

Expression of growth factors by airway epithelial cells in a model of chronic asthma: regulation and relationship to subepithelial fibrosis

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Summary

Background Growth factors produced by airway epithelial cells may be important in the pathogenesis of subepithelial fibrosis, a distinctive lesion of chronic human asthma.

Objective To examine the relationship between the development of subepithelial fibrosis and the expression of transforming growth factor- β 1 (TGF- β 1) and ligands for the epidermal growth factor receptor.

Methods BALB/c mice sensitized to ovalbumin were chronically challenged by inhalation of low levels of antigen, leading to development of subepithelial fibrosis and other changes of airway wall remodelling. Growth factor expression was assessed by immunohistochemistry and enzyme immunoassay.

Results Allergic sensitization directly correlated with airway epithelial expression of both the cleaved, potentially biologically active form of TGF- β 1 and of amphiregulin in response to allergen challenge. Accumulation of TGF- β 1 was related to remodelling of the airway wall in chronic asthma, whereas expression of amphiregulin did not exhibit a similar relationship. Production of epithelial cell-derived TGF- β 1 appeared to be regulated by IL-13, while both IL-13 and CD4⁺ T cells regulated accumulation of TGF- β 1. In contrast to results reported in high-level exposure models of airway fibrosis, eosinophils did not appear to be a significant source of TGF- β 1.

Conclusion Airway epithelial cell-derived TGF- β 1 has a potentially crucial role in the development of airway wall remodelling in asthma. Immunological mechanisms may regulate the release and accumulation of TGF- β 1.

Keywords airway remodelling, amphiregulin, IL-13, transforming growth factor- β

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Introduction

Chronic asthma is associated with a variety of structural alterations in the airway wall, including subepithelial and airway wall fibrosis, goblet cell hyperplasia/metaplasia, smooth muscle hypertrophy/hyperplasia and increased vascularity. Collectively, these changes are referred to as airway wall remodelling [1]. Airway wall thickening resulting from these changes may contribute to the development of airway hyper-reactivity and may also be related to the development of fixed airflow obstruction in chronic asthmatics [2].

Subepithelial fibrosis is a distinctive lesion of asthma that involves accumulation of extracellular matrix proteins such as type III and type V collagen, fibronectin, tenascin and β ₂ laminin, as well as various proteoglycans, in the lamina reticularis beneath the true basement membrane (reviewed in [1]). Fibrosis is associated with the presence of increased num-

bers of myofibroblasts [3], which may be largely responsible for the synthesis and accumulation of these matrix components. Airway epithelial cells express a variety of growth factors with the potential to stimulate replication of and collagen synthesis by myofibroblasts of the epithelial-mesenchymal trophic unit [4]. These growth factors could be important in the pathogenesis of subepithelial fibrosis and other morphological changes associated with airway wall remodelling in chronic asthma.

To date, however, the relationship between production of growth factors by the airway epithelium and the subsequent development of remodelling remains poorly understood. Particular attention has been focused on the expression of transforming growth factor- β 1 (TGF- β 1), which potently induces collagen synthesis by fibroblastic cells, and on ligands for the epidermal growth factor receptor (EGFR) family, which may be involved in the epithelial response to injury. Following segmental allergen challenge of asthmatics, increased levels of TGF- β 1 have been demonstrated in bronchoalveolar lavage (BAL) fluid [5] and airway epithelial cells obtained by bronchial brushing have been shown to release increased amounts of this cytokine [6]. However, immunohistochemical studies of the expression of TGF- β in

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human airways, comparing patients with asthma and non-asthmatic control subjects, have yielded contradictory results [7–10] and little is known about inflammatory and/or immunological mechanisms that regulate the production or activation of TGF- β . Expression of the EGF receptor is up-regulated in asthmatic airways [11] but assessment of the expression of EGFR ligands has also yielded conflicting data [8, 12, 13].

