

ORIGINAL ARTICLE

Inflammation-dependent and independent airway remodelling in asthma

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ABSTRACT

Background and objective: The pathology of asthma is characterized by airway inflammation (granulocytic (GA) or paucigranulocytic (PGA)) and remodelling of airway structures. However, the relationship between inflammatory phenotypes and remodelling is unclear. We hypothesized that some features of airway remodelling are dependent on granulocytic airway inflammation while others are not.

Methods: Post-mortem airway sections from control subjects ($n = 48$) and cases of asthma with ($n = 51$) or without ($n = 29$) granulocytic inflammation in the inner airway wall were studied. The thickness of the airway smooth muscle (ASM) layer, basement membrane and inner and outer airway walls, the size and number of ASM cells, the volume fraction of extracellular matrix within the ASM layer, ASM shortening and luminal mucus were estimated. Airway dimensions were compared between the three subject groups.

Results: In cases of PGA, only the thickness of the ASM layer and basement membrane was increased compared with control subjects. In cases of GA, not only the ASM and basement membrane were increased in thickness, but there was also increased inner and outer airway wall thickness and increased narrowing of the airway lumen due to ASM shortening and mucus obstruction, compared with control subjects. Granulocytic inflammation was observed more often in cases of fatal asthma.

Conclusion: These findings suggest that inner and outer wall thickening coexists with inflammation, whereas thickening of the ASM layer and basement membrane may be present even in the absence of inflammation.

SUMMARY AT A GLANCE

Findings suggest that inner and outer wall thickening coexists with airway inflammation, whereas airway smooth muscle and basement membrane remodelling may be partly independent of airway inflammation and therefore not reversible by anti-inflammatory treatment.

Remodelling of the ASM layer and basement membrane may therefore be less susceptible to anti-inflammatory therapy.

Key words: airway morphology, asthma, inflammation, stereology.

Abbreviations: ASM, airway smooth muscle; ECM, extracellular matrix; GA, granulocytic asthma; IQR, interquartile range; MOR, mucus occupying ratio; N_v, numerical density; Pbm, basement membrane perimeter; PGA, paucigranulocytic asthma; PMS, percent of smooth muscle shortening; RBMT, reticular basement membrane thickness; W_{ai}, inner wall area; W_{ao}, outer wall area.

INTRODUCTION

Asthma is a chronic respiratory disease that causes substantial morbidity and mortality and a significant public health problem. Patients with asthma are defined clinically by the presence of variable breathlessness, chest tightness, cough and wheeze. Physiologically, diagnosis is characterized by airway hyperresponsiveness, excessive airway narrowing that is inducible by stimuli that contract airway smooth muscle (ASM) or reversible by drugs that relax ASM.¹ Airway pathology in asthma includes inflammation, hypertrophy and hyperplasia of ASM cells, increased submucosal mucous glands and epithelial goblet cells and the laying down of extracellular matrix

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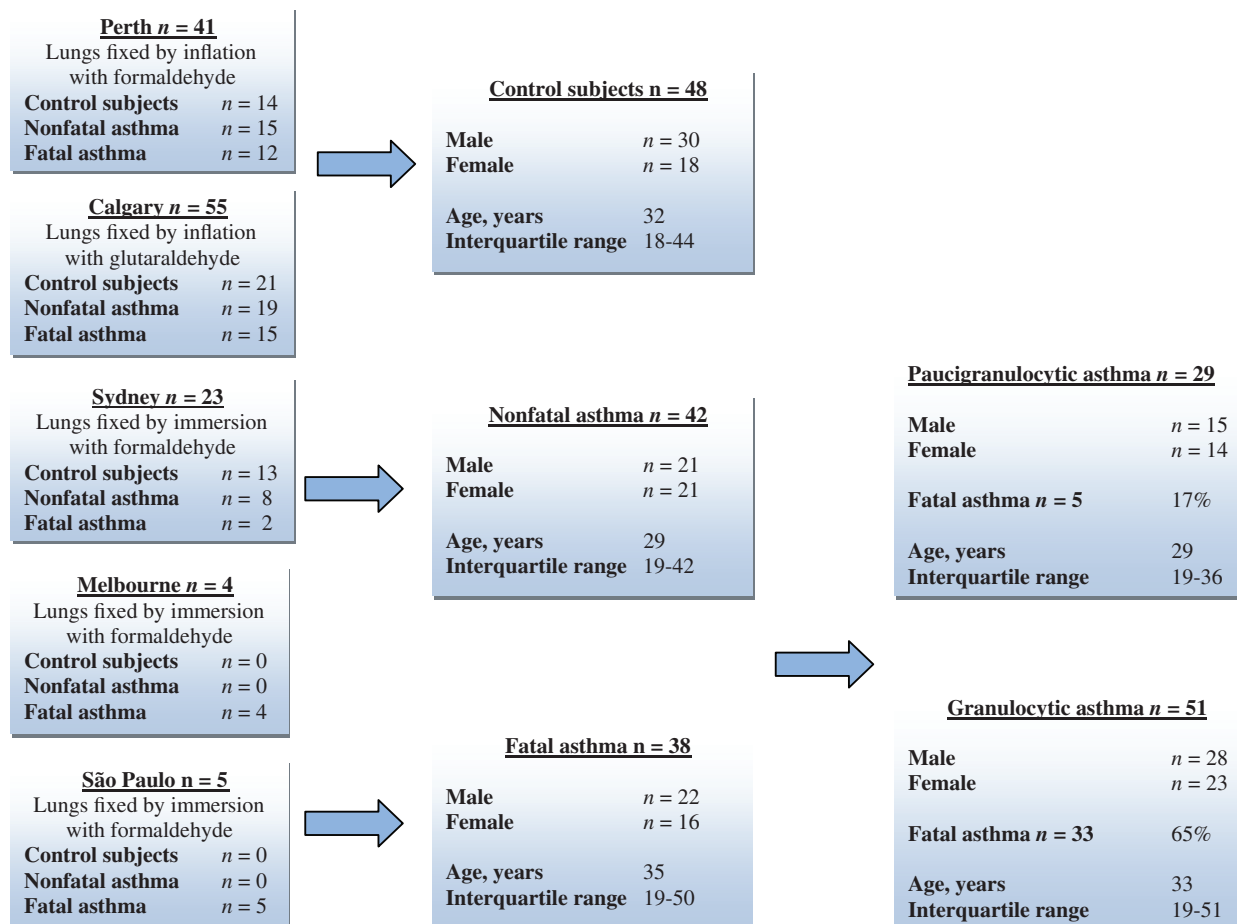


