

Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)- β 1, TGF- β 2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on α -smooth muscle actin and collagen III synthesis by primary human lung fibroblasts

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Summary

Rationale Asthmatic airway remodelling is characterized by myofibroblast hyperplasia and subbasement membrane collagen deposition. We hypothesized that cytokines and growth factors implicated in asthmatic airway remodelling are increased in bronchoalveolar lavage (BAL) fluid of asthmatics after segmental allergen challenge (SAC), and that these growth factors and cytokines increase α -smooth muscle actin (α -SMA) and collagen III synthesis by human lung fibroblasts (HLFs).

Methods Transforming growth factor (TGF)- β 1, TGF- β 2, IL-4 and IL-13 levels were measured in BAL fluid from 10 asthmatics and 9 non-asthmatic controls at baseline and then 1 day, 1 week and 2 weeks after SAC. Confluent cultures of HLFs were stimulated by exogenous addition of TGF- β 1, TGF- β 2, IL-4 or IL-13 (concentration range 0.01–10 ng/mL) over 48 h. Collagen III was measured in culture supernates and α -SMA in cell lysates by Western blot.

Results At baseline, there was no difference in BAL fluid concentrations of TGF- β 1, IL-4 and IL-13 between asthmatics and controls; however, non-asthmatics had higher concentrations of total TGF- β 2. In asthmatics, BAL fluid concentrations of all four factors increased significantly 1 day after SAC. TGF- β 1, TGF- β 2 and IL-13 concentrations returned to baseline by 1 week after SAC, but BAL fluid IL-4 concentration remained elevated for at least 2 weeks. TGF- β 1, TGF- β 2 and IL-4 significantly increased α -SMA in fibroblasts, but only IL-4 caused corresponding increases in collagen III synthesis. IL-13 had no direct effects on collagen III synthesis and α -SMA expression.

Conclusions Because IL-4 caused a dose-dependent increase in α -SMA and collagen III synthesis, it may be an important cytokine mediating asthmatic airway remodelling. TGF- β 1 and TGF- β 2 may also play a role in airway remodelling by stimulating phenotypic change of fibroblasts to myofibroblasts. Additionally, collagen III synthesis appears to be independent of myofibroblast phenotype and is apparently regulated by different growth factors and cytokines.

Keywords airway remodelling, bronchial asthma, bronchoalveolar lavage, collagen, fibroblasts, IL-4, IL-13, segmental antigen challenge, TGF- β , α -smooth muscle actin

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Introduction

It is now widely appreciated that the characteristic lesion in asthma consists of infiltrating T lymphocytes and eosinophils, with associated epithelial damage and airway remodelling. Structural remodelling of the asthmatic airway includes subepithelial fibrosis, myofibroblast hyperplasia, increased airway smooth muscle mass, goblet cell hyperplasia and

microvascular changes [1]. Subepithelial fibrosis is a unique feature of asthma and is a consequence of collagen I, III and V deposition in the subbasement membrane lamina reticularis. Myofibroblasts, cells with features intermediate between that of fibroblasts and smooth muscle cells, are thought to be important in the deposition of this collagen, and their numbers correlate with the thickness of the lamina reticularis [2, 3].

Asthma is characterized by differentiation of T lymphocytes into T helper (Th2) phenotype. IL-4 and IL-13 are important Th2 cytokines that play an important role in allergic inflammation, such as T cell differentiation towards a Th2 phenotype, isotype switching of B cells to IgE synthesis, and induction of adhesion molecule expression [4, 5]. These

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cytokines may also be important in airway remodelling. Transgenic animals selectively overexpressing IL-13 in the lungs develop airway inflammation, subepithelial airway fibrosis, mucus cell metaplasia and marked bronchial hyper-responsiveness. It has been shown that human lung fibroblasts (HLFs) express functional receptors of IL-4 and IL-13, and stimulated by these cytokines, increase the expression of VCAM-1 adhesion molecules, and the secretion of other cytokines/chemokines [6].

Transforming growth factor beta (TGF- β) has also been implicated as an important modulator of airway remodelling in asthma [7, 8]. In mammals, this growth factor is composed of three isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. These isoforms are expressed in a tissue-specific and developmentally regulated manner [9, 10] and despite structural similarity and use of the same cell receptors, there are disparities of expression and function [11–13]. *In vitro* studies have shown that mechanical or chemical damage to the epithelium leads to increased release of TGF- β 1 [14] and TGF- β 2 [15]. Redington et al. reported that TGF- β 1 levels increase in bronchoalveolar lavage (BAL) fluid of asthmatics after segmental allergen challenge (SAC) [16]. Our previous observations confirmed that epithelial cells from asthmatics release higher levels of TGF- β 1 than epithelial cells from non-asthmatic subjects [17]. TGF- β has been reported to induce collagen synthesis in fibroblasts obtained from various tissues [18–22].