We have described an improved model of inflammation of the airways in mice, which closely resembles chronic asthma [14]. Following systemic sensitization to ovalbumin (OVA), BALB/c mice are subjected to inhalational challenge with controlled low levels of aerosolized antigen for at least 6 weeks. This approach consistently elicits airway lesions typical of chronic human asthma, including recruitment of eosinophils into the airway epithelium, chronic inflammation in the lamina propria, subepithelial fibrosis, epithelial hypertrophy and mucous cell hyperplasia/metaplasia. In contrast to mouse models of asthma involving short-term challenge with high levels of allergen, in which the inflammatory response is characterized by a high density of lymphocytes and eosinophils surrounding the airways and extending to the blood vessels [15], there is no evidence of pulmonary parenchymal inflammation. The mice also exhibit marked hyper-reactivity to an inhaled cholinergic agonist, which is a consequence of abnormalities of the airways rather than of parenchymal lesions [16]. Thus the model replicates important features of the human disease [17] and significantly advances our ability to dissect the molecular processes associated with remodelling of the airways.

In the present study, we examined the expression of TGF- β 1 and of EGFR ligands in our chronic inhalational challenge model. We also compared expression of these growth factors in chronically challenged wild-type mice with gene-targeted or antibody-treated mice in which remodelling was abrogated. This approach has yielded novel temporal and spatial information about the expression of these epithelial cell-derived growth factors in the context of allergic sensitization and the induction of features of asthmatic airway remodelling. In particular, our results emphasize the role of airway epithelial cell-derived TGF- β 1 in the pathogenesis of subepithelial fibrosis and provide novel information about the immunological mechanisms regulating release and accumulation of TGF- β 1 in response to allergen challenge.

Materials and methods

Sensitization and exposure

The protocols we employed for sensitization and inhalational challenge have previously been described in detail [14, 18]. Briefly, specific pathogen-free female BALB/c mice and IL-13 $-/-$ mice on a BALB/c background [19] were sensitized by two intraperitoneal injections of 50 μ g alum-precipitated chicken egg OVA (Grade V, $\geq 98\%$ pure, Sigma Australia, Sydney; unless otherwise specified, all chemicals were obtained from this source) 21 days and 7 days before inhalational exposure. In the chronic challenge model, animals were exposed to aerosolized OVA for 30 min/day on 3 days/week for up to 8 weeks in wire flow-through cage racks in a 0.5 m³

whole-body inhalation exposure chamber (Unifab Corporation, Kalamazoo, MI, USA). Filtered air was drawn through the chamber at a flow rate of 250 L/min and an aerosol of OVA, generated from a 2.5% solution by delivery of compressed air to a sidestream jet nebulizer (Trimed, Sydney), was injected into the airstream entering the chamber. Particle concentration within the breathing zone of the mice was continuously monitored and maintained in the range 10–20 mg/m³ by controlling the airflow into the nebulizer. In experiments to deplete CD4⁺ cells, animals were administered weekly intraperitoneal injections of 0.75 mg of ammonium sulphate-purified anti-CD4 monoclonal antibody GK1.5 or isotype control monoclonal antibody β GL113 [20], beginning 3 days before inhalation exposures were commenced. In the acute challenge model, animals received a single 30 min uncontrolled exposure to aerosolized OVA, at a particle concentration estimated to be ≥ 100 mg/m³ [18].

Collection of BAL fluid and tissue samples

For collection of BAL fluid, groups of four to six animals were killed 6 h after the last inhalational challenge, by transection of the abdominal vessels under pentobarbital anesthesia. As previously described [21], the lungs were perfused with 0.9% saline to remove blood from the pulmonary capillary bed, the trachea was cannulated with a blunted #19 needle and the lungs were then lavaged with 1 mL of Dulbecco's phosphate-buffered saline (PBS) containing 0.05% fatty acid-free bovine serum albumin (BSA) as a carrier protein to minimize losses of TGF- β on to the walls of collection tubes. All tubes for handling and storage of BAL fluid had been pre-incubated at 37 °C for 4 h with a solution of PBS–0.1% BSA to block passive adsorption sites, then washed thoroughly with reverse osmosis-purified water. BAL fluid was centrifuged at 400 *g* to pellet the cells, supernatant fluid was immediately filtered through a 0.2 μ m membrane filter and proteolysis was inhibited by the addition of 0.5 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF) (ICN Biomedicals, Sydney), a water-soluble irreversible inhibitor of serine proteases. Aliquots of BAL fluid were stored at -70 °C prior to assay.

For immunohistochemistry, experimental groups comprised six animals. For studies of the time course of induction of expression of growth factors, tissues were collected at 3, 6 or 24 h after the last exposure. Otherwise, tissues were usually collected 18 h after the last exposure to aerosolized antigen, and included samples from tissue blocks collected in previously reported studies [14, 18, 20, 22]. Sections were routinely stained with haematoxylin and eosin for assessment of inflammatory changes and with Gordon and Sweet's reticulin stain for assessment of collagen deposition. Additional sections from chronically challenged animals were stained by the Picrosirius Red method and examined by polarization microscopy, which permits specific demonstration of collagen [23].

