsies*

Although there was no difference in the total biopsy area across the non-asthmatic and stratified asthmatic groups, the proportion of smooth muscle in the biopsies increased with increasing asthma severity (Table 2,  $p < 0.05$ ).

**TABLE 2** Total area and % smooth muscle area of biopsies from non-asthmatic and asthmatic subjects used for cyclin D1 immunohistochemistry

	<b>Non-asthmatic</b>	<b>Mild asthma</b>	<b>Moderate asthma</b>	<b>Severe asthma</b>
<b>N</b>	34	38	5	14
<b>Total biopsy area (mm<sup>2</sup>)</b>	0.22 (0.17, 0.34)	0.28 (0.15, 0.36)	0.33 (0.16, 0.33)	0.26 (0.17, 0.50)
<b>Muscle area (% total area)</b>	0.7 (0, 5.7)	3.0 (0, 7.8)	1.1 (0, 20.5)	19.9** (3.8, 34.9)

Results are median (interquartile range). \*\* $p < 0.01$  as compared with non-asthmatic (Kruskal-Wallis test followed by Dunn's post hoc test)

Immunostaining for cyclin D1, a marker of proliferation, revealed that the number of positive cells increased with asthma severity, with a 3.5-fold higher median value for severe asthmatics compared with non-asthmatics (Figure 1). Biopsies with the highest levels of immunostaining were obtained from 4 female patients with asthma of varying severity (two mild and two severe). The characteristics of these patients were generally unremarkable although all were on current treatment with oral steroids.

Cyclin D1 immunoreactivity was not detected in the smooth muscle bundles, but was restricted to the epithelium and sub-epithelial connective tissue (Figure 1). Further studies were therefore performed to characterize the proliferative properties of mesenchymal cells derived from a subset of all biopsies.

### ***Comparison of resection and biopsy-derived mesenchymal cells in culture***

Preliminary comparisons were made between cultures of airway myofibroblasts and smooth muscle isolated from lung resection specimens to establish phenotypic characteristics that could be used to identify the biopsy-derived mesenchymal cells. Cells from the same subject could be distinguished by FACS analysis, which showed that myofibroblasts tended to be larger than airway smooth muscle cells obtained from the same subject, with forward scatter values of  $327 \pm 26$  and  $369 \pm 26$ , respectively (n=3, p=0.07, paired t-test).

Cells from endobronchial biopsies were larger and more elongated than resection-derived airway smooth muscle (Figure 2), but were similar both in appearance and in immunostaining for phenotypic markers as myofibroblasts from lung resections (data not shown). Detailed comparisons between these biopsy-derived myofibroblasts and resection-derived airway smooth muscle cells are shown in Figure 2. Both cell types showed similar positive immunostaining for the contractile protein  $\alpha$ -SMA. However, for the collagen cross-linking enzyme proline-4-hydroxylase, immunostaining was intense and punctate for biopsy-derived cells but diffuse and light for airway smooth muscle cells. In addition, vimentin immunostaining appeared more marked for cells from endobronchial biopsies than airway smooth muscle. Labeling with the epithelial marker, cytokeratin, was not detected in any cultures. On the basis of these findings, the biopsy-derived mesenchymal cells were identified as predominantly airway myofibroblasts [26].

### ***Biopsy-derived cell culture studies***

Subsequent experiments were performed on myofibroblast cultures obtained from additional biopsies from non-asthmatics and asthmatics. Cultures were established from 22 of the 34 non-asthmatics, 20 of the 38 mild asthmatics, 4 of the 5 moderate asthmatics but only 1 of the 14 severe asthmatics with matching immunohistochemical data (Table 1). These variable proportions reflect the availability of biopsy material dedicated for cell culture, which was most limited in the severe asthmatics, rather than success in establishing cultures (approximately 75% for both the non-asthmatic and asthmatic groups). These cell culture studies were therefore not statistically powered to examine the influence of asthma severity or other patient characteristics, such as smoking status. Therefore, results from all asthmatic cultures are presented and analysed together.

### ***Proliferation - [<sup>3</sup>H]-thymidine incorporation and cell enumeration***

Under matched culture conditions, the time taken to establish confluent cultures from biopsies from non-asthmatics ( $48 \pm 4$  days, n=22) and all asthmatics ( $45 \pm 3$  days, n=25,  $p>0.05$ ) was not significantly different between groups.