In this investigation, we measured TGF- β 1, TGF- β 2, IL-4 and IL-13 in BAL fluid after SAC in asthmatics and non-asthmatic controls. We hypothesized that allergen challenge, which increases TGF- β 1 in the BAL fluid also increases TGF- β 2, IL-4 and IL-13 and that these growth factors and cytokines increase α -smooth muscle actin (α -SMA) in HLFs, and correspondingly, collagen III synthesis.

Methods

Subject characterization and bronchoscopy procedures

The Institutional Review Board of the Jefferson Medical College approved the protocol used in this study and each subject gave informed, written consent. Ten ragweed allergic asthmatics and nine non-allergic non-asthmatic controls participated in the study. Asthmatics met the National Institute of Health/National Heart, Lung, and Blood Institute expert panel criteria for the diagnosis of asthma [23]. All subjects were non-smokers and were not taking any chronic medications. Asthmatics had mild intermittent asthma and they were using inhaled β_2 -agonists on an as needed basis. Subjects were further characterized, as previously described [24, 25], for airway reactivity to methacholine and skin reactivity to ragweed allergen [short ragweed antigen, 0.001–10 U/mL in normal saline solution with 0.03% human serum albumin (1 ragweed antigen unit (RWAU) = 0.005 μ g ragweed antigen E (*Amb a I*)), Greer Laboratories, Lenoir, NC]. Subject demographics and physiologic data are listed in Table 1.

The bronchoscopy protocol (Table 2), described previously in detail [24, 25], included four bronchoscopies, on day 0 (before SAC), and then 1 day, 1 week and 2 weeks after SAC, with BAL each day. SAC was performed with 5 mL of ragweed solution at a concentration 100-fold higher than that

Table 1. Subject demographic and physiologic data

	Asthmatics	Normal controls
Number	10	9
Gender (male/female)	7/3	5/4
Age (years)	28 $\pm$ 1.5	28 $\pm$ 2.0
FEV ₁ (L)	3.6 $\pm$ 0.18	3.8 $\pm$ 0.28
FEV ₁ (% predicted)	102 $\pm$ 6.5	102 $\pm$ 3.0
FEV ₁ /FVC (%)	80.5 $\pm$ 1.7	83.0 $\pm$ 2.5
PC ₂₀ methacholine (geometric mean; mg/mL)	2.54	> 32

All values are mean $\pm$ (SEM) except for PC₂₀ methacholine that is expressed as geometric mean. FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PC₂₀, provocative concentration causing 20% fall in FEV₁.

Table 2. Bronchoscopy protocol

	Lingula	Right middle lobe	Left lower lobe
Day 1	BAL (control)	Antigen challenge	
Day 2		BAL (1 day after challenge)	Antigen challenge
Day 9			BAL (1 week after challenge)
Day 16		BAL (2 weeks after challenge)	

BAL, bronchoalveolar lavage.

needed to cause positive intradermal skin test in asthmatics. Non-asthmatic controls were given 5 mL of 10 RWAU/mL ragweed antigen. To avoid confounding from environmental ragweed, these allergen challenges were performed outside the usual ragweed season.

ELISA assay for TGF- β 1, TGF- β 2, IL-4 and IL-13

The concentrations of TGF- β 1, TGF- β 2, IL-4 (R & D Systems, Minneapolis, MN, USA) and IL-13 (Immunotech Assay, Beckman Coulter, Fullerton, CA, USA) were determined using commercial ELISAs on $\times$ 14 concentrated BAL fluid (Centriprep YM-10, Millipore, Bedford, MA, USA; 10 mL of each BAL fluid was concentrated to 0.7 mL according to the manufacturer's recommended procedure). ELISAs were performed according to the manufacturer's instructions. The minimal detectable concentrations were 2 pg/mL for TGF- β 1, 7 pg/mL for TGF- β 2 and 5 pg/mL for IL-4 and 1.5 pg/mL for IL-13. Before analysing, latent TGF- β 1 and TGF- β 2 in concentrated BAL fluid were converted to their active forms using 1 N HCl, thus the reported levels are totals of latent and active forms.

Fibroblast cell culture

Five HLF cell lines were established using macroscopically normal areas of lungs surgically resected for various diagnostic or therapeutic purposes. Passage three to five cells were cultured in Eagle's minimal essential medium supplemented with 5–10% fetal calf serum, 2 mM L-glutamine and antibiotics at 37 °C in 5% CO₂. Cultures were grown to confluency and

then serum starved in Ham's F12 medium containing ascorbic acid (40 µg/mL), 2 mM L-glutamine and antibiotics. After 24 h, the cells were stimulated by exogenous addition of TGF-β1, TGF-β2, IL-4 or IL-13 (R & D systems, Minneapolis, MN, USA) (0.01 ng/mL to 10 ng/mL for 48 h). Because the latency-associated peptide of TGF-β1 may modify its activity, the growth factor was purchased as latent TGF-β1, activated by acidification and subsequently neutralized prior to use.