Immunohistochemistry

Because inflammation and remodelling are confined to the conducting airways in the chronic exposure model, expression of growth factors was assessed by immunostaining 4 μ m

sections of formalin-fixed, paraffin-embedded tracheal tissue. TGF- β 1 was demonstrated using an affinity-purified rabbit polyclonal antibody to a peptide mapping at the carboxy terminus of the precursor form of human and mouse TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, *sc-146*). This antibody detects TGF- β 1 in the absence of its latency-associated peptide and is thus considered to react with the cleaved, potentially biologically active form of TGF- β 1 [24]. It exhibits no cross-reactivity with TGF- β 2 or TGF- β 3 and pre-incubation of the antibody with the peptide used for immunization completely inhibits its reactivity [24, 25]. EGFR ligands were demonstrated using protein A-purified rabbit antibodies to amphiregulin (Ab-1, Neomarkers, Fremont, California; raised against a 19-amino acid peptide from human amphiregulin, cross-reactive with mouse), TGF- α (Santa Cruz, *sc-9043*; raised against a recombinant 50-amino acid protein corresponding to human TGF- α , cross-reactive with mouse and rat) or EGF (Serotec, Oxford, UK; raised against purified mouse EGF).

For EGFR ligands, antigen retrieval was performed by boiling in 0.01 M citrate buffer (pH 6.0) for 10 min in a microwave. In some experiments involving immunostaining for TGF- β 1, sections were pre-treated with 25 μ g/mL proteinase K (Merck, Melbourne, Australia) in 100 mM Tris buffer (pH 8.0) containing 50 mM EDTA. Non-specific binding was blocked by incubation of sections for 15 min with 50% normal goat serum diluted in PBS. An appropriate concentration of antibody was then applied in PBS containing 0.075% Brij-35 detergent for 40 min at room temperature. Slides were washed four times with PBS after this and each subsequent incubation. Bound antibody was detected by sequential 30 min incubations with a 1:50 dilution of swine anti-rabbit immunoglobulins (Dako, Sydney) in PBS-Brij, followed by a 1:100 dilution of rabbit peroxidase-anti-peroxidase complex (Dako). Diaminobenzidine was used as the substrate and sections were lightly counterstained with hematoxylin for 10 s. Control sections stained in parallel, using non-immune rabbit immunoglobulins at the same concentration as the primary antibodies, were routinely negative.

Intensity of immunoreactivity was semi-quantitatively scored as grade 0 = no staining, grade 1 = weak staining, grade 2 = moderate staining and grade 3 = strong staining. All grading was performed by a single observer blinded to the identity of the samples and slides were presented in random order for examination.

Enzyme immunoassay for TGF- β 1

A sandwich enzyme immunoassay for TGF- β 1 was performed essentially as previously described [21]. In brief, wells were coated with 2 μ g/mL of mouse monoclonal antibody to TGF- β 1, 2 and 3 (R&D Systems, Minneapolis, MN, USA) in carbonate-bicarbonate buffer (pH 9.6) for capture, blocked with PBS-1% BSA for 60 min at 37 °C, then incubated with doubling dilutions of human TGF- β 1 (Promega, Sydney) from 31 pg/mL to 2 ng/mL in PBS-1% BSA or with test samples for 120 min at 37 °C. Latent TGF- β in the samples was activated by prior acidification with 1/20th of the sample volume of 0.8 M HCl at 4 °C for 60 min, followed by neutralization with an equal amount of Tris base. Detection was sequentially with 2 μ g/mL of a turkey polyclonal

antibody to TGF- β 1 (Collaborative Research, Waltham, MA, USA) at room temperature for 60 min, followed by 2 μ g/mL of goat anti-turkey antibody conjugated with horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD, USA) for 30 min, 1 μ g/mL of biotinylated goat anti-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min and enzyme complex amplification reagent for 15 min. The latter was prepared by assembling complexes of streptavidin (Boehringer Mannheim, Sydney) and biotinylated horseradish peroxidase in PBS-1% BSA, then cross-linking these with biotinylated goat anti-horseradish peroxidase and additional streptavidin [26]. Colour development was by incubation with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate in citrate-phosphate buffer (pH 4.0) and the reaction product was read using a Titertek Multiskan photometer (Flow Laboratories, Maclean, VA, USA) with a 405 nm filter.