[<sup>3</sup>H]-Thymidine uptake was assessed in myofibroblasts from non-asthmatic and asthmatic subjects, plated at the same cell density. Although basal [<sup>3</sup>H]-thymidine incorporation over 4hr appeared higher at passage 5 compared with all later passages, it was similar in both groups and did not then change significantly with increasing passage number in either group (Figure 3).

The passage number (median, interquartile range) at which the effects of FGF-2 (300 pM), thrombin (0.3 U/ml) and FCS (5%) were assessed was not different for

non-asthmatics (5.8, 5.1-6.6, n=18) and asthmatics (6.6, 5.3-7.6, n=26,  $p>0.05$  *cf* non-asthmatic). Significant increases in [<sup>3</sup>H]-thymidine uptake were found in response to these stimuli (Table 3,  $p<0.05$  using one-sample t-test). However, there were no significant differences in [<sup>3</sup>H]-thymidine uptake between the asthmatic and non-asthmatic groups, irrespective of the mitogen used.

**Table 3** Effect of various stimuli on [<sup>3</sup>H]-thymidine uptake in myofibroblasts from non-asthmatic and asthmatic subjects

Mitogen/stimulus	Non-asthmatic		Asthmatic		P
<b>Thrombin (0.3U/ml)</b>	155 ± 9	(20)	182 ± 12	(25)	0.08
<b>FGF-2 (300 pM)</b>	147 ± 14	(17)	177 ± 20	(21)	0.27
<b>FCS (5% v/v)</b>	211 ± 22	(14)	183 ± 15	(18)	0.27

[<sup>3</sup>H]-thymidine uptake over a 4 h incubation period between 24-28 hours after addition of stimulus are presented as mean ± SEM (n) of % uptake (dpm) in unstimulated cells. (2023 ± 634 dpm, n=18 for non-asthmatic, 1721 ± 451 dpm, n=26 for asthmatic  $p>0.05$  *cf* non-asthmatic).

Direct cell counts were obtained to extend the findings with [<sup>3</sup>H]-thymidine. In these experiments, the passage number (median, interquartile range) at which proliferation was assessed using cell enumeration was not different for non-asthmatics (6.8, 5.0-8.1, n=16) and asthmatics (8.0, 5.5-8.3, n=21,  $p>0.05$  *cf* non-asthmatic). Basal proliferation was measured for cells plated at the same initial density, then enumerated after incubation for 48h in the presence of progression factors (insulin, transferrin, selenium), but in the absence of mitogen. Under these basal conditions, cell numbers increased, but were not different between non-asthmatics ( $2.0 \pm 0.2 \times$

$10^5$  cells/well, n=20) and asthmatics ( $1.7 \pm 0.2 \times 10^5$  cells/well, n=21,  $p > 0.05$  *cf* non-asthmatic).

Incubation with FGF-2, thrombin or FCS induced increases in cell number over 48h compared with unstimulated cells. The greatest proliferative responses to both FGF-2 and FCS were measured in myofibroblasts from the same two non-asthmatic patients. Within the asthmatic group, the greatest increase in cell number to all the mitogens tested was measured in a mild asthmatic patient, who was not receiving oral steroids. This patient did not have elevated cyclin D1 immunostaining detected in the biopsy specimen from which the cells were derived. The characteristics of these subjects were otherwise unremarkable. However, cells from asthmatics had a reduced proliferative response to both thrombin and FGF-2 (Figure 4,  $p < 0.05$  *cf* non-asthmatic). This was not associated with cytolytic cell death or apoptosis as Trypan Blue exclusion was  $>98\%$  with no morphological changes observed. FCS-stimulated proliferation was much more variable between subjects and there was no significant difference between patient groups.

#### ***Effect of fluticasone propionate and salmeterol on proliferation***

The effects of the currently used asthma treatments, fluticasone propionate (FP) and salmeterol, were assessed on proliferation. FP (1 nM) had no effect on basal proliferation in cells from either non-asthmatic ( $114 \pm 11\%$  unstimulated cell number, n=9), or asthmatic subjects ( $104 \pm 9\%$ , n=7) (each group  $p > 0.05$  *cf* untreated). The degree of inhibition of the thrombin response with either 0.1 nM or 10 nM FP was similar in each group (Figure 5). In contrast, salmeterol appeared to inhibit thrombin-induced proliferation with greater effectiveness in cells from asthmatics, being evident only at 30 nM. Assessment of synergy in the effects of FP and salmeterol on the thrombin-induced proliferation was precluded by the complete inhibitory effect of

FP at 0.1 nM. However, the complete inhibition by FP was also observed in the presence of salmeterol in both groups (Figure 5).