Western blot analysis for collagen III and collagen I

Proteins in fibroblast cell culture supernatants were separated on 6% polyacrylamide gels either without further processing or after complete pepsin digestion as previously described [17]. The proteins were transferred onto nitrocellulose membranes and, probed with monoclonal anti-human collagen III (1:3000, Accurate Chemical & Scientific Co., Westbury, NY, USA) or polyclonal anti-human pro-collagen III (1:5000, Chemicon) antibodies. Protein bands were detected using enhanced chemiluminescence (ECL) after incubation of membranes with biotin-labelled secondary antibody and streptavidin-horse radish peroxidase complex. Blots were stripped and reprobed with monoclonal anti-human collagen I (Accurate Chemical & Scientific Co.) antibodies and detected with ECL as described.

Collagen band densities were quantified by densitometry (1D Image Analysis Software, v 3.0.1, Eastman Kodak, Rochester, NY, USA). Band densities were normalized to milligrams of protein in fibroblast lysates measured by BCA microassay (Pierce Chemical Co., Rockford, IL, USA) and expressed as fold increase above basal (unstimulated) levels.

Western blot analysis for α-SMA

The fibroblast cell lysates [RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 1% SDS with 0.1 mg/mL PMSF, aprotinin and 1 mM sodium orthovanadate as inhibitors)] were separated on 10% polyacrylamide gels, transferred onto nitrocellulose membranes and probed with primary antibody to α-SMA (1:1000, Cedarlane Lab., Hornby, Ontario, Canada), biotin-labelled secondary antibody, streptavidin-horseradish peroxidase complex, and developed as above. α-SMA band densities were quantified as described for collagen.

Statistical analysis

Data were analysed using Microsoft Excel'97 and SigmaStat (version 2.03, SPSS Scientific Software) for Windows. Data were tested for normal distribution and equal variance by SigmaStat. Some data, when indicated, were transformed to meet the criteria for normal distribution. Normally distributed data were tested by one- or two-way repeated measures analysis of variance, or by paired or unpaired *t*-test. Dose response linearity was tested using regression analysis.

Results

Concentration of TGF-β1, TGF-β2, IL-4 and IL-13 in BAL fluid

Figure 1 shows the concentrations of TGF-β1, TGF-β2, IL-4 and IL-13 in the × 14 concentrated BAL fluid of asthmatics

and non-asthmatic controls at baseline (0 days), 24 h (1 day), 1 week and 2 weeks after SAC. Comparing all the days together, a significant difference was observed between asthmatics and non-asthmatics in concentrations of TGF-β1 (higher in asthmatics, $P < 0.001$) and TGF-β2 (higher in non-asthmatic controls, $P < 0.05$). Before SAC (0 day), there was no difference in TGF-β1 concentration between the two groups. In asthmatics, TGF-β1 concentration significantly increased 24 h after SAC ($P < 0.001$) and returned towards baseline by 1 week after SAC. In non-asthmatics, there was no significant change in TGF-β1 concentration before and after SAC. In contrast, although the concentration of TGF-β2 did increase in asthmatics 1 day after SAC (vs. day 0, $P < 0.05$), non-asthmatics had higher TGF-β2 concentrations at baseline, 1 week and 2 weeks after SAC ($P < 0.05$) than asthmatics.

There was no significant difference in BAL fluid concentrations of IL-4 and IL-13 between asthmatic and non-asthmatic subjects at baseline. Both cytokines increased significantly in BAL fluid 24 h after SAC ($P < 0.01$) in asthmatics. IL-13 returned to the baseline value at 1 week after SAC, but IL-4 concentration remained elevated at 1 and 2 week time-points compared with baseline ($P < 0.05$). In non-asthmatics, there was no change in IL-4 and IL-13 concentrations after SAC compared with baseline concentration.