Statistical analysis

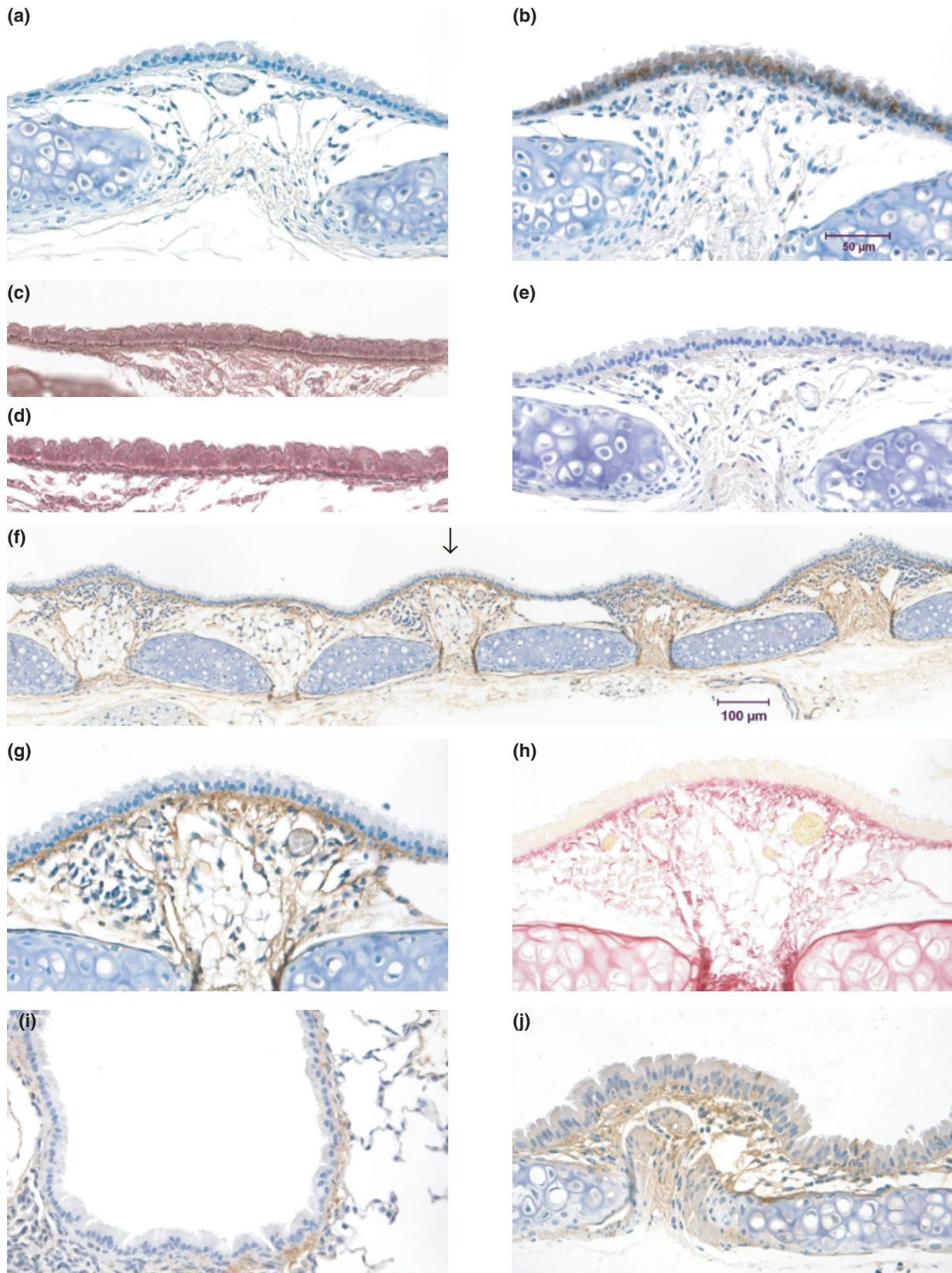
For immunohistochemistry data, comparisons employed a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison procedure or a Mann-Whitney test as appropriate. For enzyme immunoassay data, comparisons were by a one-way ANOVA. The software package GraphPad Prism 3.03 (GraphPad Software, San Diego, CA, USA) was used for all data analysis and preparation of graphs.

