Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics

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Eosinophil-derived TGF-β has been implicated in remodeling events in asthma. We hypothesized that reduction of bronchial mucosal eosinophils with anti–IL-5 would reduce markers of airway remodeling. Bronchial biopsies were obtained before and after three infusions of a humanized, anti–IL-5 monoclonal antibody (mepolizumab) in 24 atopic asthmatics in a randomized, double-blind, placebo-controlled study. The thickness and density of tenascin, lumican, and procollagen III in the reticular basement membrane (RBM) were quantified immunohistochemically by confocal microscopy. Expression of TGF-β1 mRNA by airway eosinophils was assessed by in situ hybridization, and TGF-β1 protein was measured in bronchoalveolar lavage (BAL) fluid by ELISA. At baseline, airway eosinophil infiltration and ECM protein deposition was increased in the RBM of asthmatics compared with nonasthmatic controls. Treating asthmatics with anti–IL-5 antibody, which specifically decreased airway eosinophil numbers, significantly reduced the expression of tenascin, lumican, and procollagen III in the bronchial mucosal RBM when compared with placebo. In addition, anti–IL-5 treatment was associated with a significant reduction in the numbers and percentage of airway eosinophils expressing mRNA for TGF-β1 and the concentration of TGF-β1 in BAL fluid. Therefore eosinophils may contribute to tissue remodeling processes in asthma by regulating the deposition of ECM proteins.

Introduction

Allergic asthma is characterized by eosinophilic airway inflammation and structural changes in the airway wall termed remodeling (1). This remodeled phenotype, which may be the consequence of excessive repair processes following repeated airway injury, includes increased deposition of ECM proteins in the reticular basement membrane (RBM) (2) and in the bronchial submucosa (3). These ECM proteins include procollagen III (the mature precursor for collagen III) and the proteoglycans, tenascin, and lumican (2, 4–6). In addition to their structural role, ECM proteins may also influence cellular function including adhesion, differentiation, and survival (7, 8). Other features of remodeling include increased smooth muscle mass (9), goblet cell hyperplasia (10), and new blood vessel formation (11).

There is increasing evidence that the eosinophil may be important in the pathophysiology of airways remodeling. For example, thickening of the subepithelial basement membrane was associated with increases in bronchial mucosal eosinophils in severe asthmatics (12). Tissue eosinophilia and eosinophil degranulation have been associated with several fibrotic syndromes (13–15), and the cell is a source of several molecules implicated in tissue remodeling processes such as TGF-α (16), TGF-β (17), VEGF (18), matrix metalloperoxidase-9 (19), tissue inhibitor of metalloproteinase-1 (20), and IL-13 (21). TGF-β is a potent regulator of fibroblast/myofibroblast function with the production of several ECM proteins including collagens, proteoglycans, and tenascin (22, 23).

Our group reported that eosinophil-derived TGF-β1 is temporally associated with myofibroblast formation and the expression of tenascin and procollagen-I by fibroblasts in allergic inflammation in vivo in human atopic skin (24). Direct evidence for the production of these repair markers was obtained in vitro by coculturing

eosinophils and fibroblasts. In addition, eosinophil-induced myofibroblast formation was associated with the generation of tenascin transcripts and protein product, which was could be inhibited by neutralizing antibodies to TGF-β. Interleukin-5 is essential for the terminal differentiation of the committed eosinophil precursor (25). It is also involved in eosinophil migration and priming (26) and prolongs the survival of the cell in tissues (27). More recently, monoclonal antibodies to IL-5 have been prepared and administered as a single intravenous infusion to both mild atopic (28) and chronic, severe asthmatics (29). These antibodies have produced no appreciable effects on late asthmatic reaction, airway hyperresponsiveness, or other clinical outcomes including lung function. However, although anti–IL-5 almost totally ablated eosinophils in the blood and sputum (28), tissue eosinophils were reduced rather than depleted (30), possibly as a result of downregulated IL-5Rα expression of airway eosinophils (31, 32). Our own group has been able to partially and selectively deplete eosinophils from the airway mucosa of mild atopic asthmatics using three intravenous infusions of an anti–IL-5 monoclonal antibody given at monthly intervals (30). In the present study, we have used these biopsy samples to show that, even with the modest reduction in bronchial mucosal eosinophils achieved (median decrease of 55%), this was associated with significant decreases in the deposition of the ECM proteins within the reticular basement membrane.