α-SMA in HLFs

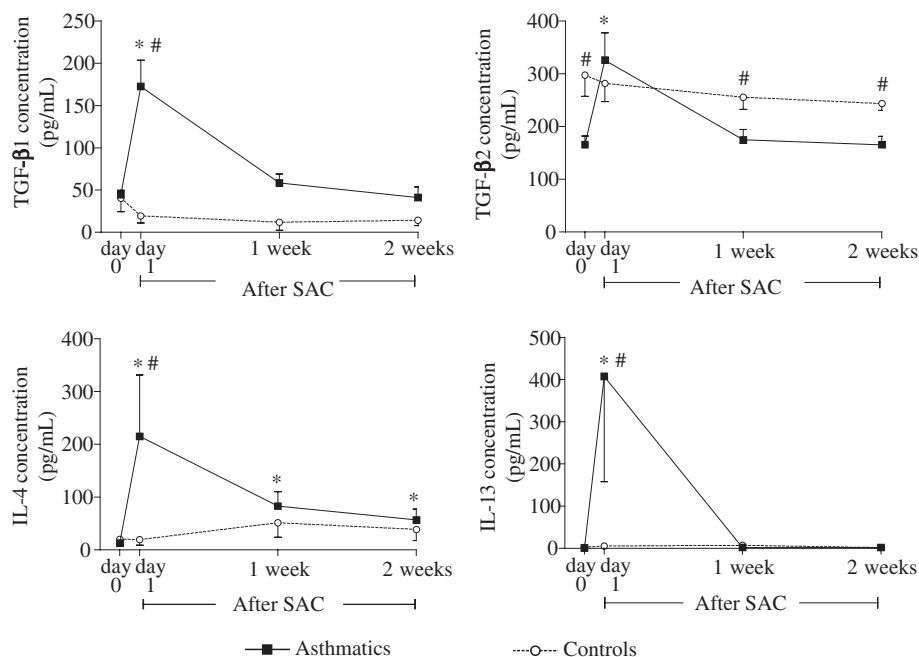
α-SMA expression increased in a dose-dependent manner when confluent cultures of HLFs were stimulated by exogenous addition of TGF-β1 ($r = 0.768$; $P < 0.001$) or IL-4 ($r = 0.620$; $P < 0.01$). TGF-β2 also induced a significant increase in α-SMA expression at 1 ng/mL ($P < 0.01$) and 10 ng/mL ($P < 0.001$) concentrations. There was no increase in α-SMA expression when HLFs were stimulated with IL-13 (Fig. 2).

Collagen synthesis by HLFs

Increasing concentrations of IL-4 increased collagen III synthesis in a concentration-dependent manner ($r = 0.54$; $P = 0.03$). Compared with control, TGF-β2 increased collagen III synthesis at 0.1 and 1 ng/mL concentration ($P < 0.05$ for both) but not at 10 ng/mL. In fact, there was a trend towards decreased collagen III synthesis when fibroblasts were stimulated with 10 ng/mL of TGF-β2 ($P = 0.053$ when compared with collagen synthesis with 0.1 and 1 ng/mL of TGF-β2). There was no significant change in collagen III synthesis by HLF stimulated with increasing concentrations of TGF-β1 and IL-13. However, there was a slight increase in collagen III synthesis (one- to twofold over control) at 0.1 ng/mL of TGF-β1 ($P = 0.07$) (Fig. 3). There was no significant increase in collagen I synthesis (Fig. 4).

Co-treatment with TGF-β1 and basic fibroblast growth factor (bFGF)

The growth factor bFGF is released from airway epithelial cells prior to the release of other growth factors such as TGF-β [15]. To determine whether TGF-β1 effects on fibroblasts are modified by prior exposure to this growth factor, co-treatment with bFGF (10 ng/mL) and TGF-β1 (dose range



* $P < 0.05$ (compared to day 0); # $P < 0.05$ (comparison between asthmatics and controls)

Fig. 1. Mean concentration ($\pm$ SEM) of TGF- β 1, TGF- β 2, IL-4 and IL-13 in $\times 14$ concentrated bronchoalveolar lavage (BAL) fluid of asthmatics ($n = 10$) and non-asthmatics ($n = 9$) at baseline (day 0), 24 h (day 1), 1 week and 2 weeks after segmental allergen challenge (SAC). Before SAC (day 0), there was no difference in $\times 14$ BAL fluid concentrations of TGF- β 1, IL-4 and IL-13 between asthmatics and non-asthmatic controls. However, TGF- β 2 was higher in non-asthmatic subjects at baseline. In asthmatics, BAL fluid concentrations of all four factors increased significantly 1 day after SAC and returned to baseline by 1 week, except IL-4, which remained elevated at one and 2 week time-points (compared with day 0, $P < 0.05$). Data within each cytokine or growth factor (note that levels differ) were separately analysed using two-way repeated measures ANOVA. * $P < 0.05$ (compared with day 0); # $P < 0.05$ (comparison between asthmatics and non-asthmatic controls).