Results

Immunostaining for TGF- β 1

Naïve animals exhibited no immunostaining for TGF- β 1 in either the airway epithelium or the connective tissue of the lamina propria (Fig. 1a). However, intense immunoreactivity was readily demonstrable after treatment of sections with proteinase K (Fig. 1b). This was inferred to be a consequence of proteolytic cleavage of latent TGF- β 1 complex in the airway epithelial cells, rather than a result of antigen retrieval, because no immunoreactivity was observed after boiling in citrate buffer or treatment with acid. The result was consistent with previous evidence that the anti-peptide antibody used in these experiments detects an epitope on TGF- β 1, which is unmasked after cleavage of the latency-associated peptide from the intracellular complex [24].

As previously reported [14], whereas non-sensitized BALB/c mice that had been chronically exposed to low mass concentrations of aerosolized OVA exhibited no evidence of subepithelial fibrosis, systemically sensitized chronically challenged animals developed a zone of collagenization in the subepithelial region of the trachea (Figs 1c and d). Immunostaining for TGF- β 1 was virtually absent in non-sensitized chronically challenged mice (Fig. 1e). In contrast, sensitized chronically challenged mice exhibited a prominent band of immunoreactivity for TGF- β 1 in the subepithelial region, detectable without antigen retrieval (Figs 1f and g). This corresponded to the zone of collagenization that was clearly demonstrated by Picrosirius Red staining (Fig. 1h). Immunoreactivity was also associated with collagen fibrils deeper in the lamina propria of the trachea and with subepithelial fibrosis in intrapulmonary airways (Fig. 1i).



The cytoplasm of airway epithelial cells of sensitized chronically challenged mice exhibited immunoreactivity for TGF-β1 at 3 h after challenge, but this was transient and

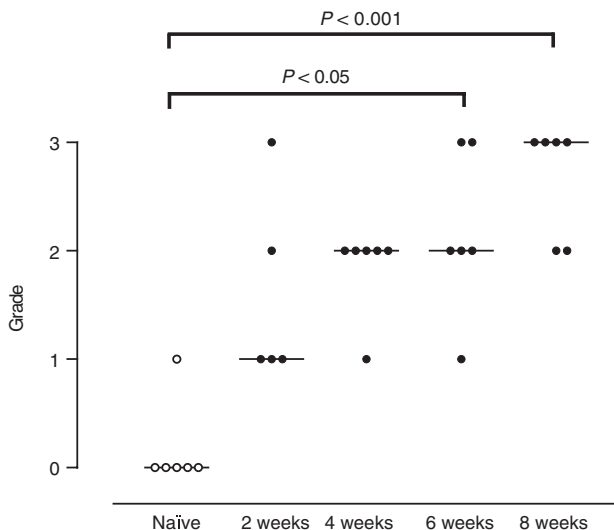
declined to virtually undetectable levels by 24 h. In contrast, subepithelial staining for TGF-β1 was stable for at least 24 h after challenge (Table 1). There was no evidence of

Table 1. Expression of TGF- β 1 in the airways of mice following antigenic challenge

	Epithelium			Subepithelial zone		
	3 h	6 h	24 h	3 h	6 h	24 h
Sensitized chronic	2 (1–2.5)	1.5 (1–2)	0 (0–1)*	2 (1.5–3)	2 (2–2.5)	2 (2–3)
Non-sensitized acute	0 (0)	0 (0)	0.5 (0–1)	0 (0)	0 (0)	0 (0)
Sensitized acute	1 (0.5–2)	1.5 (0.5–2)	0.5 (0–1.5)	2 (0.5–2.5)	3 (2–3)	2 (1.5–3)

Data are presented as median (interquartile range) for each group, $n = 6$.

* $P < 0.01$ compared with the 3-h group.

**Fig. 2.** Intensity of immunoreactivity for active TGF- β 1 in the subepithelial zone of naïve (open circles) and sensitized BALB/c mice (closed circles) chronically challenged with aerosolized ovalbumin for 2, 4, 6 or 8 weeks. Bars show median values.

immunoreactivity for TGF- β 1 in any non-epithelial cells. Notably, eosinophils within the airway epithelium and the lamina propria did not stain for TGF- β 1.