Methods

Study population and design

Normal nonatopic human volunteers were recruited by advertisement. The asthmatics were part of a study that has been described in detail elsewhere (30). Briefly, 24 mild atopic asthmatic volunteers, treated only with β_2 agonists as required, completed the study. Eleven were randomized to receive mepolizumab and 11, placebo. Both groups were well matched in terms of age, sex, forced expiratory volume (FEV_1), peak expiratory flow rate (PEFR), and histamine PC_{20}.

Seven to 14 days after screening, fiberoptic bronchoscopy with endobronchial biopsy and bronchoalveolar lavage (BAL) were performed, and 2 days later the study medication (placebo or active drug) was given. This was administered as an intravenous infusion over 30 minutes in a double-blind fashion. The second and third infusions of study drug were given 4 and 8 weeks following the first infusion. Bronchoscopy was repeated 2 weeks after the final drug infusion.

Bronchial mucosal biopsies were taken from lower-lobe segmental and subsegmental carinae. Whether the pretreatment biopsies were taken from the right or left side was determined randomly. The post-treatment biopsies were then taken from the other side. Bronchial biopsies were also taken from the 10 normal (nonatopic, nonasthmatic) subjects.

The study was approved by the ethics committees of the Royal Brompton & Harefield NHS Trust and the London Chest Hospital, and all volunteers gave informed consent prior to participation.

Tissue processing, immunohistochemistry, confocal microscopy

Bronchial mucosal eosinophils. Bronchial mucosal biopsies were fixed in 4% paraformaldehyde (VWR, Leicester, UK) before being mounted in OCT (optimum cutter) temperature (Bayer UK Ltd., Basingstoke, United Kingdom) and snap-frozen in isopentane (BDH) precooled with liquid nitrogen. Briefly, 6-μm–thick sections were incubated with a mouse monoclonal antibody against major basic protein (MBP) (BMK 13; in-house) and developed using a modification of the alkaline phosphatase antialkaline phosphatase (APAAP) technique as described previously (33). Appropriate isotype controls were included (Dako Cytomation, Cambridge, United Kingdom). The number of cells per square millimeter was determined by counting whole sections in a blinded fashion at ×400 magnification using an Olympus BH-2 microscope (Olympus, New York, New York, USA).

Reticular basement membrane: tenascin, procollagen III, and lumican. Tissue sections were pretreated with PBS for 30 minutes and incubated overnight at room temperature in a humidified chamber with a mouse antibody against tenascin (Monosan, Uden, The Netherlands), rabbit antibody against procollagen-III (Chemicon International, Harrow, United Kingdom), or lumican (a generous gift of Peter Roughley, Shriner's Hospital for Crippled Children, McGill University, Montreal, Quebec, Canada). After extensive washing, sections were incubated with either a FITC-conjugated goat antimouse Ab (Dako Ltd.) or FITC-conjugated swine antirabbit Ab (Dako Ltd.) at a 1:30 dilution for 1 hour. Sections were then incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Gillingham, United Kingdom) at 1 μg/mL for 5 minutes as a counterstain (to avoid measurement of intracellular protein) and mounted in fluorescent mounting medium (Dako Ltd.). Appropriate isotype controls were included (IgG1, Dako Ltd.). Images were acquired using a Leica TCS SP confocal microscope (Leica Microsystems Ltd., Heidelberg, Germany). The microscope settings were standardized to allow comparison of immunoreactivity intensity among different sections.

