

Epithelial Expression of Profibrotic Mediators in a Model of Allergen-Induced Airway Remodeling

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Airway remodeling, including subepithelial fibrosis, is a characteristic feature of asthma and likely contributes to the pathogenesis of airway hyperresponsiveness. We examined expression of genes related to airway wall fibrosis in a model of chronic allergen-induced airway dysfunction using laser capture microdissection and quantitative real-time PCR. BALB/c mice were sensitized and subjected to chronic ovalbumin exposure over a 12-wk period, after which they were rested and then harvested 2 and 8 wk after the last exposure. Chronic allergen-exposed mice had significantly increased indices of airway remodeling and airway hyperreactivity at all time points, although no difference in expression of fibrosis-related genes was found when mRNA extracted from whole lung was examined. In contrast, fibrosis-related gene expression was significantly upregulated in mRNA obtained from microdissected bronchial wall at 2 wk after chronic allergen exposure. In addition, when bronchial wall epithelium and smooth muscle were separately microdissected, gene expression of transforming growth factor- β 1 and plasminogen activating inhibitor-1 were significantly upregulated only in the airway epithelium. These data suggest that transforming growth factor- β 1 and other profibrotic mediators produced by airway wall, and specifically, airway epithelium, play an important role in the pathophysiology of airway remodeling.

Keywords: airway hyperresponsiveness; airway remodeling; asthma; laser capture microdissection; transforming growth factor- β 1

Chronic structural changes in the airway of subjects with asthma, often termed airway remodeling, include goblet cell metaplasia, deposition of extracellular matrix (ECM) in the submucosa, smooth muscle hyperplasia/hypertrophy, and hyperplasia of fibroblasts/myofibroblasts (1). There is evidence that airway remodeling plays an important role in the pathophysiology of airway hyperresponsiveness (AHR) (2, 3), which is a characteristic feature of asthma (1), and may be related to the development of fixed airflow obstruction in individuals with long-standing chronic asthma (4).

In addition to inflammatory cells, including macrophages, mast cells, and eosinophils in the lung, resident structural cells such as fibroblasts, smooth muscle, and epithelial cells have been shown to release significant amounts of mediators (5), and likely play an important role in the chronicity of asthma (4). Transforming growth factor- β 1 (TGF- β 1) has numerous activities relating to fibrosis, which include inducing the expression of matrix pro-

teins, tissue inhibitors of matrix metalloproteinases (TIMPs), as well as plasminogen activator inhibitor-1 (PAI-1) (6). Although TGF- β 1 has been established as the major profibrotic cytokine in the pathogenesis of pulmonary fibrosis (7), its role in airway remodeling in asthma is less certain, with some studies suggesting that TGF- β 1 suppresses AHR (8, 9), whereas others suggest that TGF- β 1 is associated with subepithelial fibrosis and AHR (10, 11). In addition, studies in human subjects with asthma which have examined TGF- β 1 mRNA and protein have found conflicting results as to its role in the pathogenesis of asthma (9, 12–16).

We have recently described a model in which airway dysfunction and aspects of airway remodeling develop in mice after chronic exposure to allergen (17), where AHR is *sustained* for at least 8 wk after final exposure to allergen, well beyond the resolution of acute inflammatory events. This model reflects airway structural changes in asthma, including goblet cell metaplasia, subepithelial fibrosis, and increased α -SMA staining, and is accompanied by AHR. In addition, there is no obvious inflammation of the parenchyma. The phenotypic characteristics of this model, we believe, are similar to those of subjects with asthma, who demonstrate airway remodeling and sustained AHR, despite attenuation of the airway inflammatory infiltrate by anti-inflammatory corticosteroid therapy (18–20). The purpose of this study was to determine whether profibrotic genes were upregulated in the remodeled airways. Furthermore, we sought to determine whether airway epithelial or mesenchymal cells were involved in this process. We applied the technique of laser capture microdissection (LCM) (21) to collect airway tissue as well as individual cell types from the airway and examined the expression of important profibrotic genes at 2 and 8 wk after the last allergen challenge. We demonstrate that marked specific gene upregulation is seen in airway tissue, but not whole lung mRNA, and that this regulation can be detected in epithelial and not smooth muscle cells of the airway. These findings illustrate the importance of examining specific tissues within the lung when investigating the pathogenesis of remodeling.

MATERIALS AND METHODS

Animals

Female BALB/c wild-type mice, aged 10–12 wk, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mice were housed in environmentally controlled specific pathogen-free conditions for 1 wk before study, and for the duration of the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University, and conformed to NIH guidelines for the care and use of laboratory animals.

Sensitization

Mice were sensitized with intraperitoneal ovalbumin (OVA) as described by us previously (22). Briefly, intraperitoneal OVA injections involved precipitating 10% aluminum potassium sulfate with 0.05% OVA, adjusting to pH 6.5, centrifuging, and then resuspending the pellet in 5 ml saline, followed by a 200- μ l intraperitoneal injection containing $\sim$ 80 μ g of OVA.

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Challenge

Sensitized mice were subjected to chronic periods of allergen exposure, as described by us previously (17), which involved six 2-day periods of intranasal OVA challenges (100 μ g in 25 μ l saline), each separated by 12 d for a total of 12 challenges over a 10-wk period. Control mice were subjected to the same sensitization protocol but received saline challenges. Mice were studied 2 and 8 wk after the final exposure to either allergen or saline. The following outcome measurements were made: (1) *in vivo* airway responsiveness to intravenous methacholine; (2) levels of TIMP-1 and TGF- β 1 (active and total) in bronchoalveolar lavage (BAL) fluid; (3) airway morphometry, using a computer-based image analysis system; and (4) quantitative gene expression in the whole lung and in the airway using LCM and quantitative real-time (Q-RT) PCR. Separate groups of 15 mice were studied in each treatment arm of each protocol.