Because the development of airway wall remodelling is related to the total duration of antigenic challenge in this model of chronic asthma [14], we investigated the relationship between the intensity of subepithelial staining for TGF- β 1 and the period of inhalational exposure. Immunoreactivity for TGF- β 1 was apparent after only 2 weeks of exposure to antigen and the median grade was significantly increased by 6 weeks (Fig. 2).

To further examine the relationship between sensitization, challenge and expression of TGF- β 1, we compared the patterns and intensity of immunoreactivity in non-sensitized and sensitized mice subjected to an acute inhalational challenge with a high mass concentration of aerosolized

Table 2. Concentrations of active TGF- β 1 in bronchoalveolar lavage fluid of mice following antigenic challenge

	TGF- β 1 (ng/mL)
Naïve	5.14 $\pm$ 0.34
Non-sensitized acute	11.84 $\pm$ 2.17
Sensitized acute	6.45 $\pm$ 2.34
Non-sensitized chronic	4.32 $\pm$ 0.90
Sensitized chronic	8.04 $\pm$ 3.15

Data are presented as mean $\pm$ standard error for each group, $n = 4$ –6. Differences between groups were not statistically significant.

OVA. Non-sensitized animals exhibited no evidence of immunostaining in the subepithelial region at any time-point after challenge. Nor was there staining of epithelial cell cytoplasm at 3 and 6 h after challenge, but weak staining was seen at 24 h. In contrast, sensitized animals exhibited moderate immunoreactivity of airway epithelial cells at 3 and 6 h after acute challenge, as well as immunoreactivity in the subepithelial region and in the connective tissue of the lamina propria (Fig. 1j), which peaked at 6 h after challenge (Table 1).

Quantification of TGF- β 1 in BAL fluid

The concentrations of active TGF- β 1 in BAL fluid samples from naïve mice, as well as in samples collected 6 h after acute or chronic challenge of non-sensitized or sensitized mice, exhibited considerable variability, with absolute values ranging from 4 to 17 ng/mL. There were no significant differences between experimental groups (Table 2). Acidification revealed the presence of additional latent TGF- β 1 in all samples, with approximately 80% of the total content being active TGF- β 1 (data not shown).

Regulation of expression of TGF- β 1

To assess the relationship between TGF- β 1 and airway wall remodelling, we compared immunoreactivity for TGF- β 1 in

Fig. 1. (a) Immunoperoxidase staining demonstrating absence of immunoreactivity for TGF- β 1 in the tracheal epithelium and connective tissue of naïve mice. (b) Intense reactivity of the airway epithelium of naïve mice following pre-treatment with proteinase K to activate latent TGF- β 1. Scale bar applies to this and all other images except the low-magnification montage. (c) Reticulin-stained section showing normal airway epithelium and thin subepithelial collagenous zone in a non-sensitized BALB/c mouse chronically challenged with aerosolized ovalbumin (OVA) for 8 weeks. (d) Reticulin-stained section showing epithelial hypertrophy and subepithelial fibrosis in a chronically challenged sensitized BALB/c mouse. (e) Almost complete absence of immunoreactivity for TGF- β 1 in a chronically challenged non-sensitized BALB/c mouse. (f) Digital montage of consecutive low-magnification microscopic fields of the trachea from a chronically challenged sensitized BALB/c mouse, showing immunoreactivity for TGF- β 1 in the subepithelial region and associated with collagen fibrils deeper in the lamina propria. (g) Higher magnification of the region indicated by the arrow in (f), demonstrating marked subepithelial immunoreactivity for TGF- β 1. (h) Picrosirius Red staining of a consecutive section showing the zone of subepithelial fibrosis, which exhibited yellow birefringence under polarized light. (i) Immunoreactivity for TGF- β 1 in subepithelial connective tissue surrounding the main bronchus in a chronically challenged sensitized BALB/c mouse. (j) Moderate immunoreactivity of airway epithelium and marked immunoreactivity of connective tissue for TGF- β 1 in a sensitized BALB/c mouse at 6 h after acute challenge with a high mass concentration of aerosolized OVA.