#### ***Release of GM-CSF, IL-8 and PGE<sub>2</sub>***

Basal GM-CSF supernatant levels (pg/ml, interquartile range) were 144 pg/ml (108-153) in the non-asthmatic group (n=12) and 150 pg/ml (94-177) for the asthmatic group (n=14, p>0.05 *cf* non-asthmatic). Cell supernatant levels of IL-8 and PGE<sub>2</sub> in the absence of inflammogen were below the level of detection in each group.

GM-CSF, IL-8 and PGE<sub>2</sub> levels increased in response to IL-1 $\alpha$ . However, in marked contrast to reduced proliferation in the cells from asthmatics, IL-1 $\alpha$ -dependent cytokine levels were increased in this group compared with non-asthmatics. IL-1 $\alpha$  induced a 10-fold increase in GM-CSF supernatant levels from asthmatic cells, and only a three-fold increase in cells from non-asthmatics (Figure 6). There was no significant difference in cell number in either non-asthmatic or asthmatic cells in the presence of IL-1 $\alpha$  (data not shown). Increases were also noted with IL-1 $\alpha$ -dependent IL-8 production from asthmatics compared with the non-asthmatic group (Figure 6). This difference was modest compared with the difference for GM-CSF. Cell supernatants from a single non-asthmatic subject had notably elevated GM-CSF, but not IL-8 levels. The same two asthmatic patients, one mild and one moderate, showed the highest levels of both GM-CSF and IL-8 (Figure 6). Asthma status did not have a detectable effect on IL-1 $\alpha$ -stimulated PGE<sub>2</sub> levels (data not shown).



## DISCUSSION

This study compared biopsies obtained from patients with a range of asthma severity and treatment with those derived from non-asthmatic subjects, with the primary aim being to explore differences in proliferation in the airway wall *in situ*. Additionally, the proliferative and inflammatory capacities of myofibroblasts derived from these biopsies were assessed *in vitro*. This is one of the largest biopsy studies undertaken in subjects recruited from a longitudinal asthma and control cohort since the majority of subjects (81 of 92 studied) were from the MESCA cohort, in which data has been collected from participants every 7 years since age 7 [19, 20].

In biopsies, clear evidence was obtained for an increase in the percentage area occupied by the smooth muscle bundle with increasing asthma severity. This finding agrees with those from several groups of investigators [28-30]. Cyclin D1 was used as a marker of *in situ* proliferation, since it is elevated in cultured airway smooth muscle cells upon mitogen stimulation and is essential for proliferation of cultured fibroblasts and muscle cells [31, 32]. In keeping with previous studies assessing proliferation with either Ki67 or proliferating cell nuclear antigen expression [29], cyclin D1 staining of muscle cells was rarely detected and we did not observe any differences with asthma status. However, cyclin D1 immunostaining provided clear evidence of hyperproliferation of cells in other airway wall compartments contiguous with muscle bundles in biopsies from asthmatic subjects. This finding directed further investigations of the function of biopsy-derived cells *in vitro*.

There are a number of possible explanations for the absence of detectable proliferation specifically within smooth muscle bundles *in situ*. A short-lived period of muscle proliferation early in the course of the disease may have established asthma severity, such that ongoing proliferation of these cells is present only at very low

frequencies in chronic asthma [33], or the smooth muscle cells may have reached senescence at the time of biopsy. However, the *in vitro* observations that cultured airway smooth muscle cells isolated from asthmatic biopsy patients are responsive to mitogen stimulation, and are hyperproliferative in culture [5] argue against these asthmatic cells being senescent. Alternative additional mechanisms have been proposed for the increased amount of smooth muscle in the asthmatic airway that may not be detected using cell proliferation markers [34]. These include a decreased rate of muscle apoptosis [35] or migration and differentiation of circulating fibrocytes [36]. Alternatively, mesenchymal cells may undergo proliferation in the subepithelial region outside mature muscle bundles in response to micro-environment changes within the airway wall [37]. This proposed mechanism is speculative and requires the cells to subsequently differentiate to adopt a smooth muscle phenotype, and to migrate in order to contribute to the increase in smooth muscle volume.

Given this context, and evidence of increased cellular cyclin D1 immunostaining with increasing asthma severity within the biopsies from the MESCA cohort, it was of particular interest also to examine cells derived from this collagen-rich sub-epithelial region. It was therefore necessary to phenotype these cells, and explore their responsiveness to mitogens and inflammatory mediators. Such studies have the potential to elucidate inherent differences in the characteristics with asthmatic status, or to provide evidence for changes in function that may occur selectively in the complex milieu of the asthmatic airway wall.