Measurements were analyzed using the Scion Image Analysis software package (Scion Corp., Frederick, Maryland, USA). The thickness and density of immunoreactivity in the RBM area was calculated by taking multiple measurements over the length of the biopsy (40–90 measurements) at 20-μm intervals. At each measurement, a line was drawn perpendicular to and across the band of immunoreactivity in the RBM. The image analysis software calculated the length of the line (thickness) and the mean density along the line (pixels per square microns). The values were averaged
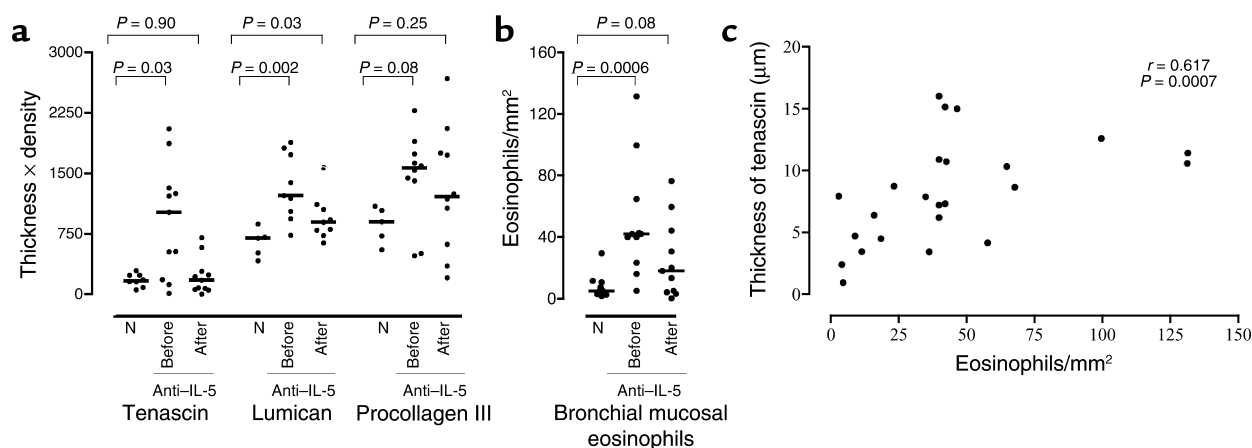
Figure 1
(**a**) A comparison of tenascin, lumican, and procollagen III expression in the reticular basement membrane between normal controls (*n* = 10) and asthmatic individuals (*n* = 11) before and after anti–IL-5. N denotes normal subjects. The crossbars represent the median values. (**b**) A comparison of the number of MBP⁺ eosinophils in the bronchial mucosa of normal controls and asthmatic subjects at baseline. (**c**) The correlation between eosinophil numbers and thickness of tenascin in the reticular basement membrane at baseline.

over the whole length of the RBM to give mean thickness and density of immunoreactivity. The product of thickness and density ($t \times d$) was taken as a measure of expression of the matrix proteins in the RBM. Thickness × density is a derivative of an equation: mass = density × volume. Therefore, $t \times d$ is proportional to the mass (or, alternatively, total expression) of matrix protein per unit length of RBM in each biopsy. The intraobserver error for tenascin thickness was ±8.7%, and tenascin density was ±7.3%. This is consistent with previous studies measuring the thickness of tenascin immunoreactivity in the RBM (5, 34).

In situ hybridization and immunohistochemistry. The cDNA fragment encoding human TGF-β1 was inserted into a pGEM RNA expression vector (Promega UK Ltd., Southampton, United Kingdom). ³⁵S-labeled riboprobes were prepared as previously described (33). TGF-β1 mRNA, double immunohistochemistry/in situ hybridization (ISH) was performed as previously described (33). Sections were first stained with anti-EG2 (Pharmacia Biotech, Milton Keynes, Bucks, United Kingdom) using the APAAP technique, followed by

ISH (33). For autoradiography, slides were dipped into K-5 emulsion (Ilford Ltd., Basildon, United Kingdom) and exposed at 4°C for 3 weeks in complete darkness in a desiccated environment, and the slides were then developed in D-19 developing solution (Eastman Kodak Co., Rochester, New York, USA). Eosinophils were red and TGF-β1 mRNA⁺ signals appeared as silver grains (33). Whole sections were counted in duplicate by investigators unaware of the patients' clinical status, using an eyepiece graticule as previously described (33). Results were expressed as the total number of positive cells per square millimeters of biopsy. The percentage of eosinophils that were TGF-β1 mRNA⁺ was also calculated.