Airway Responsiveness

Airway responsiveness was measured on the basis of the response of total respiratory system resistance (R_{RS} , cm H₂O/ml/s) to saline and increasing (10, 33, 100, and 330 μ g) intravenous doses of methacholine ($n = 15$ per group). Mice were anesthetized (Avertin; Aldrich Chemical Co., Milwaukee, WI; 240 mg/kg intraperitoneally) and the trachea cannulated and attached to a ventilator (RV5; Voltek Enterprises, Inc., Toronto, ON, Canada) designed to deliver constant inspiratory flow. R_{RS} was measured using the flow-interrupter technique, as modified for use in mice, and described in detail elsewhere (17). Sustained hyperreactivity (rate of increase in R_{RS} for a given increase in Mch dose) and maximal bronchoconstriction (maximum R_{RS}) were measured.

BAL

After airway physiology measurements, BAL was performed as described previously (17). Total and active TGF- β 1 levels and TIMP-1 levels were assessed by ELISA immunoassay (R&D Systems, Minneapolis, MN).

Preparation of Lung for Assessment of Gene Expression

Five mice per group, as well as three mice that had received no intervention, had their lungs removed and inflated with a 50% vol/vol mixture of Tissue-Tek OCT (Sakura Finetek, Torrance, CA) in 10% RNase-free sucrose. The right lung was tied off and stored at -70°C for later examination of gene expression from the whole lung. The left lung was frozen on dry ice and then sectioned transversely across the first generation bronchus and stored at -70°C . It was subsequently sectioned at 6–10 μ m, mounted on clean glass slides, and stored again at -70°C . The cryostat blade was cleaned between each block, paying attention to RNase-free conditions.

LCM

Immediately before LCM being performed, the frozen sections were thawed for 10 s, and then stained with Histogene (Arcturus, Mountain View, CA), according to the manufacturer's protocol. LCM was performed using the Pixcell II microscope (Arcturus) (21), using a 7.5- μ m laser beam, with varying pulse power (40–100 mW) and width (0.7–2.0 ms). Three tissue groups were captured, using ExtracSure HS LCM caps (Arcturus). "Airway" tissue consisted of epithelial cells from the large airways as well as underlying smooth muscle, fibroblasts, and ECM to a depth of ~ 20 μ m beneath the epithelium (Figure 1). "Epithelium" consisted of epithelial cells from the epithelial layer of the large airways, and "smooth muscle" consisted of blocks of smooth muscle beneath the epithelium. Approximately 500–3,000 laser pulses were applied for each tissue group, using consecutive frozen sections. The identity of the cells captured was confirmed by examining the surface of the cap itself under the microscope.

Gene Expression in Microdissected Tissue

Extraction of RNA from microdissected tissue was performed according to the manufacturer's protocol by a column-based method (PicoPure RNA isolation kit; Arcturus), followed by one round of linear amplification (RiboAmp RNA Amplification kit; Arcturus). Reverse transcription with the Super-Script First-Strand Synthesis System (Invitrogen Inc., Burlington, ON, Canada) using random hexamers according to the manufacturer's protocol was performed. Q-RT-PCR analysis for

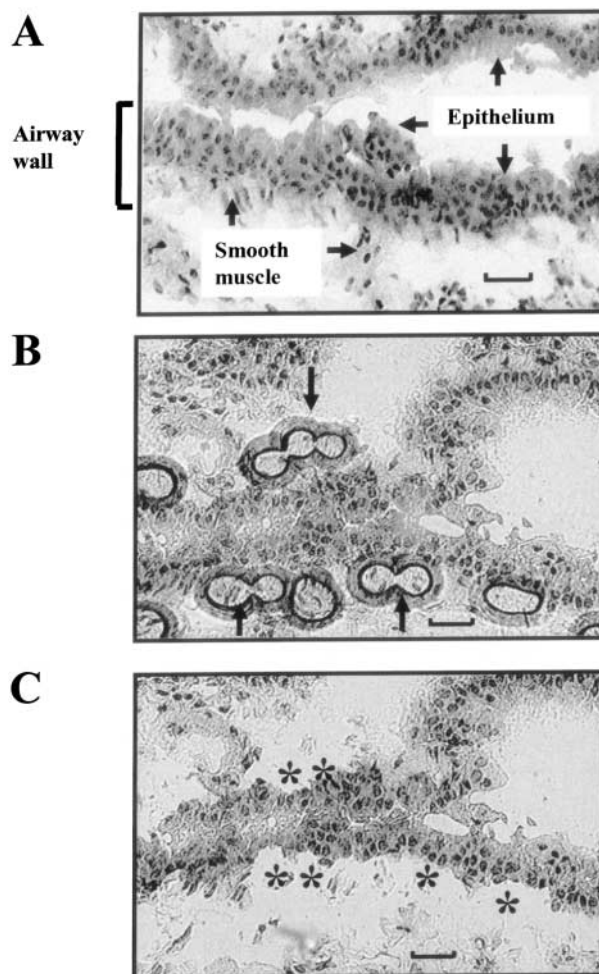


Figure 1. Laser capture microdissection. (A) Representative photograph of airway wall showing epithelium and smooth muscle. (B) Arrows point to blocks of smooth muscle, identified by elongated parallel nuclei, that have been targeted by laser beam. (C) Asterisks identify areas where smooth muscle was removed. Bars indicate 50 μ m.

cDNA was carried out using the ABI PRISM 7700 Sequence Detection System instrument and software as described by the manufacturer (Applied Biosystems, Foster City, CA). Oligonucleotide PCR primer pairs (Mobix, Hamilton, ON, Canada) and fluorogenic probes (Applied Biosystems) were designed from the published sequences using Primer Express software (Perkin-Elmer, Boston, MA) and are shown in Table 1. Samples were normalized against β 2 microglobulin to control for varying RNA content of each sample. Two other housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ribosomal protein L32, were also assayed. PCR premixes (Applied Biosystems) containing all reagents except templates (universal master mix, 900 nM each forward and reverse primer, and 200 nM Taqman probe in a final volume of 25 μ l) were prepared and aliquoted into 96-well plates. PCR was performed for 40 cycles, consisting of a denaturation step at 95°C for 15 s and a combined annealing and extension step at 58°C for 2 min. The relative standard curve method was used for quantitation (ABI Prism 7700 sequence detection system User Bulletin No. 2, 1-36; Applied Biosystems). A section from a mouse in the 8-wk allergen challenge group was used to generate the standards for the Q-RT-PCR. Each Q-RT-PCR run included the standard curve, water as a no-template control, and all samples were run in duplicate. Samples with a coefficient of variation of $> 10\%$ were retested.