Comparison of morphological and immunohistochemical characteristics revealed that the biopsy-derived cells were similar to resection-derived myofibroblasts, but could be distinguished from airway smooth muscle cells. Although we cannot completely exclude the presence of smooth muscle cells in the

biopsy cultures or that the proportion of these cells was unchanged with asthma severity, the predominant phenotype of the cells characterised in these cultures was myofibroblast-like [26].

Assessment of proliferative markers showed no evidence of increased basal proliferation of myofibroblasts cultured from the asthmatic biopsies despite increased expression of cyclin D1 in sub-epithelial connective tissue areas of the histological sections. A possible explanation for this discrepancy is that detection of asthma-related changes in proliferation may be less apparent when cells are maintained in culture isolated from the asthmatic microenvironment of the intact airway wall (cell interactions, mitogenic and inflammatory milieu, drug treatments).

When cultured in the presence of mitogens, we obtained unequivocal evidence in myofibroblasts from asthmatics of a reduced proliferative response to thrombin and FGF-2. Critically, this is in contradistinction with mitogen-induced hyperproliferation described in airway smooth muscle cultured from asthmatics [5]. Phenotypic differences in mitogenic signalling between mesenchymal cell types, with different cell populations derived from different locations within the airway may contribute to varying proliferative responses under culture conditions. Elsewhere, characteristics of proximal and distal fibroblasts, including mitogenic responses, have previously been shown to vary significantly [38].

When current asthma treatments were assessed on the myofibroblast mitogenic response, the  $\beta_2$ -adrenoceptor agonist salmeterol did not inhibit proliferation in cells from non-asthmatics, in agreement with a recent report [39]. However, in the current study a small attenuation of the thrombin-mediated response was evident in asthmatic-derived myofibroblasts but only at the highest concentration examined. This apparent increased sensitivity of asthmatic myofibroblasts to

salmeterol may reflect their relatively reduced proliferative response to thrombin compared with myofibroblasts from non-asthmatics. In contrast, the glucocorticoid FP was found to exert similar anti-proliferative effects in myofibroblasts from both non-asthmatic and asthmatic patients. These findings using cell enumeration were in contrast to previous studies where dexamethasone was reported to increase [<sup>3</sup>H]-thymidine incorporation in primary asthmatic, but not normal bronchial fibroblasts [7], an observation confirmed in fibroblasts from mild, but not severe asthmatics [9].

Alterations in regulation of release of cytokines and mediators from myofibroblasts could either perpetuate or minimise the inflammatory process and remodelling associated with asthma. Although there was no difference in basal secretion, IL-1 $\alpha$ -stimulated levels of inflammatory cytokines were greater with asthma, consistent with previous reports in airway smooth muscle [17, 18]. The production of GM-CSF may be regulated by IL-1-induced PGE<sub>2</sub> synthesis and activation of EP2 receptors [40, 41]. Although we showed that PGE<sub>2</sub> production was maintained in the asthmatic myofibroblasts, downregulation of EP2 receptors may contribute to impairment of the negative feedback loop regulating GM-CSF release, as has been demonstrated in fibroblasts following fibrotic lung injury [42]. The cell supernatant concentrations of GM-CSF measured were up to two orders of magnitude higher than measured in BAL following segmental lung allergen challenge [43] so that even allowing for dilution in the BAL, this increased GM-CSF production could be functionally significant. Future studies could evaluate receptor and cytokine expression and downstream signalling pathways for differential regulation of cytokine production by these biopsy-derived mesenchymal cells in asthma.

In this community-based MESCA cohort, the recruitment process precluded controlling for potential confounding factors such as atopy, smoking status or asthma

therapy in either the biopsy or the cell culture study. Furthermore, availability of biopsies dedicated for the *in vitro* study varied according to subject numbers within each group and the suitability of these biopsy samples for cell culture. *Post-hoc* stratification within the *in vitro* study would have led to increasingly smaller subsets of data and a reduction in the statistical power.

The effects of prior drug treatment on cell function must also be considered, since the majority of asthmatic subjects were using both  $\beta_2$ -adrenoceptor agonists and inhaled glucocorticoids. However, a direct effect persisting after cells have been isolated and maintained in culture over many weeks is unlikely, although it is possible that a selection pressure exerted by anti-asthma drugs in the pro-mitogenic environment of the asthmatic airway may persist in cultured populations.