Bronchoalveolar lavage fluid TGF-β1. BAL fluid was passed through a 50-μm filter to remove mucus, centrifuged at 800 *g* at 4°C, and the supernatant was stored at 80°C until use. BAL fluid was concentrated 5–30 times at room temperature using Centiplus YM-10 concentrators (Amicon Inc., Beverly, Massachusetts, USA) according to the manufacturer's instructions. TGF-β1 levels in the concentrated BAL

Table 1
The effect of mepolizumab on tenascin, lumican, and procollagen III expression

		Mepolizumab			Placebo			
		Before	After	*P* value	Before	After	*P* value	*P* value^A
Bronchial eosinophils		42.0 (5.0–131.3)	17.9 (4.5–37.3)	0.007	36.2 (2.9–131.2)	61.7 (38.4–86.0)	0.06	0.009
Tenascin	Thickness	10.3 (8.1–12.2)	5.2 (4.7–6.2)	0.02	6.2 (4.2–8.6)	7.8 (7.0–9.8)	0.19	0.008
	Density	96.7 (26.3–137.0)	34.0 (11.7–50.0)	0.01	30.5 (18.2–42.3)	46.3 (30.3–76.6)	0.1	0.003
Lumican	Thickness	10.8 (8.9–11.9)	9.0 (8.2–10.4)	0.3	9.8 (9.4–10.5)	10.1 (9.2–10.8)	1.0	0.16
	Density	135.5 (101–147)	102.3 (89–118)	0.05	127.5 (95–141)	120.3 (96–246)	0.98	0.05
Procollagen III	Thickness	10.4 (9.7–11.1)	10.2 (7.9–11.6)	0.43	9.9 (8.5–11.4)	10.8 (9.8–13.2)	0.08	0.22
	Density	155.8 (135–169)	140.0 (103–160)	0.1	151 (135.0–179)	177.6 (151–189.5)	0.28	0.05

Median value (interquartile range). At baseline (before mepolizumab vs before placebo), there were no significant differences in the density or thickness of tenascin, lumican, or procollagen III. ^ADenotes *P* value between groups.

fluid were then determined using a commercial ELISA kit (R & D Systems Europe Ltd., Abingdon, United Kingdom).

Statistical analysis. Nonparametric statistical methods were used for within-group paired comparisons (Wilcoxon signed rank test) and for between-group comparisons to test the effect of mepolizumab with that of placebo treatment or normal subjects (Mann-Whitney U test). Correlations were analyzed using Spearman's rank correlation coefficient. A P value of ≤ 0.05 was considered to be significant.

Results

Tenascin, lumican, and procollagen III in normal subjects and mild asthmatics. First we sought to confirm that eosinophil infiltration of the bronchial mucosa in asthma was associated with increased deposition of ECM proteins. The total expression (thickness × density) of tenascin ($P = 0.03$) and lumican ($P = 0.002$) was significantly increased in mild asthmatics when compared with normal subjects, but the changes for procollagen were more variable and did not achieve significance (Figure 1a). Eosinophil numbers were also significantly higher in asthmatics versus normal subjects ($P = 0.0006$; Figure 1b). Immunoreactivity of tenascin and lumican was principally localized to the subepithelial area within the RBM. In the asthmatics, at baseline, there was a positive correlation between the thickness (Figure 1c), density, and total expression of tenascin and the bronchial mucosal eosinophil numbers ($P = 0.0007$, $r = 0.617$; $P = 0.039$, $r = 0.3661$; $P = 0.02$, and $r = 0.42$, respectively). These associations were not observed with lumican. There was also no significant correlation between the thickness, density, or total expression of tenascin or lumican and baseline PEFR, FEV_1, or histamine PC_{20}.