Gene Expression in Whole Lung Tissue

The frozen right lung samples were homogenized in 10 ml of Trizol (Invitrogen Inc.) and the RNA extracted according to the manufactur-

TABLE 1. MOUSE PRIMERS AND PROBES USED IN SEMI-QUANTITATIVE REAL-TIME PCR REACTIONS

	Forward Primer	Reverse Primer	Taqman Probe 6-FAM-5' end; TAMRA-3' end (*MGB instead of TAMRA)
	5'	→	3'
β2-Microglobulin	CATACGCCTGCAGAGTTAAGCA	GATCACATGTCTCGATCCCAGTAG	ACAGTATGGCCGAGCCCAAGACCG
TGF-β1	AAACGGAAGC-GCATCGAA	GGGACTGGCGAG-CCTTAGTT	CCATCCGTGGC-CAGATCCTGTCC
PAI-1	TGCATCGCC-TGCCATTG	CTTGAGATAGGA-CAGTGCTTTTCC	TGTGGAGGGT-GCCATGGGCC
CTGF	TCCCAGAGAAGGG-TCAAGCT	TCCTTGGGCTCG-TCACACA	CCTGGGAAATGCTGC-AAGGAGTGG
TIMP-1	GTGGGAAATGCC-GCAGAT	GGGCATATCCACA-GAGGCTTT	CCGTACGCCTACAC-CCCAGTCATG
MMP-9	CATGCACTGGGGGC-TTAGATCATTC	CGAGGGTAGCTAT-ACAGCGGGTAC	TGGAGCGCTTCCGG-GCAGCGGT
MMP-2	CACCTGGTTTCA-CCCTTTCTG	CGAGCGAAGG-GCATACAAA	CCCAGATACCTGCAC-CACCTTAAGTGTGC
Fibronectin	AAATGCACATATTGATTACACATCCA	TTGTGTGGCTGGTCCATAGC	CGGATATTCTGAACACA*
Procollagen1α2	GTGTGCAATATGATCCAATAAGTCTC	CCACAAAATAAAGTCTGCAAAACA	TCCTTTGGCCCTCCCAAAA
α-SMA	CCAGAGCAAGAGAGGGATCCT	TGTCGTCCCAGTTGGTGATG	ACGCTGAAGTATCCGATAGAACACGGCA
L32	AAGCCATTGTAGAAAGAGCAGCAC	GCCTGGCGTTTGGGATTG	CTGGCCATCAGAGTC*
Myosin Heavy Chain 11	AGCGCCAGAACAAAGGAAGTCTG	TCCAGCGCCGCAACA	CTTTGACAGCACCTTCT*
GAPDH	GCTACACTGAGGACCAGTTGTC	GCCAGCCCCGGCAT	CGACTTCAACAGCAACTCCCACTCTTCC

Definition of abbreviations: α-SMA, α-smooth muscle actin; CTGF, connective tissue growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of metalloproteinase-1.

er's instructions. Extracted RNA was DNase-treated (Qiagen DNase, Mississauga, ON, Canada), and the concentration determined with a microgel bioanalyzer (Agilent 2100; Agilent Technologies, Palo Alto, CA). Reverse transcription of 1 µg RNA and Q-RT-PCR were performed as described for the laser capture microdissected samples. RNA extracted from the right lung of an 8-wk allergen challenged lung was used as the standard.

Lung Histology and Morphometry

Lungs from an additional five mice per group were dissected, inflated with 10% buffered formalin, and processed as described by us in detail previously (17). Three-micrometer-thick transverse sections were cut and stained with hematoxylin and eosin, picrosirius red (to demonstrate collagen), and periodic acid Schiff (to demonstrate goblet cells). Immunohistochemistry using an antibody against α-smooth muscle actin (α-SMA, clone 1A4; DakoCytomation, Mississauga, ON, Canada), to identify contractile elements was performed with the Animal Research Kit (DakoCytomation). In addition, sections were stained with antibodies to phosphorylated Smad2 (phospho-Smad2; Cell Signaling Technology, Beverly, MA), using the streptavidin-biotin method with horseradish peroxidase, and 0.4 mg/ml aminoethylcarbazole (Sigma, Oakville, ON, Canada) as the chromogen. The nuclei were counterstained with hematoxylin, which, with the scarlet color of aminoethylcarbazole, resulted in reddish-black staining of positive nuclei. The purpose of the phospho-Smad2 staining was to detect cells in which TGF-β1 (or other family members) have signaled. Negative control sections were included in each set of slides. Morphometric quantification of the stained lung sections was performed using a customized digital image analysis system (Northern Eclipse; Empix Imaging Inc., Mississauga, ON, Canada), as described by us in detail previously (17, 23).

Electron Microscopy

The lungs of a further five mice per group were inflated with 2% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 24 h. Subsequently the left lung was sectioned through the hilum to obtain the first generation bronchus in cross-section, which was then dissected free from the parenchyma. The tissue was then rinsed in sodium cacodylate buffer, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in graded ethanol solutions, and embedded in Spurr's resin. Ultrathin sections (90 nm) were cut and placed on a 200-mesh thin bar copper grid and stained with uranyl acetate and lead citrate. The specimens were examined with a Philips CM10 transmission electron microscope.

Statistical Analysis

Reported values are expressed as mean and SEM. Comparisons between control mice and mice receiving allergen exposure, with respect to airway reactivity, BAL TGF-β1 (active and total) and TIMP-1, indices of airway remodeling and gene expression in airway tissue, were made using ANOVA or independent *t* tests depending on the number

of groups being compared. *Post hoc* multiple comparison testing was performed using Bonferroni's test to assess for significant effects. All comparisons were two-tailed, and *P* values < 0.05 were considered to be significant.