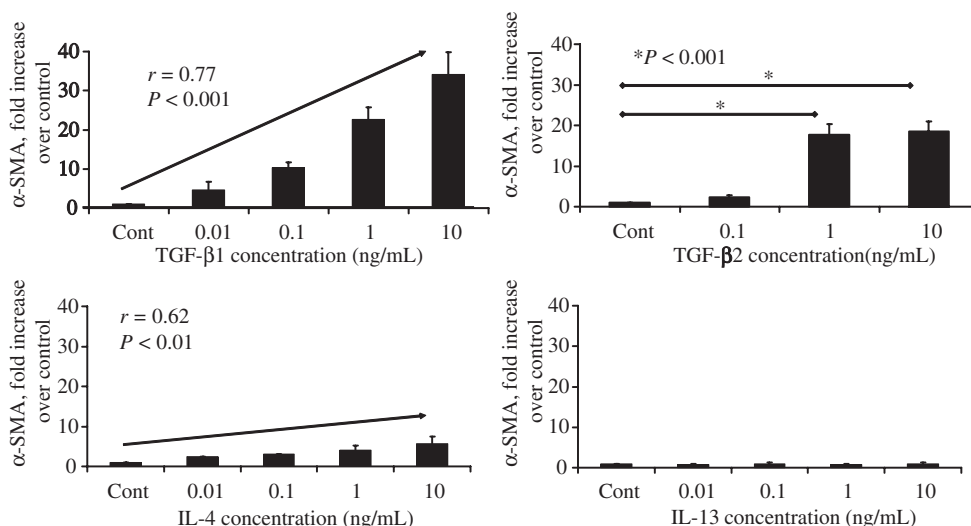


Fig. 2. Stimulation of α -smooth muscle actin (SMA) expression (mean $\pm$ SEM of α -SMA as a fold increase over control) in fibroblasts treated with TGF- β 1, TGF- β 2, IL-4 or IL-13 (concentration range 0.01–10 ng/mL). Averaged results from the five fibroblast lines studied. α -SMA expression increased linearly when fibroblasts were stimulated with increasing concentrations of TGF- β 1 ($r = 0.768$; $P < 0.001$) or IL-4 ($r = 0.620$; $P < 0.01$). Compared with control, TGF- β 2 increased α -SMA expression at 1 and 10 ng/mL concentrations ($P < 0.001$, one-way repeated measures ANOVA).

0.01–10 ng/mL) on α -SMA and collagen III synthesis was examined in three experimental designs. Fibroblasts were either treated with both growth factors simultaneously, or one growth factor was added 24 h before the other. Forty-eight hours after initial stimulation, the supernates and cell lysates were analysed for collagen III and α -SMA, respec-

tively, and compared with stimulation with TGF- β 1 alone (Fig. 5). Fibroblasts expressed less α -SMA at all concentrations of TGF- β 1 when treated with both growth factors simultaneously ($P = 0.03$). There was a trend towards decreased α -SMA expression when cells were treated first with bFGF ($P = 0.07$, two-way repeated measures ANOVA).

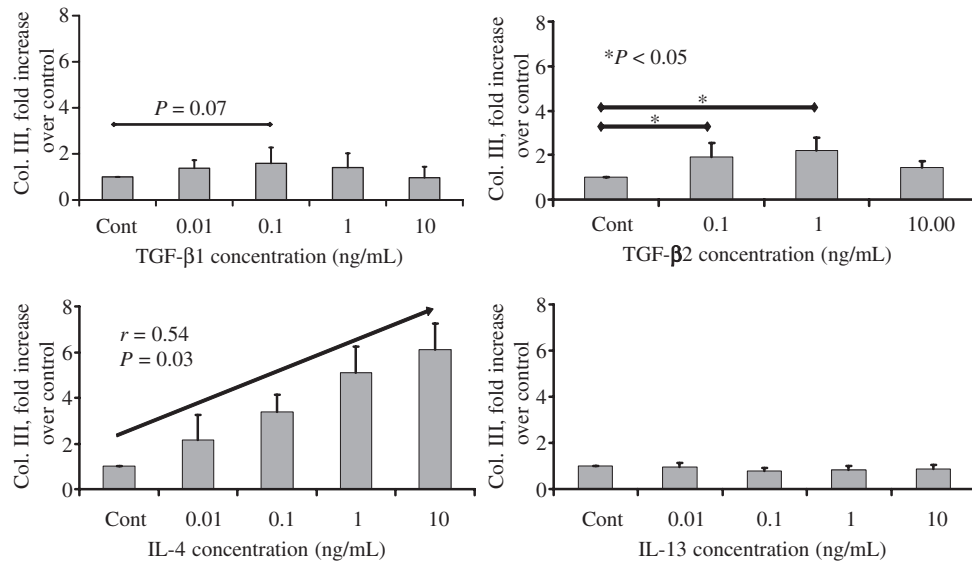


Fig. 3. Mean ($\pm$ SEM) of collagen III band density, expressed as a fold increase over control, for confluent fibroblast cultures stimulated with TGF- β 1, TGF- β 2, IL-4 and IL-13 (0.01–10 ng/mL). Averaged results from the five fibroblast lines studied. IL-4 increased collagen III synthesis in a dose-dependent manner ($r = 0.54$, $P = 0.03$). TGF- β 2 increased collagen III synthesis at 0.1 and 1 ng/mL concentrations ($P < 0.05$ for both, one-way repeated measures ANOVA). At 10 ng/mL concentration of TGF- β 2, there was a trend towards decreased collagen III synthesis compared with that at 0.1 and 1 ng/mL ($P = 0.053$). At all the concentrations of TGF- β 1 and IL-13, there was no statistically significant difference in collagen III synthesis compared with control ($P > 0.05$, one-way repeated measures ANOVA). However, there was a trend towards increased collagen III synthesis with 0.1 ng/mL concentration of TGF- β 1 ($P = 0.07$).