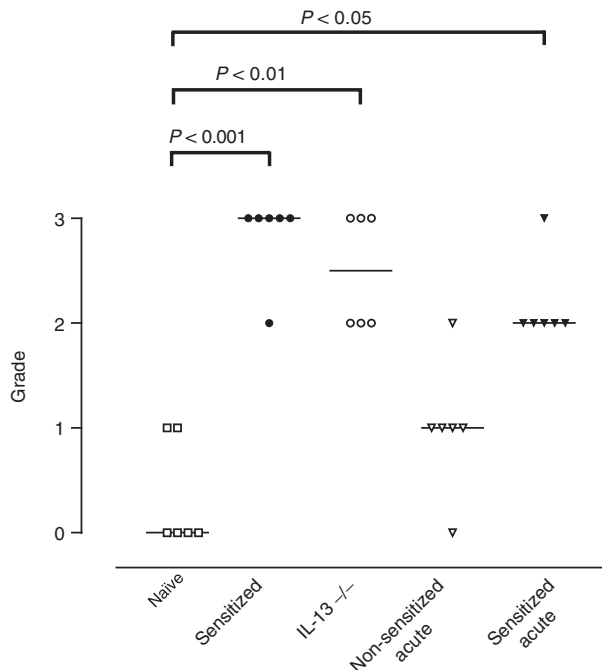


Fig. 5. Intensity of immunoreactivity for amphiregulin in the epithelium of naïve wild-type BALB/c mice (open squares); sensitized wild-type BALB/c mice (closed circles) and IL-13 $-/-$ mice (open circles), chronically challenged with aerosolized ovalbumin (OVA) for 6 weeks and tested 3 h after the final exposure; as well as non-sensitized (open triangles) and sensitized wild-type BALB/c mice (closed triangles) acutely challenged with a high mass concentration of OVA. Bars show median values.

In the first instance, we employed immunohistochemistry to detect growth factor expression within the airway wall, because expression of mRNA for TGF- β 1 correlates poorly with immunoreactivity for protein [27, 28]. Moreover, immunohistochemistry provided valuable spatial information and permitted assessment of expression of the cleaved, potentially biologically active protein, a consideration of particular importance in the case of TGF- β 1, which is primarily regulated post-translationally [29]. We used an antibody to TGF- β 1 directed against an epitope that is masked by the latency-associated peptide derived from the precursor molecule, which is initially transcribed. Antibodies that are selectively immunoreactive with the cleaved form of TGF- β 1 exhibit staining patterns quite distinct from those seen using antibodies to the latency-associated peptide, as well as a rapid change in staining pattern following exposure to a stimulus leading to activation of TGF- β 1 [30, 31]. This differential pattern of staining and the corresponding altered pattern following injury have been demonstrated for the antibody we employed, which is thus considered to react with the active form of TGF- β 1 [24]. Moreover, our observation that proteolytic pre-treatment revealed immunoreactivity for TGF- β 1 in normal airway epithelium is consistent with the notion that this antibody reacts with an epitope on active TGF- β 1 that is masked by latency-associated peptide.

In sensitized animals, airway epithelium was the major site of induction of expression of growth factors following antigenic challenge. Epithelial expression of both TGF- β 1 and amphiregulin was transient, and in the case of TGF- β 1 was

associated with the appearance of extracellular staining in the subjacent matrix, suggestive of secretion. Airway epithelial cells produce large amounts of latent TGF- β 1 [27, 32, 33] (as well as TGF- β 2 and TGF- β 3 [32]) and our data indicate that in sensitized animals, allergen challenge is able to trigger activation of latent TGF- β 1, leading to immunoreactivity for the cleaved form of TGF- β 1 in the cytoplasm of the epithelial cells and in the subjacent extracellular matrix. These observations are analogous to the activation of TGF- β 1 in mammary gland tissues demonstrated by Barcellos-Hoff (1994) following radiation injury, leading to cytoplasmic immunoreactivity of epithelial cells and adjacent connective tissues. The mechanisms involved in the alteration of the epithelial response in sensitized as compared with non-sensitized animals are at present unknown. However, a recent study demonstrated selective activation and nuclear translocation of NF- κ B in airway epithelial cells of sensitized animals in a short-term model of allergen exposure [34], which could be relevant to this altered response.