Effect of anti–IL-5 (mepolizumab) on bronchial mucosal eosinophils and tenascin, lumican, and procollagen III expression in asthma. As previously reported (30), there was a significant decrease in the number of bronchial mucosal eosinophils in asthmatics treated with anti–IL-5 antibody (mepolizumab) compared with placebo ($P = 0.009$; Table 1). This was associated with a significant decrease in both the thickness ($P = 0.008$) and density ($P = 0.003$) of tenascin immunoreactivity and in the density of lumican ($P = 0.05$) and procollagen III ($P = 0.05$) immunoreactivity in the RBM when compared with placebo (Table 1). The total expression (thickness × density) is shown in Figure 2. There were significant decreases in tenascin and lumican in the mepolizumab-treated group both within the treated group ($P = 0.01$ and 0.05, respectively) and when compared with placebo ($P = 0.004$ for tenascin and 0.008 for lumican). There was no significant reduction in procollagen III expression in patients treated with anti–IL-5, but there was a significant reduction when compared with placebo ($P = 0.007$), with a trend to increased expression in the placebo group. There was no significant difference between tenascin expression

in asthmatics after mepolizumab therapy and the expression of this ECM protein in nonasthmatic. nonatopic controls (Figure 1a).

Figure 3 shows representative photomicrographs of tenascin and lumican immunoreactivity in the bronchial mucosa from subjects before and after treatment with mepolizumab. Examples of procollagen III immunoreactivity are also shown. There was no immunoreactivity observed in sections using appropriate isotype controls for tenascin, lumican, and procollagen.

Effect of anti–IL-5 treatment on TGFβ1 expression by eosinophils in asthma. At baseline, a median of 86% of $TGF\beta1mRNA^+$ cells in the bronchial mucosa of the asthmatic subjects were identified as eosinophils. Following mepolizumab treatment, the numbers of $TGF\beta1^+$ eosinophils decreased significantly ($P = 0.03$), compared with placebo (Figure 4a). Similarly, a median of 76% of eosinophils were TGF-β1mRNA$^+$ at baseline and this decreased significantly, compared with
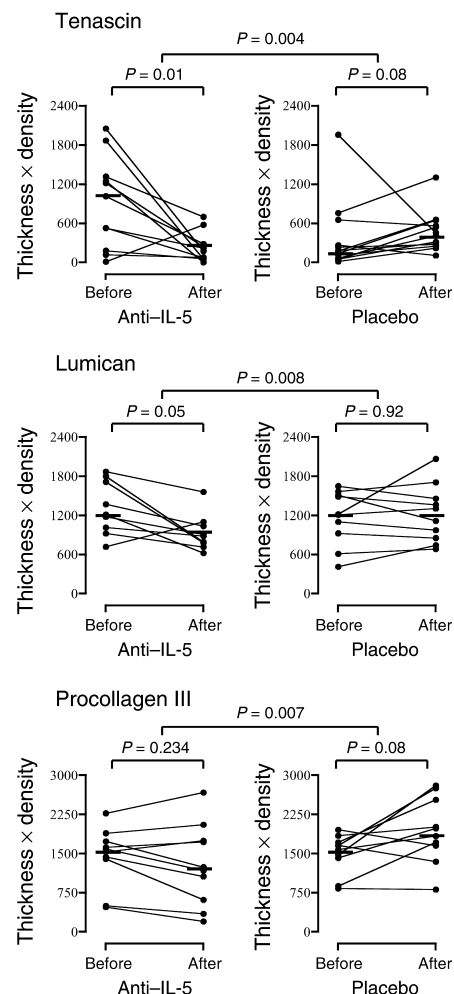