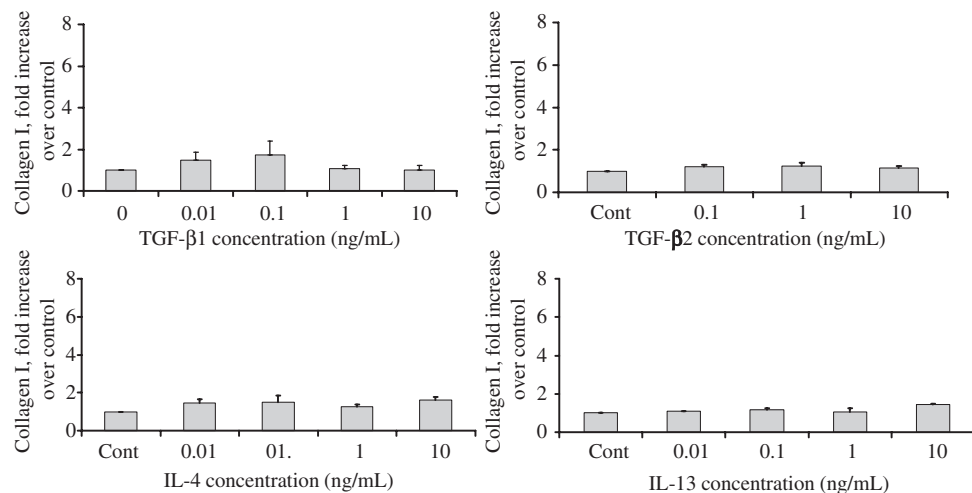


Fig. 4. Mean ($\pm$ SEM) of collagen I band density, expressed as a fold increase over control, for confluent fibroblast cultures stimulated with TGF- β 1, TGF- β 2, IL-4 and IL-13 (0.01–10 ng/mL). Averaged results from the five fibroblast lines studied. At all the concentrations of TGF- β 1, TGF- β 2, IL-4 and IL-13, although there was some increase in collagen I synthesis, none achieved statistically significant differences compared with control.

Analysis of each TGF- β 1 concentration separately revealed that bFGF significantly decreased α -SMA expression at 1.0 and 10 ng/mL of TGF- β 1 when added before TGF- β 1. However, if fibroblasts were treated first with TGF- β 1, there was no significant difference in α -SMA expression.

With respect to collagen III synthesis, there was a trend towards increased synthesis when fibroblasts were treated simultaneously with 10 ng/mL of TGF- β 1 and bFGF ($P = 0.06$, t -test). Unlike the effects on α -SMA, all other experimental designs for co-treatment of fibroblasts with bFGF and TGF- β 1 had no significant effect on collagen III synthesis.

Discussion

Novel findings in this study are: (1) previous investigators have looked at BAL fluid levels of various growth factors and cytokines at short time-points after antigen challenge (few minutes to 1 day) [16, 26, 27]; however, we measured BAL fluid concentration of TGF- β 1, TGF- β 2, IL-4 and IL-13 acutely after antigen-induced lung injury (1 day after SAC) and then during the recovery phase from that injury (1 and 2 weeks after SAC). We did not find any significant differences in BAL fluid concentrations of these factors between asthmatics and non-asthmatic subjects at baseline (before