Another potential influence on the outcomes of the study is smoking status. Acute exposure of fibroblasts to cigarette smoke extract has been shown to stimulate GM-CSF and IL-8 production [44, 45], and smoking may alter the therapeutic response to glucocorticoids in asthma [46]. The possibility that a distinct asthma phenotype is associated with smoking could not be tested in the current study. Despite the size of the cohort, numbers within each subject group were insufficient to stratify prospectively by smoking status. Nevertheless, we observed striking differences in proliferative indices and in the production of specific cytokines between the non-asthmatic and asthmatic subject groups. Given that the proportion of smokers did not vary between groups in either biopsy or culture studies, these current findings are of particular interest given the wider literature where smokers have been excluded.

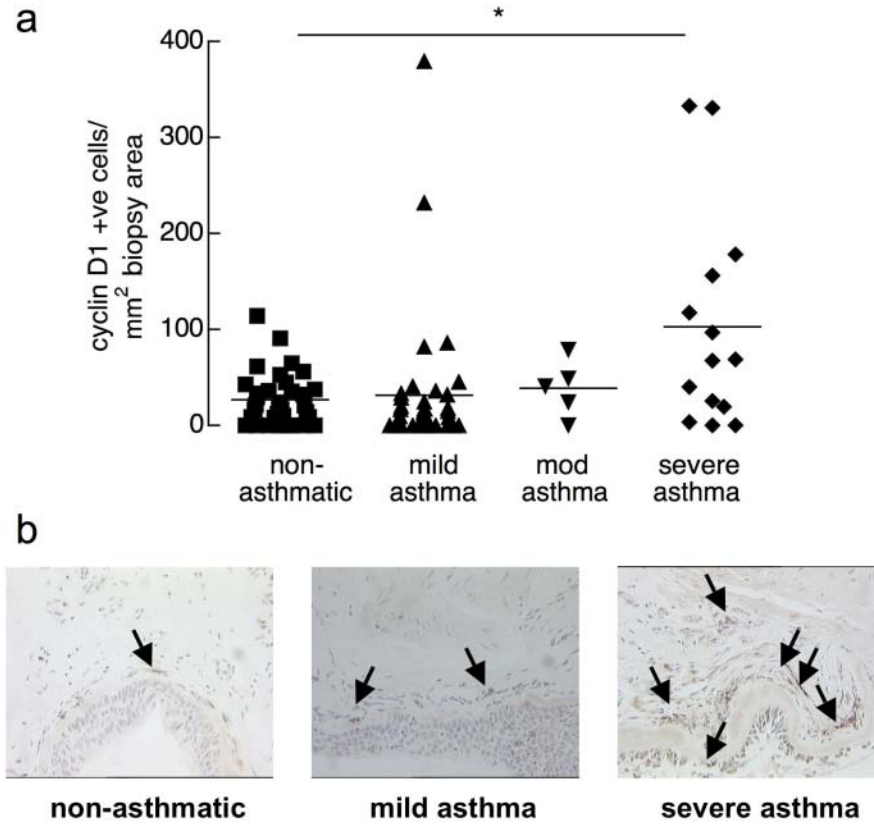
In summary, immunostaining for cyclin D1 in airway biopsy specimens from asthmatics provided evidence of persistent increased proliferation in the epithelium and sub-epithelial connective tissue. Proliferation was not apparent within airway

smooth muscle bundles, despite established muscle accumulation in asthma. Biopsy-derived cells were characterized as myofibroblasts, with cells from asthmatics showing a hypoproliferative response to mitogens known to be elevated in the asthmatic airway, and sensitivity to inhibitory effects of glucocorticoids and  $\beta$ -agonists. Increased cytokine production, particularly of GM-CSF, by cells derived from asthmatics supports their potential contribution to airway inflammation. This study offers insights into the contribution of myofibroblasts to inflammation, airway remodelling and AHR associated with asthma.

## Figure 1

- a. Quantitation of cyclin D1 positive cell numbers in biopsies from subjects with increasing asthma severity. Individual and median values from non-asthmatic (n=34), mild asthmatic (n=38), moderate asthmatic (n=5) and severe asthmatic (n=14) subjects are shown. \*p<0.05, Kruskal-Wallis test.
- b. Immunohistochemistry for cyclin D1 in endobronchial biopsies from a non-asthmatic (left), a mild asthmatic (centre) and a severe asthmatic (right) subject. Arrows indicate cyclin D1 positive cells. Magnification x 40.