RESULTS

Chronic Allergen Exposure Results in Airway Remodeling and Sustained Airway Hyperresponsiveness

Goblet cells were significantly increased in the airway epithelium at 2 and 8 wk after chronic exposure to allergen compared with saline control mice (*P* < 0.001), as we have previously shown (17), although there were significantly less goblet cells at the 8 wk time-point than at the 2 wk time-point (*P* < 0.01) (Table 2). Similarly, at 2 and 8 wk after the final allergen exposure, there were significantly greater numbers of α-SMA positive cells (Table 2) present as well as increased ECM deposition (Figure 2B) compared with saline controls (*P* < 0.01). Ultrastructural examination confirmed the increase in goblet cells, fibroblasts, and collagen after prolonged allergen exposure (Figure 2D). In addition, the increased fibroblasts showed marked activation with enlarged nuclei, prominent nucleoli and increased fibronexin (data not shown).

Chronic allergen exposure resulted in sustained airway hyper-reactivity (slope of increase in R_{RS} [cm H₂O/ml/s] for a given increase in methacholine dose [mg/ml]), which persisted at 2 wk (mean 4.94 [± 0.36]) and 8 wk (mean 3.25 [± 0.3]) after final allergen exposure, as compared with saline control mice (2.23 [± 0.27] and 2.35 [± 0.2], respectively; *P* < 0.001). There was also a significant increase in the mean maximum inducible bronchoconstriction (maximum R_{RS}) at 2 (8.72 [± 0.4]) and 8 (7.06

TABLE 2. MORPHOMETRY OF AIRWAY WALL

	Saline Control	Time after Final Chronic Allergen Exposure	
		2 wk	8 wk
Goblet cells/mm	0 (0)	66.4 (10.5)*	14.2 (8.6)*†
Picrosirius red staining (%)	26.8 (6.7)	55.1 (3.0)*	51.1 (3.8)*
α-SMA staining (%)	7.2 (2.1)	28.9 (4.0)*	19.2 (2.9)*

Definition of abbreviation: α-SMA, α-smooth muscle actin.

Results are mean (SEM).

* *P* < 0.001 compared with saline control.

† *P* < 0.01 compared with 2 wk.

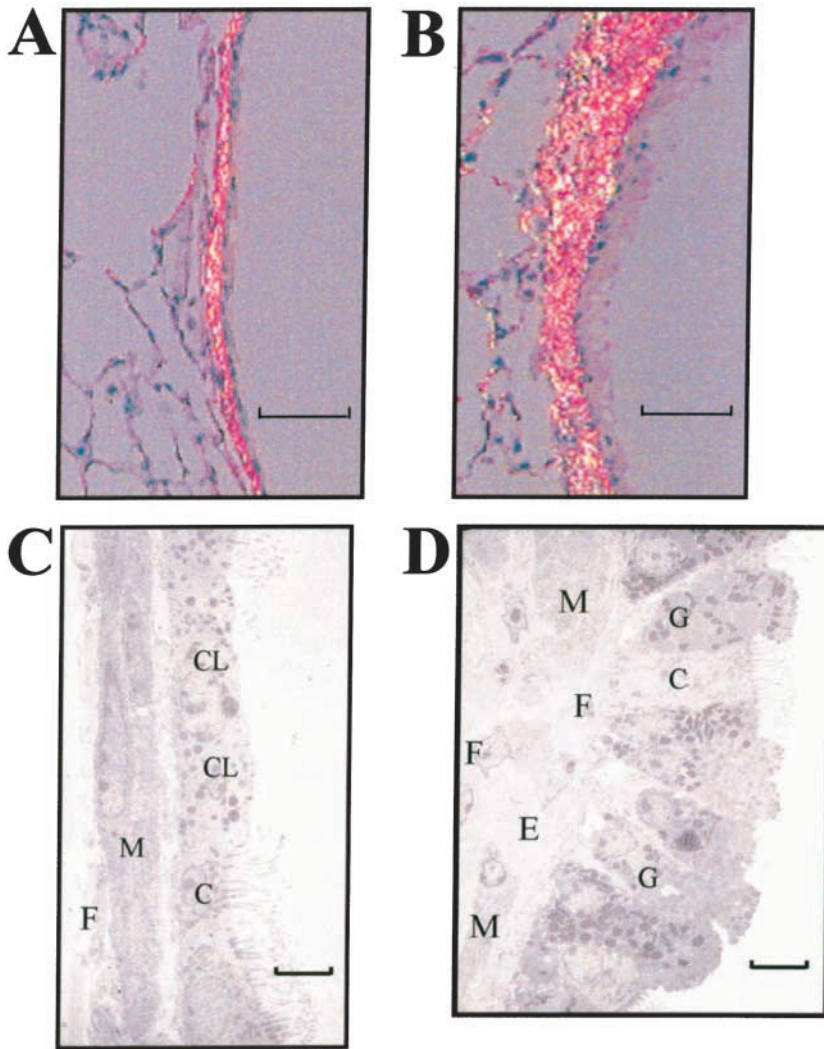


Figure 2. Cell types and extracellular matrix in airway wall following chronic exposure to saline or allergen. Airway wall stained with picrosirius red and viewed with polarized light to demonstrate increased extracellular matrix at 2 wk after final chronic exposure to allergen (*B*) compared with saline (*A*) (Bars indicate 100 μ m). Ultrastructural examination of airway wall at 2 wk after final chronic exposure to allergen revealed increased fibroblasts, extracellular matrix, and goblet cells (*D*) compared with airway wall after saline exposure (*C*), in which there were scanty fibroblasts and goblet cells (Bars indicate 5 μ m). F, fibroblasts; E, extracellular matrix; M, smooth muscle; G, goblet cells; C, ciliated cells; CL, clara cells.

[$\pm$ 0.28] weeks after final allergen exposure, compared with saline control mice (4.7 [$\pm$ 0.43] and 5.28 [$\pm$ 0.31]) at these same time points ($P < 0.001$).