Figure 2
The effect of anti–IL-5 on the total expression (thickness × density) of tenascin, lumican, and procollagen III in the bronchial subepithelial reticular basement membrane. The crossbars represent the median values.
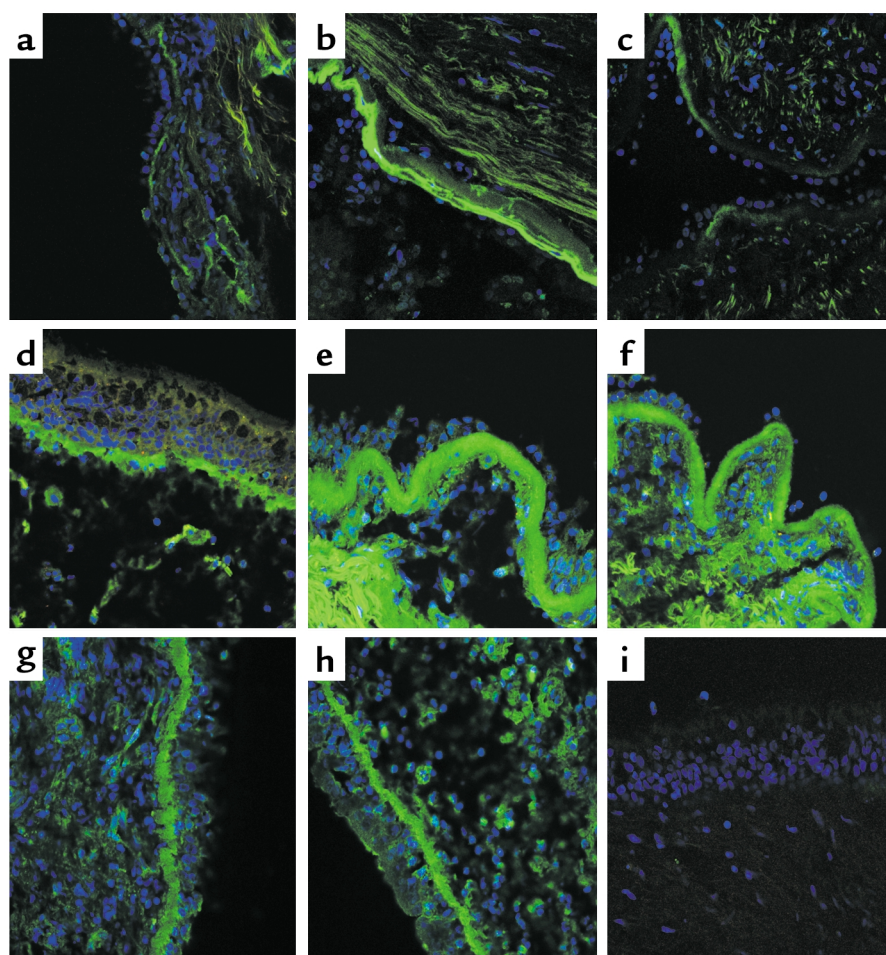
placebo, in the mepolizumab-treated group (*P* = 0.04) (Figure 4b). There was also a significant decrease in the concentration of TGFβ1 in BAL fluid in the mepolizumab-treated group when compared with placebo (*P* = 0.042, Figure 4c).

Discussion
We have shown that specific reduction of eosinophil numbers in the bronchial mucosa in mild asthmatic subjects using an anti–IL-5 monoclonal antibody (mepolizumab) is associated with a significant decrease in the expression of the ECM proteins tenascin and lumican in the reticular basement membrane of the airway mucosa. Furthermore, these changes were associated with significant reductions in TGF-β1 expression by airway eosinophils. These data support a novel role for the eosinophil and suggest a link between eosinophilic infiltration and airway remodeling in asthma.

We confirmed previous reports that the thickness of tenascin and lumican within the RBM was significantly greater in asthmatic individuals than in normal subjects (6, 34). With tenascin, mepolizumab reduced RBM expression to values observed in nonasthmatic, nonatopic individuals. We also showed that tenascin thickness, density, and total expression correlated with bronchial mucosal eosinophil numbers. Our findings are consistent with those previously reported in animal studies. In an IL-5 gene knockout mouse model of airways remodeling, abrogation of eosinophil infiltration following repeated allergen challenge was associated with an absence of structural changes, including fibronectin production and mucus gland hyperplasia observed in the WT controls (35). In a separate animal model of atopic asthma, prophylactic treatment of ovalbumin-sensitized BALB/c mice with anti–IL-5 significantly reduced the subepithelial fibrosis produced by repeated allergen challenge (36). However, these authors did not report the effects of anti–IL-5 treatment on established fibrosis.