Figure 1



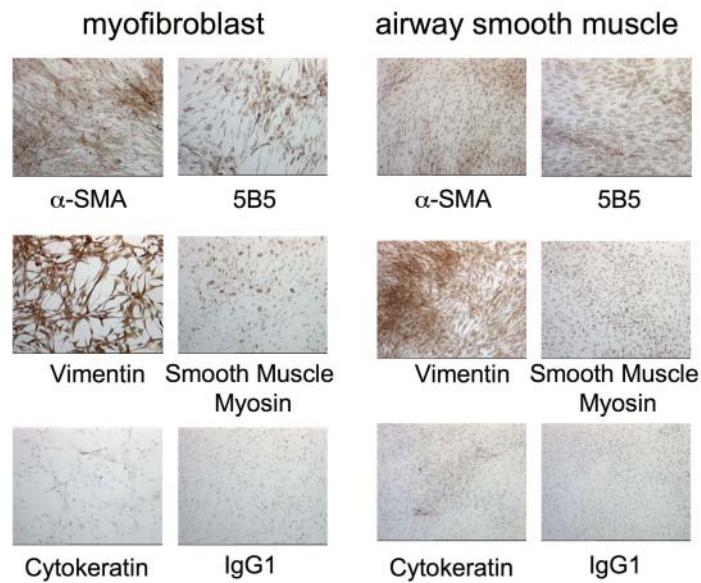
c.

**Figure 2** Comparative immunohistochemistry for human airway myofibroblast and smooth muscle cultures derived from an endobronchial biopsy and a lung resection specimen, respectively. Immunostaining was performed for proline-4-hydroxylase



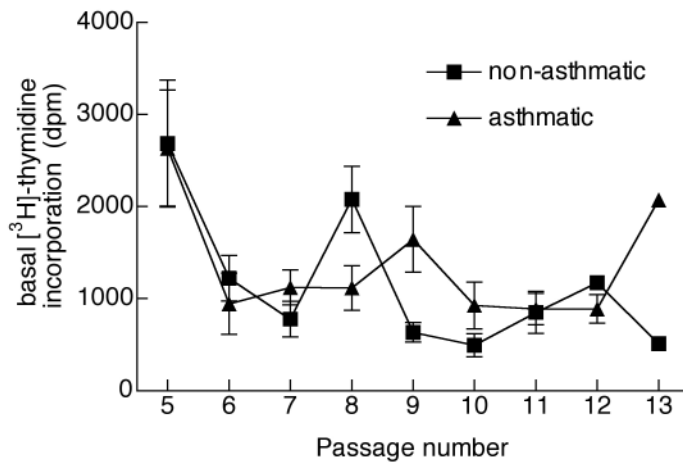
(clone 5B5),  $\alpha$ -SMA, vimentin, smooth muscle myosin and pan cytokeratin. The pattern shown is representative of n=3 contrasts.