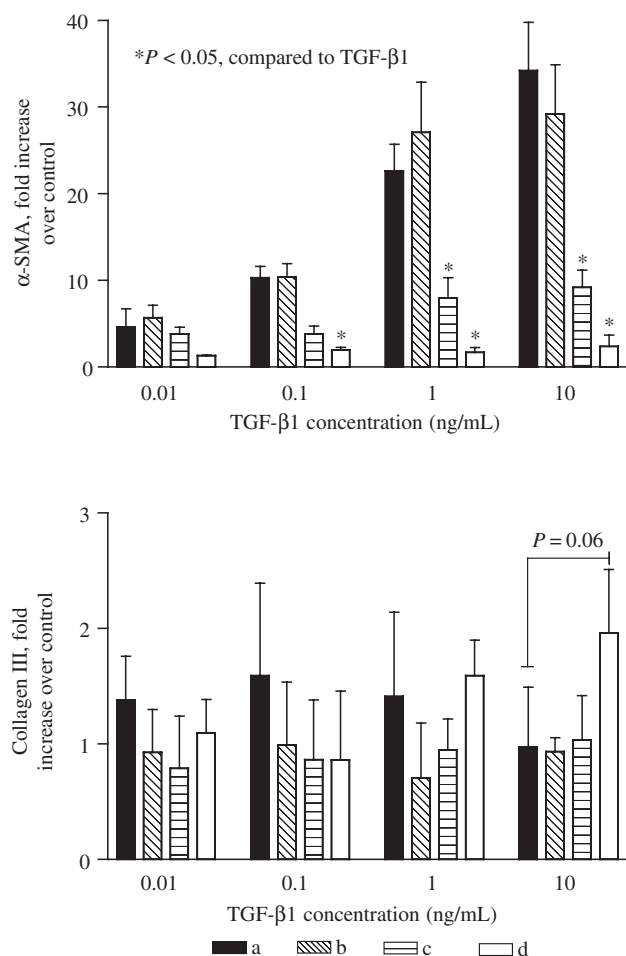


Fig. 5. Effect of co-treatment of fibroblasts with TGF- β 1 (0.01–10 ng/mL) and basic fibroblast growth factor (bFGF) (10 ng/mL) on α -smooth muscle actin (SMA) and collagen III synthesis. All fibroblasts at confluence were serum starved for 24 h and treated as follows: (a) Increasing concentrations of TGF- β 1 for 48 h. (b) Increasing concentrations of TGF- β 1 first, and 24 h later bFGF. Samples were harvested after 24 h of treatment with bFGF. (c) bFGF added first and 24 h later TGF- β 1. Samples were harvested after 24 h of treatment with TGF- β 1. (d) TGF- β 1 and bFGF added simultaneously for 48 h. α -SMA and collagen III band density (mean $\pm$ SEM, fold increase over control) were tested for statistical significance using two-way repeated measures ANOVA. Averaged results from five fibroblast lines studied. As a group, TGF- β 1 and bFGF added simultaneously decreased α -SMA expression ($P = 0.03$) compared with TGF- β 1 alone. bFGF first followed by TGF- β 1 24 h later showed a trend towards significantly decreased α -SMA ($P = 0.07$). No significant differences were noted in collagen III synthesis between the four different treatment designs (a, b, c or d); please note the difference in collagen III scale with Figs 2–4. However, there was a trend towards increased collagen III synthesis when fibroblasts were treated simultaneously with 10 ng/mL of TGF- β 1 and bFGF ($P = 0.06$, t -test).

SAC) except for total TGF- β 2, which was higher in non-asthmatics. In asthmatics, all the factors increased significantly 24 h after SAC and then returned to baseline 1 week later except IL-4, which remained elevated for at least 2 weeks (Fig. 1).

(2) Myofibroblast hyperplasia and subepithelial fibrosis are two of the principal histologic features of asthmatic airway remodelling. Collagen III is an important component of the subepithelial fibrosis [28]. Previous studies using HLFs have mainly looked at collagen I (and not collagen III) synthesis [29, 30]. Moreover, most of the studies have examined

indirect markers of collagen synthesis rather than specific collagen proteins [29–32]. We measured α -SMA and collagens III and I protein synthesis by HLFs after stimulation with TGF- β 1, TGF- β 2, IL-4 or IL-13. TGF- β 1, TGF- β 2 and IL-4 increased α -SMA expression, but only IL-4 increased collagen III synthesis in a dose response manner (Figs 2 and 3). (3) We report dissociation between myofibroblast phenotype and collagen III synthesis. Brewster et al. [2] reported a correlation between the number of myofibroblasts and the depth of subbasement membrane collagen in asthmatic bronchial biopsies, and thus proposed that myofibroblasts are responsible for collagen deposition. We find that only IL-4 increased α -SMA and correspondingly collagen III synthesis. TGF- β 1 and TGF- β 2 significantly increased α -SMA expression, and thus myofibroblast phenotype, but this did not result in similarly increased collagen III synthesis. bFGF decreased TGF- β 1-mediated α -SMA expression when added simultaneously with, or 24 h before TGF- β 1. In contrast, there was a trend towards increased collagen III synthesis when fibroblasts were simultaneously stimulated by bFGF and TGF- β 1. These data suggest separate transcriptional regulation of α -SMA expression and collagen III synthesis, possibly by additional growth factors and cytokines.

Overall, our results are consistent with the results of other studies examining BAL fluid concentrations of these factors. Previous studies have shown significant increases in BAL fluid IL-4 and IL-13 concentration after antigen challenge in asthmatics [26, 27]. This study adds that IL-4 concentration remained elevated for a longer duration than IL-13. Redington et al. have reported significantly higher TGF- β 1 levels in BAL fluid of asthmatics at 24 h after allergen challenge [16]. Unlike our results, in their study the basal concentration of TGF- β 1 was significantly higher in asthmatics than control subjects. However, asthmatics in the Redington study had more severe disease than in our study (mean forced expiratory volume in 1 s 83.3% predicted vs. 102% respectively). Studies examining TGF- β 1 mRNA and immunoreactivity in bronchial biopsies have found that the increase in basal TGF- β 1 is directly related to the severity of asthma [33]. Interestingly, we find that TGF- β 2 is significantly higher in non-asthmatics at baseline. However, the TGF- β 1 and TGF- β 2 levels are for total TGF- β , and not just the active fractions. The measurement of total TGF- β is thought to provide an approximation of the active fraction, but it is possible that active TGF- β levels may differ, particularly in asthmatics [34].