Compared with lumican or procollagen III, there was a particularly close correlation between baseline eosinophils and tenascin expression and a more marked decrease in tenascin expression in response to anti–IL-5 therapy. With procollagen III, mepolizumab did not significantly decrease the expression of this ECM protein, although the changes observed were significantly different than with placebo. This suggests a particularly strong association between eosinophils and tenascin deposition, as previously

shown in allergen-induced tenascin formation in atopic skin (24). It is possible that longer periods of treatment may be required to reverse procollagen and lumican expression to the same degree as that observed with tenascin. Evidence from studies investigating the effect of inhaled corticosteroids on RBM thickness indicates that although short-term treatment is associated with a significant decrease in tenascin thickness in the RBM (5), longer-term studies are required to show a reduction in collagen III thickness (37, 38). In the placebo group, there were nonsignificant increases, over the study period, in eosinophils, tenascin, and procollagen, raising a further possibility that these changes reflect the natural history of untreated mild atopic asthma and that anti–IL-5 may have prevented such an increase.

The value for tenascin expression before placebo was lower than before mepolizumab, albeit not significantly lower. Because the treatment groups were otherwise well matched both in terms of clinical and histopathological features and were selected randomly, this difference is most likely the consequence of the relatively small numbers and the large standard deviation of the baseline values for tenascin.

TGF-β1 is a potent differentiation factor for the formation of myofibroblasts in vivo and in vitro (39, 40) and has been shown to upregulate the expression by fibroblasts of an array of ECM proteins, including tenascin (41). Consistent with previous studies (42, 43), we have shown that most of the TGF-β1 mRNA+ cells in the submucosa in these mild atopic asthmatics were eosinophils. Selective depletion of eosinophils with anti–IL-5 significantly reduced the number of TGF-β1 mRNA+ eosinophils in the bronchial mucosa and decreased TGF-β1 concentrations in BAL fluid. In addition, the proportion of cells expressing TGF-β1 mRNA decreased significantly in the mepolizumab-treated group. This suggests that anti–IL-5 therapy not only decreased the number of eosinophils producing TGF-β1, but also reduced the degree of activation of these cells for TGF-β1 expression. The fact that the majority of TGF-β1mRNA+ cells were eosinophils, and that levels of TGF-β1 in BALF were also reduced supports our hypothesis that eosinophils were an important source of TGF-β1 in the bronchial mucosa of asthmatics.

We did not quantify bronchial mucosal myofibroblasts in the present study. The myofibroblast marker α-smooth muscle actin is also expressed by a large number of other cells in the bronchial mucosa, and the ultrastructural identification of myofibroblasts using electron microscopy as used by Gizycki and co-workers (44) was beyond the scope of this study. Our previous in vitro work, however, has demonstrated that coculture of eosinophils and fibroblasts is associated with myofibroblast differentiation and the generation of tenascin transcripts and protein, an effect that is inhibited by antibodies directed against TGF-β (24). We suggest, therefore, that eosinophil-derived TGF–β1 may influence the expression of tenascin and other ECM proteins via the activation of myofibroblasts.