Whole Lung Homogenates Show No Significant Differences in Gene Expression

When mRNA was extracted from whole lung and subjected to Q-RT-PCR, expression of TGF- β 1, PAI-1, and TIMP-1 in whole

lung homogenates was not significantly different between the 2 or 8 wk after allergen exposure groups or the saline controls (Figure 3).

Gene Expression Is Upregulated in Microdissected Airway Wall after Chronic Allergen Exposure

Gene expression of TGF- β 1, PAI-1, TIMP-1, CTGF α -SMA, fibronectin, and procollagen 1 α 2 was significantly increased in

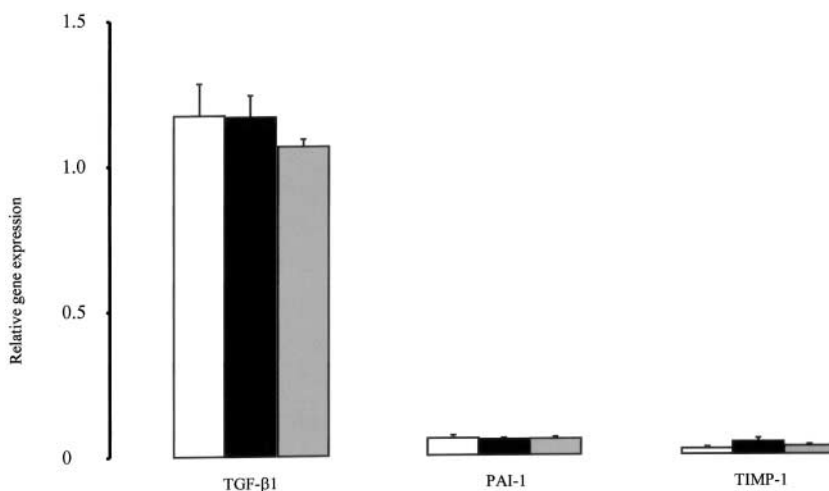


Figure 3. Gene expression profile of whole lung. Semi-quantitative gene expression in whole right lung. No significant difference was detected between the different groups. Gene expression normalized to β 2-microglobulin. Bars represent mean and SEM. TGF- β 1, transforming growth factor- β 1; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of matrix metalloproteinase-1. Open bars, 2 wk after saline exposure; black bars, 2 wk after chronic allergen exposure; gray bars, 8 wk after chronic allergen exposure.

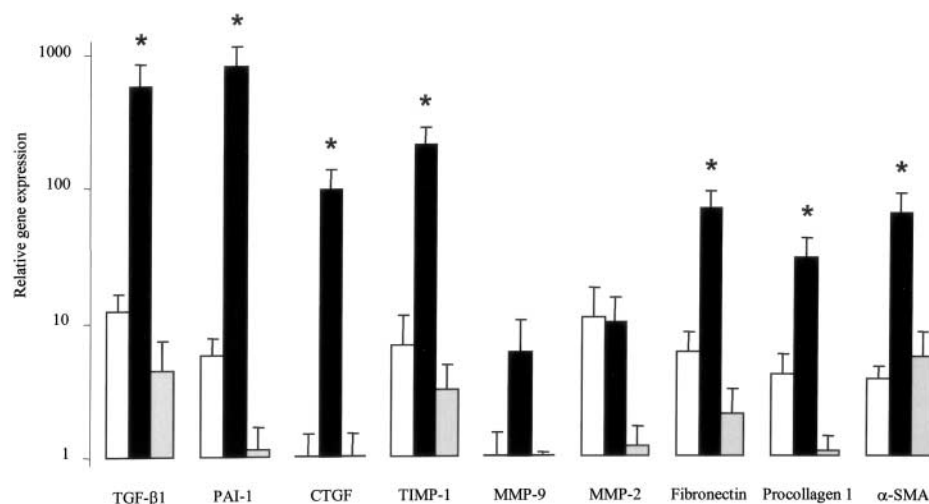


Figure 4. Gene expression profile of microdissected airway wall. The expression of TGF-β1, PAI-1, CTGF, TIMP-1, fibronectin, procollagen 1, and α-SMA were significantly increased 2 wk after final chronic allergen exposure (black bars), compared with saline control (open bars). Gray bars, 8 wk after chronic allergen exposure. Note that the x axis has been logarithmically transformed. Gene expression normalized to β2-microglobulin. Bars represent mean and SEM. * $P < 0.05$ compared with postsaline control.

the microdissected airway wall 2 wk after chronic allergen exposure, compared with saline controls ($P < 0.05$) (Figure 4). This was in marked contrast the lack of significant results when whole lung was examined. There was no significant difference in gene expression of matrix metalloproteinase (MMP)-9 or MMP-2, nor in gene expression between the group with no intervention and the saline control groups (data not shown). The upregulation in fibrosis-related genes in the airway wall seen at 2 wk after chronic allergen exposure was no longer evident at 8 wk after exposure. Therefore, only the airway wall at 2 and not 8 wk after allergen exposure was further microdissected.

Gene Expression of TGF-β1 and PAI-1 Are Upregulated in Microdissected Epithelium after Chronic Allergen Exposure

To determine which airway cells type(s) showed upregulated gene expression at 2 wk after chronic allergen exposure, airway epithelium and smooth muscle were (separately) microdissected (Figure 1B). Gene expression of TGF-β1 and PAI-1 was significantly increased in the epithelial cell layer compared with saline controls ($P < 0.05$) (Figure 5), and there was a trend to increased gene expression of CTGF ($P = 0.09$). In contrast, there was no significant difference in gene expression in smooth muscle cells recovered from the airways of allergen-exposed and saline-exposed mice (Figure 5).