In contrast to our findings, a recent study by Richter et al. [29] did not find a significant increase in α -SMA actin or collagen synthesis when fibroblasts were stimulated with IL-4. The discrepancy in collagen synthesis may derive from their measurement of collagen I mRNA vs. our measurement of collagen III protein. In agreement with Richter et al. [29], we did not see increased collagen I from IL-4 stimulation. However, consistent with our results for collagen III and α -SMA, other investigators have reported that IL-4 is fibrogenic using fibroblasts from different organs [35–39], and that it induces α -SMA expression and myofibroblast-like differentiation of fibroblasts [40, 41]. Despite homology in structure, widely overlapping functions and sharing of a receptor subunit with IL-4 [42], IL-13 did not similarly increase α -SMA and collagen III synthesis. However, IL-13

may contribute towards airway remodelling by modulating airway epithelial cell function with subsequent effect on fibroblasts, and by recruiting inflammatory cells into the airways [4, 29]. Therefore, model systems that include epithelial cells [29], or *in vivo* studies [42, 43], show a fibrogenic effect for IL-13, but this possibly occurs by indirect action on fibroblasts. Instead of increasing collagen synthesis, IL-13 may also act to decrease collagen degradation via down-regulation of matrix metallo-proteinases and up-regulation of tissue inhibitor of metalloproteinases [44].

Our findings of moderate increases in collagens III and I synthesis in response to TGF- β 1 and TGF- β 2 may appear to contradict some previously published studies. However, these discrepancies in collagen synthesis may be explained by our use of a direct measure of collagen protein vs. earlier assessment of indirect markers of collagen (collagen mRNA, or bacterial collagenase susceptible radioactivity). Studies correlating TGF- β levels and subbasement membrane collagen deposition in asthmatics have been contradictory: Vignola et al. [45] reported a significant correlation between the number of epithelial or submucosal cells expressing TGF- β and basement membrane thickness, whereas Redington et al. [16] found no correlation between TGF- β 1 concentration in BAL fluid and the depth of subepithelial collagen deposition. It is possible that TGF- β markedly increases the mRNA for collagen synthesis, but not all is translated into increased protein. In addition, fibroblasts are heterogeneous in several parameters, including collagen synthesis as well as in response to TGF- β [46, 47]. TGF- β receptor number, which is dependent on fibroblast density, modulates response to TGF- β in *in vitro* experiments. Some reports use low cell density in order to enhance response to TGF- β [31], although increases in collagen III and I are only approximately twofold greater than the increases we observed. Fibroblasts plated at low density have increased functional expression of TGF- β receptors and promotion of signal transmission [48]. Failure to demonstrate large increases in collagen synthesis in our experiments is unlikely due to insufficient receptor stimulation because there a robust increased α -SMA expression was observed. Low sensitivity of our Western blots is disputed by increased collagen synthesis observed when fibroblasts were stimulated with IL-4.

Our *in vitro* experiments were conducted with fibroblasts obtained from non-asthmatic lungs and so results should be cautiously extrapolated to asthmatics. Although there is evidence that lung fibroblasts in some disease states may be inherently different [49], we did not find any difference in cell-cytoskeleton-matrix protein gene expression between fibroblasts from asthmatic and non-asthmatic subjects (unpublished data). Furthermore, the fibroblasts were from lung parenchyma, and may differ from bronchial airway fibroblasts. However, in preliminary studies our laboratory did not find any differences in α -SMA or collagen III synthesis between parenchymal and bronchial fibroblasts (data not shown). The concentrations of growth factors used in our *in vitro* experiments are in accordance with the levels seen in the BAL fluid after SAC, for example, in asthmatics, the mean concentration of IL-4 in un-concentrated BAL fluid 1 day after SAC was 0.015 ng/mL (BAL fluid was 14-fold concentrated before ELISA). Therefore, an *in vitro* concentration range from 0.01 to 10 ng/mL covers the measured level and

accounts for dilution as BAL was performed after instilling 150 mL of saline.

In summary, we propose that because IL-4 causes a dose-dependent increase in α -SMA and collagen III synthesis and its BAL fluid concentration remains elevated for a long time after allergen challenge, it may be one of the important factors mediating airway remodelling in asthma. TGF- β 1 and TGF- β 2 may also play a role in airway remodelling by promoting myofibroblast phenotype. Additionally, collagen synthesis appears to be independently regulated from myofibroblast phenotype, and may involve different growth factors and cytokines.

Acknowledgements

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