Figure 2



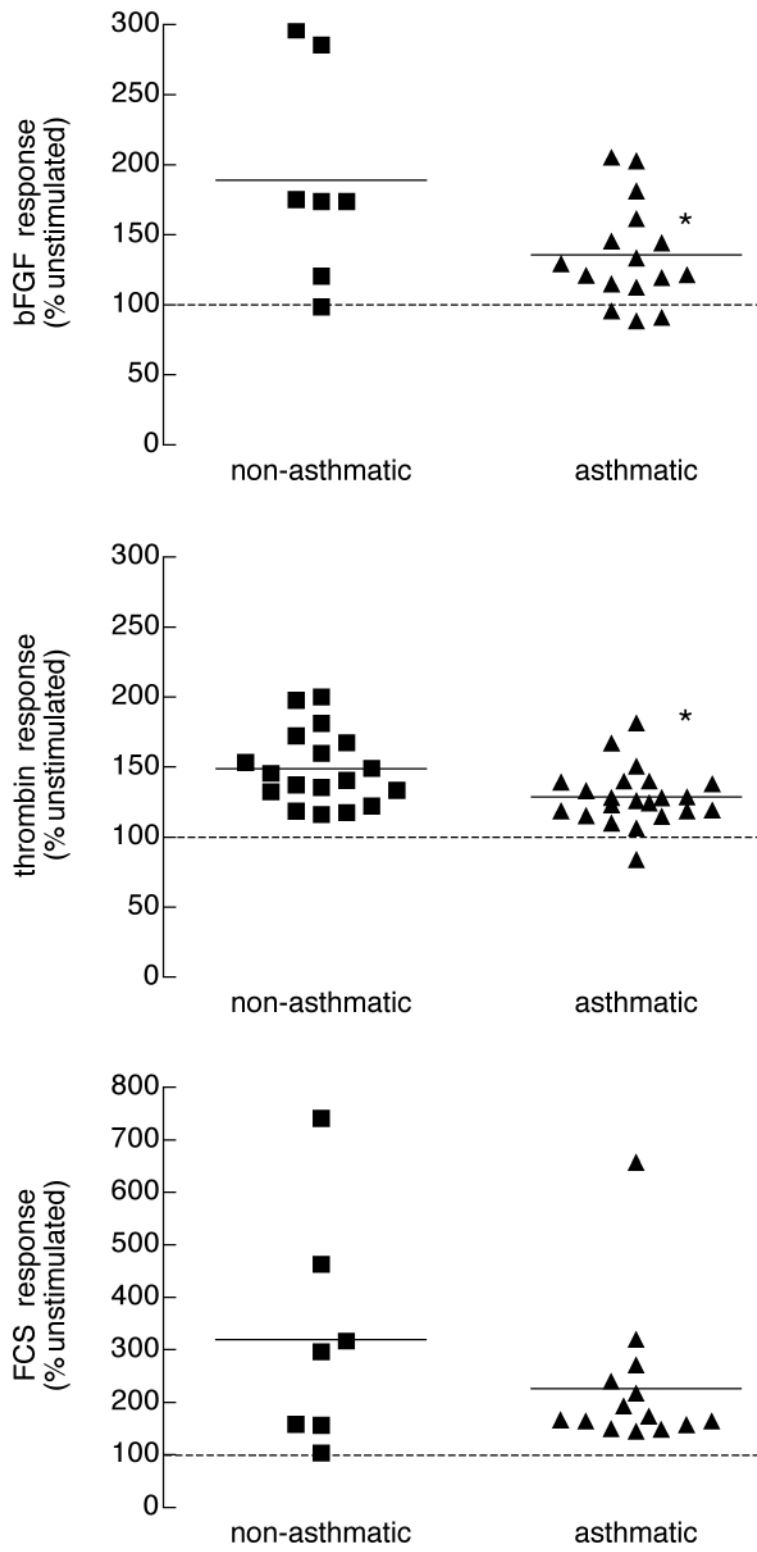
**Figure 3** Effect of passage number on basal [ $^3$ H]-thymidine uptake in myofibroblasts from non-asthmatic and asthmatic subjects. Results are presented as the mean  $\pm$  SEM of the [ $^3$ H]-thymidine uptake over a 4 h incubation period for non-asthmatic (up to n=19 cultures at each passage) and asthmatic (up to n=25)  $p > 0.05$  cf non-asthmatic) cells, plated at the same cell density.

Figure 3



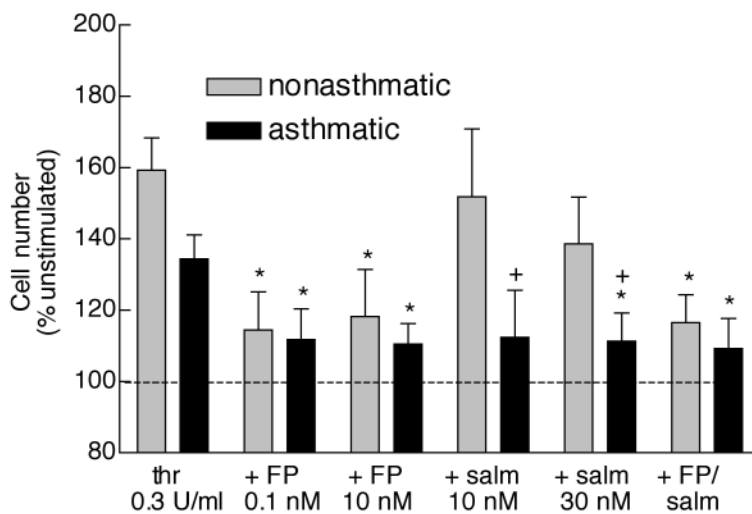
**Figure 4** Effect of FGF-2 (300pM), thrombin (0.3 U/ml) and FCS (5%) on proliferation of myofibroblasts from non-asthmatic and asthmatic subjects. Confluent cells were serum-deprived for 24 hours, then stimulated with mitogen for 48 hrs in the presence of essential progression factors. Cell numbers were determined by haemocytometry. Individual data points represent the average response from 1-6 replicates within a given culture, expressed as a % of the unstimulated cell number. The mean response is also shown for FGF-2 (n=7 non-asthmatic, 17 asthmatic), thrombin (n=18, 21) and FCS (n=7, 14). \*p<0.05 as compared with non-asthmatic, unpaired t-test