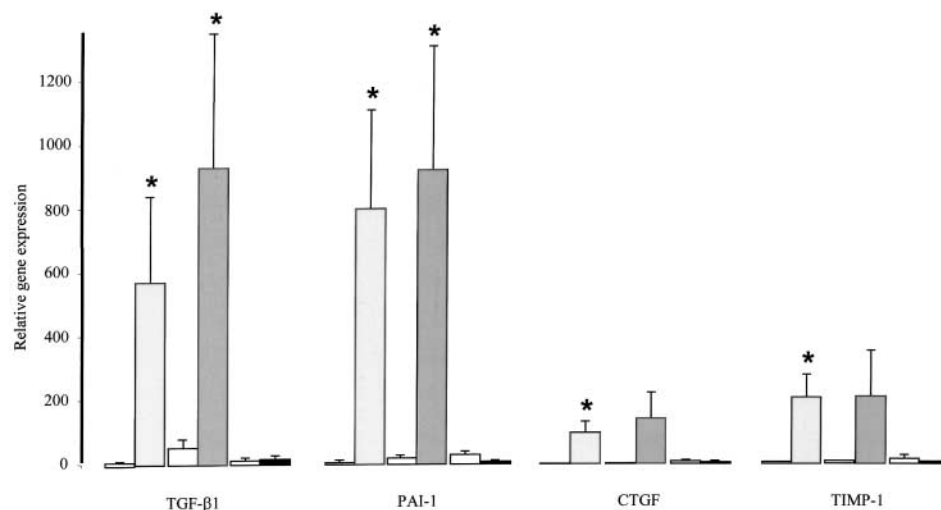


Figure 5. Comparison of gene expression profile of microdissected airway wall, epithelium, and smooth muscle. TGF-β1 and PAI-1 gene expression were significantly increased at 2 wk after final chronic allergen exposure compared with saline control in the epithelial layer, but not the smooth muscle. Gene expression normalized to β2-microglobulin. Bars represent mean and SEM. * $P < 0.05$. Open bars, all tissues after saline exposure; light gray bars, total airway wall after allergen exposure; dark gray bars, epithelium after allergen exposure; black bars, muscle after allergen exposure.

TGF-β1 and TIMP-1 Levels Are Increased in BAL Fluid after Chronic Allergen Exposure

In an effort to correlate protein expression with gene expression at 2 wk after chronic allergen exposure, we measured protein levels of TGF-β1 and TIMP-1 in the BAL fluid and found them to be significantly increased compared with saline controls. Furthermore, levels of active as well as total TGF-β1 were found to be increased (Table 3).

Phospho-Smad2 Is Increased after Chronic Allergen Exposure

Immunohistochemistry for phospho-Smad2 in the 2-wk-post-saline control sections was mostly negative, with only the occasional alveolar cell nucleus being positively stained (Figure 6A). In contrast, sections from the 2-wk-post-chronic allergen exposure group showed numerous positively stained nuclei (Figure 6B). Over 99% of airway epithelial cell nuclei were positive, and numerous smooth muscle cells, fibroblasts, endothelial cells, and alveolar cells were also positive. The majority of the infiltrating inflammatory cells were also positive.

DISCUSSION

In this mouse model of chronic allergen exposure, airway remodeling was present by 2 wk after the final allergen challenge and

TABLE 3. BRONCHOALVEOLAR FLUID LAVAGE: CYTOKINE LEVELS 2 WK AFTER FINAL CHRONIC ALLERGEN EXPOSURE

	Saline Control	Allergen
TGF- β 1 (total)	7.7 (7.5)	94.4 (32.3)*
TGF- β 1 (active)	6.0 (3.9)	40.8 (14.8)*
TIMP-1	27.6 (5.8)	193.5 (40.2)†

Definition of abbreviations: TGF- β 1, transforming growth factor- β 1; TIMP-1, tissue inhibitor of metalloprotease-1.

Results are pg/ml expressed as mean (SEM).

* $P < 0.05$ compared with saline control.

† $P < 0.01$ compared with saline control.

was associated by sustained AHR. At this time point, in contrast to the examination of whole lung homogenates, in which there was no evidence of gene regulation to account for tissue remodeling (Figure 3), gene expression of TGF- β 1, PAI-1, CTGF, TIMP-1, α -SMA, procollagen 1, and fibronectin was markedly upregulated within the microdissected airway wall (Figure 4), evidence of the presence of a dynamic remodeling process. Although airway remodeling was still present 8 wk after the final allergen challenge, gene expression was comparable to that in saline-challenged animals, indicating a low rate of turnover of ECM, which was consistent with the low levels of profibrotic genes also present at this time (Figure 4). Having detected upregulated gene expression in the airway wall, we examined samples of pure cell populations of airway epithelium and smooth muscle at the 2 wk time point (Figure 1), and identified the airway epithelium as the main source of gene expression of TGF- β 1, PAI-1, CTGF, and TIMP-1 (Figure 5).

The upregulated gene expression at the 2 wk time point was accompanied by elevated levels of TGF- β 1 (active and inactive) and TIMP-1 protein recovered in BAL fluid. Upon activation of type I TGF- β receptors, Smad proteins are phosphorylated and translocate to the nucleus. The nuclear immunoreactivity of epithelial cells for phospho-Smad2 at the 2 wk time point is evidence that TGF- β or Activin (another member of the TGF- β superfamily) have been active in the cell (24), in contrast to saline controls, in which only occasional cells in the parenchyma were positive (Figure 6). In another model of allergic airway inflammation (25), as well as in a study of bronchial biopsies from subjects with asthma (26), bronchial epithelial cells and fibroblasts also showed strong immunoreactivity for phospho-

Smad2. In the latter study, the presence of cells positive for phospho-Smad2 staining correlated with the extent of airway remodeling and AHR (26). We take the positive phospho-Smad2 staining and the increased levels of active TGF- β 1 found in the BAL fluid as evidence that the upregulation of TGF- β 1 gene expression is related to an increase in secretion of active TGF- β 1. In further support of these observations, Kumar and coworkers (10), using a different murine model of chronic allergen exposure, demonstrated the presence of active TGF- β 1 protein within the airway epithelium, which was related to the development of airway remodeling.