We attempted to confirm epithelial release of active TGF- β 1 by assessment of concentrations of this growth factor in BAL fluid following antigen challenge, at the time-point corresponding to the peak of epithelial immunoreactivity for TGF- β 1. However, no significant increase in levels was demonstrable in either acutely or chronically challenged sensitized animals. The absence of a significant increase following acute challenge is consistent with results of another recent study comparing acute and chronic challenge [35]. However, in a model involving uncontrolled exposure to high levels of aerosolized antigen, those authors noted increased BAL fluid levels of TGF- β 1 following chronic challenge for 36 weeks. Our results emphasize the differences between models employing low level as compared with high-level exposure to antigen. In high-level challenge models, eosinophils appear to be the major source of TGF- β 1 in BAL fluid [36] but whereas accumulation of modest numbers of intra-epithelial eosinophils is a feature of our model, we found no evidence of expression of TGF- β 1 by eosinophils following chronic low-level allergen challenge. The reasons for the absence of a demonstrable increase in BAL fluid concentrations of TGF- β 1 despite increased epithelial and subepithelial immunoreactivity for this cytokine are unclear, although an intriguing possibility is that release of TGF- β 1 might occur in a polarized fashion, which would be consistent with the localized development of fibrosis in the subepithelial region.

Progressive accumulation of subepithelial TGF- β 1 correlated with the development of airway wall remodelling in our model of chronic asthma. Notably, in animals that were subjected to inhalational challenge over a period of 8 weeks, there was accumulation of TGF- β 1 in the subepithelial region from 2 weeks onwards. This preceded the development of significant subepithelial fibrosis, which we have previously shown progressively increases from 4 weeks onwards [14], and strongly suggests a pathogenetic role for this growth factor. This inference is supported by data from the associated study of IL-13 $-/-$ mice and of mice treated with anti-CD4, both of which do not develop epithelial hypertrophy, goblet cell hyperplasia/metaplasia or subepithelial fibrosis [20, 22]. In IL-13 $-/-$ mice, the content of latent TGF- β 1 in the cytoplasm of airway epithelial cells was markedly reduced and there was no evidence of activation of this growth factor by

epithelial cells following allergen challenge. Thus the failure of these animals to accumulate immunoreactive TGF- β 1 in the subepithelial zone might be related both to deficient synthesis and defective activation. In contrast, airway epithelial cells of anti-CD4-treated mice contained abundant latent TGF- β 1, but the absence of subepithelial accumulation of immunoreactive TGF- β 1 in these animals indicates that CD4⁺ T cells provide a critical signal for the activation and release of this growth factor. We speculate that the CD4⁺ T lymphocyte-derived signal might be IL-13 itself, a notion that is consistent with the data of Lee et al. on IL-13 regulation of expression and secretion of TGF- β 1 in transgenic mice [25], as well as published reports on the ability of IL-13 to stimulate release of TGF- β by airway epithelial cells [37, 38]. In several respects, our results are complementary to the findings of Lee et al., who demonstrated increased expression of TGF- β 1 in mice overexpressing IL-13, while we have shown decreased expression in mice deficient in this cytokine. However, a significant difference between the two studies is that although they observed epithelial cell expression of TGF- β 1, maximal expression was seen in macrophages and was associated with marked fibrosis of the pulmonary parenchyma.

Airway epithelial cells of naïve animals expressed moderate amounts of TGF- α , lesser amounts of epidermal growth factor and little amphiregulin. Inhalational challenge of sensitized animals with allergen elicited increased immunoreactivity for amphiregulin in airway epithelial cells. However, immunostaining for amphiregulin was not seen in eosinophils, which have also been suggested to be important sources of EGFR ligands [39, 40]. Interestingly, we did not observe increased airway epithelial immunoreactivity for the other EGFR ligands examined. In this context, it is noteworthy that while various EGFR ligands have been demonstrated to be expressed by normal human airway epithelium [41], expression of TGF- α , EGF or heparin-binding EGF did not appear to be up-regulated after allergen challenge of asthmatics [13], but expression of amphiregulin was not examined. We would stress, however, that in contrast to the results for TGF- β 1, expression of immunoreactive amphiregulin did not correlate with changes of airway wall remodelling in our chronic exposure model of asthma. Expression was undiminished both in IL-13 $-/-$ mice and in anti-CD4-treated animals. These results indicate that increased expression of amphiregulin is not in itself sufficient for induction of any of the changes of airway wall remodelling and highlight the important contribution of TGF- β 1 to the development of structural changes in the airways.

In conclusion, our findings emphasize the potentially crucial role of airway epithelial cell-derived TGF- β 1 and other growth factors in the development of airway wall remodelling in asthma. Furthermore, they also provide novel information about immunological regulation of the release and accumulation of TGF- β 1 at the epithelial barrier in response to antigenic challenge.

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