It is also possible that the observed effect of anti–IL-5 on matrix protein expression was mediated by the effect of the drug on IL-5 itself, rather than through the reduction in eosinophil numbers. Although IL-5 has been shown to influence airway smooth muscle contraction (45), it is not known to directly release growth factors from smooth muscle or other cells appropriate to the remodeling changes studied. In addition, the consistent correlation between eosinophil numbers and tenascin thickness before and after mepolizumab therapy makes a direct action of IL-5 unlikely — at least for tenascin. It is equally unlikely that the changes in ECM protein expression could be explained by the action of anti–IL-5 on other cell types in the bronchial mucosa. There were no significant changes in the numbers of other cells in response to anti–IL-5. Although there was a trend towards a
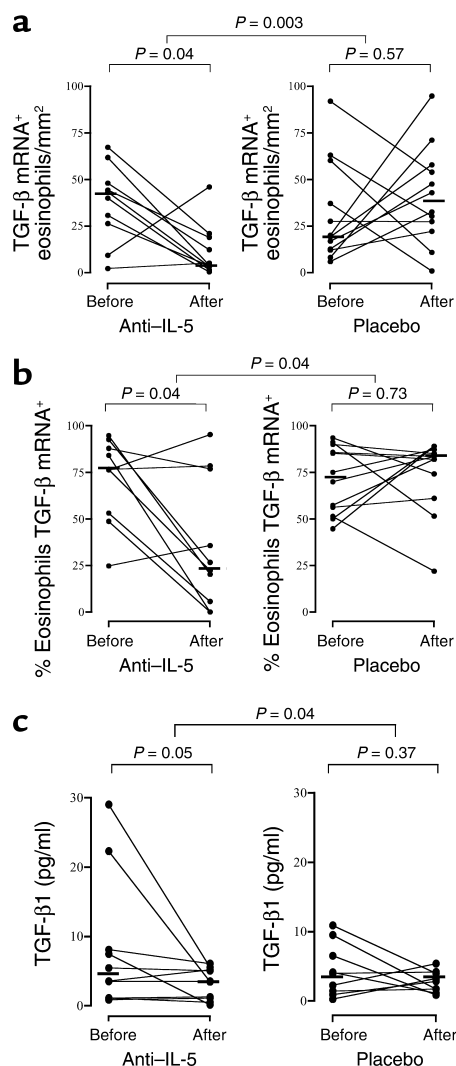

Figure 4
The effect of anti–IL-5 on (**a**) TGF-β1mRNA+ eosinophils in the bronchial mucosa, (**b**) the percentage of eosinophils expressing TGF-β1mRNA in the bronchial mucosa, and (**c**) the concentration of TGF-β1 in bronchoalveolar lavage fluid. The crossbars represent the median values.

reduction in basophil numbers, this did not correlate with changes in ECM protein expression.

The relevance of our findings in terms of asthma pathophysiology is unclear because the effect of airways remodeling on lung function in asthma is uncertain. Deposition of ECM proteins in the airway wall may have a mechanical effect, amplifying the degree of narrowing associated with a given degree of smooth muscle contraction (46). A number of studies have reported a correlation between the thickness of the RBM and airways hyperreactivity (AHR), FEV_1, and other markers of asthma severity (6, 34, 42, 47). We found no significant correlation between baseline PC_{20}, FEV_1, and the thickness, density, and expression of tenascin, lumican, or procollagen III. Our subjects were mild asthmatics, and the study was not powered to detect significant correlations in clinical variables such as FEV_1 or AHR. Furthermore, although we observed a significant reduction in the expression of tenascin, lumican, and procollagen III in the RBM, we did not measure overall RBM thickness in these biopsies, and it is possible that eosinophil depletion was not associated with so marked a reduction in the expression of other matrix proteins such as fibronectin and types I and V collagen.

Although anti–IL-5 administration significantly reduced the numbers of tissue eosinophils in the bronchial submucosa, it did not deplete them to the same extent as that reported previously in sputum and blood (28). GM-CSF, IL-3, and eotaxin are also involved in eosinophil homeostasis, and there is evidence that inhibition of these, in conjunction with IL-5 blockade, may be associated with more complete eosinophil depletion from the bronchial mucosa (48, 49). Equally, studies showing the most marked effects of corticosteroids on RBM thickness have tended to be of longer duration, and so sustained eosinophil depletion may be required to achieve a maximal effect (50–52).

In summary, we have shown that the selective reduction of eosinophils in mild asthmatics by anti–IL-5 treatment is associated with a significant decrease in expression of ECM proteins in the airway RBM. Furthermore, this reduction was accompanied by a decrease in TGF-β1 expression by airway eosinophils. These data provide strong evidence of a causal association between eosinophils and airway remodeling in mild atopic asthma.

Acknowledgments

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