Figure 4



**Figure 5** Effect of fluticasone propionate (FP) and salmeterol (salm) on thrombin (0.3 U/ml)-induced proliferation of myofibroblasts from non-asthmatic and asthmatic subjects. FP (0. 1-1nM) or salm (10-30nM) or both (FP 0.1 nM, salm 30 nM) were added to serum-deprived cells 30 min prior to thrombin addition. Cell numbers were determined by haemocytometry after 48 hrs. Data presented are the mean  $\pm$  SEMsem of the responses expressed as percentages of the unstimulated cell number (in the absence of mitogen). 1-4 replicates were performed for each culture (n=9 non-asthmatic, 7 asthmatic). \*p<0.05 as compared with thrombin alone within group, +p<0.05 as compared with non-asthmatic under same conditions.

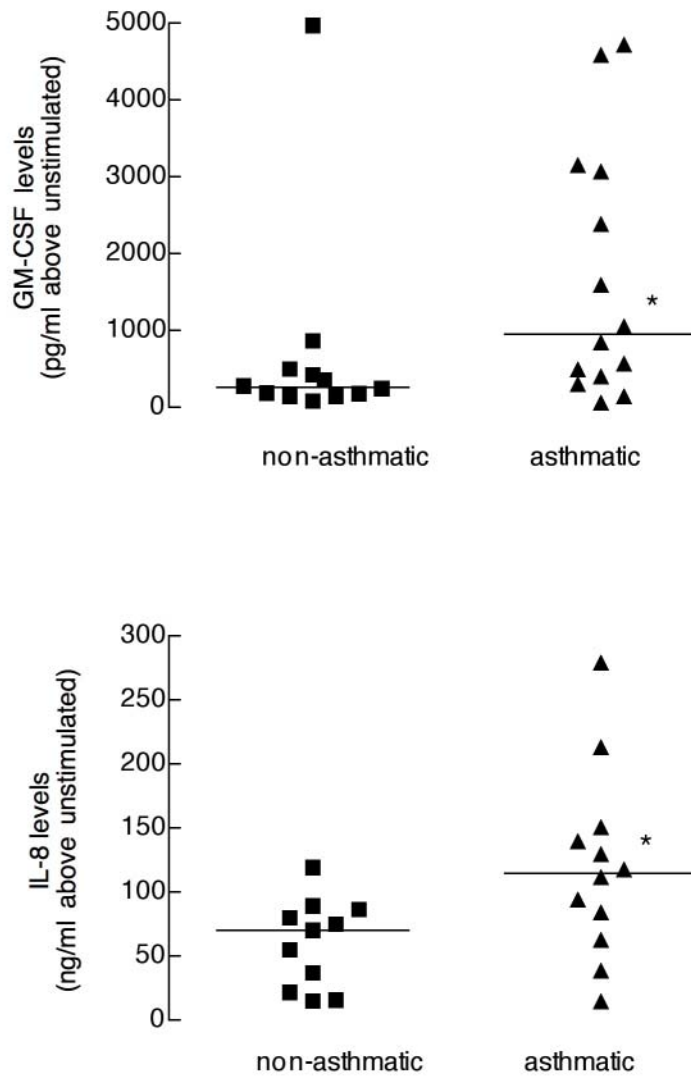
**Figure 5:**



**Figure 6** Effect of IL-1 $\alpha$  (1 ng/ml) on supernatant levels of inflammatory mediators for myofibroblasts from non-asthmatic and asthmatic subjects. Confluent cells were serum-deprived for 24 hours, then incubated with IL-1 $\alpha$  for 48 hrs. Supernatant levels of GM-CSF and IL-8 were measured by ELISA. Individual data points represent the average increase in response to IL-1 $\alpha$  above unstimulated levels from 1-11 replicates within a given culture. The median response for each group is also shown for GM-

CSF (n=12 non-asthmatic, 14 asthmatic) and IL-8 (n=11, 12). \*p<0.05 as compared with non-asthmatic, unpaired t-test on log transformed data.

Figure 6:



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