AHR can be divided into a variable component, attributed to fluctuating degrees of immune-mediated airway inflammation, and a sustained component that is relatively independent of acute inflammatory events and which persists despite corticosteroid therapy (18, 19, 27). Airway remodeling, which is also relatively resistant to treatment with corticosteroids, may be responsible for this sustained component of AHR (2, 3). This model was therefore designed to explore the mechanisms involved in airway remodeling after the acute inflammation had subsided (17).

The findings of upregulated expression of profibrotic mediators in the airway epithelium alongside active tissue remodeling, despite resolution of the acute immune-mediated inflammatory response, suggest that the epithelium is playing a prominent role in the maintenance of the remodeling process in this model. This is in keeping with a study in which epithelial cells in explanted lung depleted of inflammatory cells produced large quantities of biologically active TGF- β 1, resulting in pulmonary fibrosis (28). Further evidence that expression of TGF- β 1 may be independent of inflammatory cells is that increased expression of TGF- β 1 in individuals with asthma (20) and in animal models of fibrosis (29) is not suppressed by high doses of corticosteroids.

We did not examine the initiating mechanisms responsible for gene upregulation in the epithelium, as we only examined gene expression after the onset of airway remodeling. However, there is substantial evidence that Th2-mediated airway inflammation is involved in this process (10, 30, 31). Previous publications from our group have shown that Th2 cytokines are critical for the development of airway remodeling and AHR in this animal model (32), but, once these are established, Th2-mediated inflammation is not required for the persistent airway remodeling or sustained AHR (33, 34). Thus, the findings in this paper are consistent with our previous published observations.

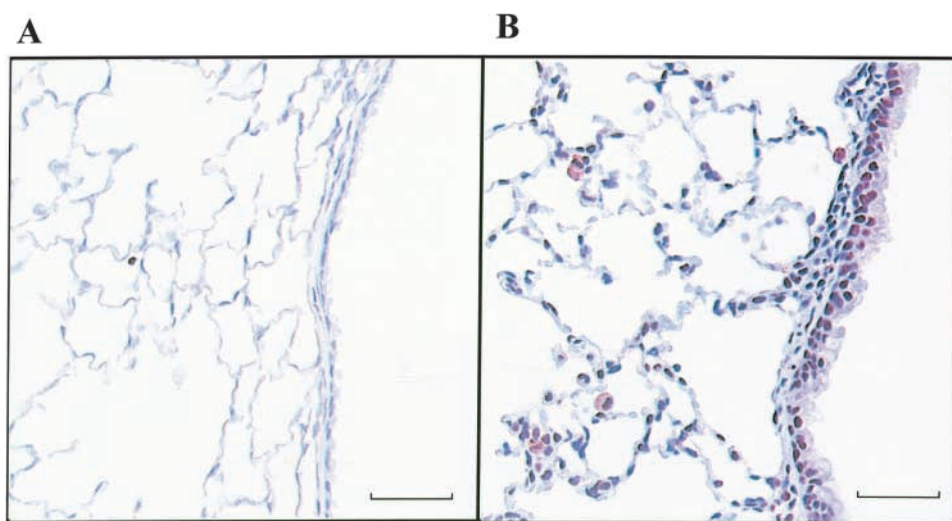


Figure 6. Phospho-Smad2 immunohistochemistry. Representative sections of airway wall and adjacent parenchyma stained with phospho-Smad2 antibody 2 wk after final chronic saline (A) and chronic allergen (B) exposure. The color developed with aminoethylcarbazole was dark red. Almost 100% of epithelial cell nuclei are positively stained in the 2 wk after chronic allergen exposure group (arrowheads); in contrast, the saline control group shows little positive staining. Bars indicate 100 μ m. Inflammatory cells with positive nuclei are marked by arrows.

TGF- β 1 is an obvious candidate to play a central role in airway remodeling. It promotes the synthesis and secretion of ECM, is chemotactic for both monocytes and fibroblasts, induces the expression of α -SMA, TIMPs, and PAI-1, downregulates expression of MMP-1 and -3, and induces hyperplasia and hypertrophy of airway smooth muscle cells (35). Transient overexpression of active TGF- β 1 in the rodent lung induces a chronic progressive fibrotic response (7), and these, and other animal studies, have established TGF- β 1 as the major profibrotic cytokine in the pathogenesis of pulmonary fibrosis.

There are conflicting results from different studies of the role of TGF- β 1 in airway remodeling, and this is at least partly due to different types of animal models being used (36). In a study of acute allergen exposure (three challenges over 3 d), the expression of latent TGF- β 1 provided a protective role during OVA-induced airway inflammation (8), and transgenic mice with impaired TGF- β signaling in T cells showed increased AHR upon antigen challenge compared with wild-type mice (37). In contrast, we observed that increased TGF- β 1 was associated with remodeling and AHR. Our model is fundamentally different to those involving short-term allergen challenge protocols, in that AHR and tissue remodeling are *sustained* for at least 8 wk after final exposure to allergen. Thus, different mechanisms are likely to be active in these models.

Our findings are in keeping with other animal studies, in which the accumulation of active TGF- β 1 within the airway wall was correlated with the degree of airway remodeling (10), and TGF- β 1 administered intratracheally to BALB/C mice produced airway fibrosis with increased AHR (11). In studies in human subjects with asthma, increased TGF- β 1 mRNA and protein have been demonstrated within the bronchial mucosa and BAL fluid, and their levels correlated with the thickness of the lamina reticularis (12–14). Cell culture studies have demonstrated that epithelial cells obtained from individuals with asthma before and after allergen challenge are able to stimulate lung myofibroblast collagen production (38). Our results extend this understanding by providing novel information with regard to the levels of gene expression of specific cells (epithelium and smooth muscle) within the airway wall in an *in vivo* model of airway remodeling.

Although we did not specifically look for epithelial expression of other profibrotic mediators such as interleukin-1, platelet-derived growth factor, or insulin-like growth factor-1, there is evidence that these are induced by TGF- β 1 (39, 40). In addition, the marked upregulation of CTGF expression suggests that TGF- β 1 is responsible for the observed subepithelial fibrosis, because collagen synthesis mediated by cytokines other than TGF- β 1 does not induce CTGF (41, 42). Eosinophils may also be a major source of TGF- β and their numbers correlate with subepithelial fibrosis in some studies (12, 43–45). However, because the acute inflammatory infiltrate had largely subsided at the time of sampling, it is unlikely that they would be playing a significant role at this time in this particular model. This is consistent with the findings of Kumar and colleagues (10), who concluded that eosinophils were not a significant source of TGF- β in their chronic model of allergen challenge.

Gene expression of MMP-2 and MMP-9 were not significantly different at any time point compared with controls, and with the increased expression of TIMP-1 this suggests that an environment favoring deposition rather than degradation of fibrillar collagen is present, which is in agreement with the increased TIMP:MMP ratios noted in sputum and BAL fluid of individuals with asthma (46, 47). PAI-1, through its inhibition of plasmin, results in activation of TIMPs (48), and it has been strongly correlated with the degree of ECM accumulation (49). In addition, PAI-1-deficient mice have recently been shown to have

less deposition of ECM after OVA challenge than wild-type controls (50).

We did not find airway smooth muscle to be a significant source of profibrotic cytokines at the 2 wk postexposure time point, although it has previously been shown that CTGF expression is upregulated in airway smooth muscle cells *in vitro* by TGF- β (51). Heavy chain myosin expression is normally confined to muscle and, unlike α -SMA, has not been described as being expressed in activated fibroblasts. Significantly higher levels of myosin heavy chain gene expression were present in the smooth muscle than in the epithelium (mean relative gene expression [SEM] 10.8 [$\pm$ 5.8] versus 1.8 [$\pm$ 1.0], respectively [$P < 0.05$]). Although it cannot be concluded that the microdissected airway smooth muscle sample was entirely homogeneous, the data suggests that it contained at least some smooth muscle and was not significantly contaminated by fibroblasts or epithelium.

The failure to detect upregulation of α -SMA and myosin heavy chain II expression in airway smooth muscle is in agreement with another study that examined microdissected bronchial smooth muscle from subjects with asthma and normal subjects (52), and found no difference in the expression of genes encoding for structural and contractile proteins. However, limiting quantities of RNA prevented the examination of other genes such as rho A, calponin, smooth muscle γ -actin, or desmin, which would have allowed a more comprehensive measure of the role of airway smooth muscle. In addition, this model may not be fully representative of the smooth muscle changes present in asthma, because we were unable to demonstrate conclusively that the increased α -SMA staining on light microscopy represented increased airway smooth muscle cells and/or myofibroblasts.

This study emphasizes the importance of targeting specific cell types that may be important in the process of airway remodeling. Because this is a process mainly confined to the airway wall, it makes biological sense to examine the gene expression of individual cell types within the airway wall, which might otherwise be masked by examination of the whole lung. The utility of this approach is illustrated by another study in which the examination of whole lungs infected with *Mycobacterium tuberculosis* failed to detect the specific gene profile found when granulomas were microdissected from surrounding lung and examined separately (53).

The use of LCM with T7-based RNA amplification has been validated as a method to amplify starting mRNA by $\sim 1,000$ times while maintaining representation of the starting mRNA population which is essential for quantification (54–57). We elected to use LCM with quantitative RT-PCR instead of *in situ* hybridization (ISH), although the technical demands of LCM (the requirement for fresh frozen tissue for optimal RNA integrity, time-consuming microdissection and RNA processing, the need for an operator experienced in scope optics) mean that LCM is not necessarily more convenient. However, LCM with quantitative RT-PCR has advantages over ISH in that it allows the examination of several different genes simultaneously and also provides a quantitative measurement.

The choice of β 2-microglobulin, an internal reference gene to normalize gene expression, was based on a review of the literature, which indicated that it was relatively stable, unlike two other commonly used housekeeping genes, GAPDH and β -actin (58). 18S rRNA was not employed as a housekeeping gene since the samples in this study were enriched for mRNA with loss of rRNA. For increased confidence in the validity of the internal reference used, a second housekeeping gene, the ribosomal protein, L32, was also assayed. The levels of L32 showed good correlation with those of β 2-microglobulin ($R^2 = 0.87$), in contrast to the poor correlation of GAPDH levels with β 2-microglobulin ($R^2 = 0.15$). In addition, the correlation of L32

with $\beta 2$ -microglobulin was excellent in all the tissues examined, airway wall, smooth muscle and epithelium, and, when the genes of interest were normalized to L32, the results were similar to those presented using $\beta 2$ -microglobulin as the reference gene (data not shown).

In conclusion, we have demonstrated significant upregulation of gene expression of profibrotic factors (TGF- $\beta 1$, PAI-1, CTGF, TIMP-1) and structural proteins (α -SMA, procollagen 1, fibronectin) in the airway wall at 2 wk after the last allergen challenge, after the acute inflammatory response had subsided. MMP-2 and MMP-9 mRNA was not significantly elevated, suggesting a prevailing nondegradative environment. When we examined microdissected airway epithelium and smooth muscle, we found significantly increased levels of mRNA for TGF- $\beta 1$ and PAI-1 in the epithelium, with no difference in gene expression in the smooth muscle compared with controls. TGF- $\beta 1$ expression by the epithelium appears to play an important role in the maintenance of airway remodeling in this model and, although it is recognized that these findings cannot be directly extrapolated to human asthma, they may be related to the airway remodeling seen in individuals with asthma, which is relatively resistant to anti-inflammatory treatment. The secretion of TGF- $\beta 1$, or other profibrotic mediators induced by TGF- $\beta 1$ by the airway epithelium may be relevant targets for future therapeutic options in asthma.

Conflict of Interest Statement: M.M.K. has no declared conflicts of interest; R.L. has no declared conflicts of interest; P.B. has no declared conflicts of interest; R.E. has no declared conflicts of interest; J.W. has no declared conflicts of interest; M.J.S. has no declared conflicts of interest; G.M. has no declared conflicts of interest; M.P. has no declared conflicts of interest; M.D.I. has no declared conflicts of interest; and J.G. has no declared conflicts of interest.

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