Figure 1 Flow chart showing study centres, tissue fixation, case classification, gender and the percentage of fatal asthma cases within each of the inflammatory asthma groups.

(ECM) below the basement membrane, all contributing to increased airway wall thickness.²⁻⁴

Airway inflammation and remodelling appear to arise early in the course of asthma,^{5,6} although the relationship between them remains to be determined. It is well established that lung inflammation induced experimentally in animal models produces features of airway remodelling consistent with human patients.⁷ However, other animal studies suggest that airway remodelling and inflammation occur in parallel rather than sequentially⁸ and that remodelling can occur in the absence of any detectable inflammation.⁹ In asthma subjects, inhaled corticosteroids produce partial reduction in airway wall thickness determined by computed tomography, suggesting an unresponsive component in the absence of inflammation.¹⁰

We hypothesized that there is inflammation-dependent and -independent airway remodelling in asthma. We compared features of airway remodelling in asthmatic subjects with or without granulocytic inflammation with controls.

METHODS

Subjects

Subjects included in the study were derived from five post-mortem studies of asthma, as previously described.³

Subjects were categorized as: non-asthmatic controls; nonfatal asthma-died suddenly of non-respiratory causes with a confirmed history of asthma; and fatal asthma-died from asthma with a confirmed history (Fig. 1). Approval for this study was obtained from the Sir Charles Gairdner Hospital Human Research Ethics Committee (HREC-2007-195).

Tissue preparation

Tissue samples were fixed in inflation or immersion using formaldehyde or glutaraldehyde and embedded in paraffin. Airways were systematically sampled or obtained during diagnostic dissection at the discretion of the pathologist. Consecutive sections of 0.5, 5 and 30 μ m thickness were cut from each block and stained using the Masson's trichrome technique, haematoxylin and eosin, and haematoxylin, respectively.

Airway measurements

Airway dimensions and ASM parameters were estimated using stereological software (newCAST version 4.2.1, Visiopharm, Hoersholm, Denmark) on airways cut in cross-section, as previously described.³ On 30- μ m sections, the area of the ASM layer was measured by planimetry, the

Table 1 Subject characteristics

	Controls <i>n</i> = 48	Paucigranulocytic asthma <i>n</i> = 29	Granulocytic asthma <i>n</i> = 51
Gender (% male)	62	52	55
Age (years)	32	29	33
Median (IQR)	(18–44)	(19–36)	(19–51)
Height [†] (m)	1.74	1.70	1.69
Median (IQR)	(1.64–1.81)	(1.63–1.75)	(1.60–1.80)
BMI (kg/m ²)	23	28	26
Median (IQR)	(19–27)	(22–35)	(22–28)
Ever smoked [†] (%)	59	52	51
Nonfatal/fatal asthma, <i>n</i> [‡]	—	24/5	18/33
Age at onset of asthma [†] , years median (IQR)	—	10 (2–28)	10 (4–22)
Duration of asthma [†] , years median (IQR)	—	16 (10–27)	17 (8–27)
Corticosteroid use (oral or inhaled) [†] , <i>n</i> (%)	—	10 (77)	12 (80)
Asthma severity [†] , mild-moderate/severe	—	9/10	13/20

Categories of inflammation are based on inflammatory cell counts from large or small airways.

[†]Data not available for all cases.

[‡]*P* < 0.001; median (IQR).

IQR, interquartile range

numerical density (N_V) of ASM cell nuclei was estimated using the optical disector approach³ and the total number of ASM cells per mm of airway length was calculated. The volume fractions of ASM and ECM were estimated using point counts (0.5 μ m sections) and the mean cell volume (V_c) was calculated as the inverse of N_V , with a correction for the volume fraction of ASM within the ASM layer. On 5- μ m sections, the inner (W_{Ai}) and outer (W_{Ao}) airway wall areas and the reticular basement membrane thickness (RBMt) were measured.⁴ Percent of smooth muscle shortening (PMS) was calculated by comparing the measured outer perimeter of the ASM layer with its calculated, relaxed perimeter.¹¹ Luminal mucus was measured and expressed as the mucus occupying ratio (MOR).¹² On the same slide, eosinophil and neutrophil numbers were counted in the inner airway wall and cell densities calculated. The basement membrane perimeter (Pbm) was measured and used to define airway size.¹¹

Data analysis

Analyses were undertaken using SigmaPlot (Version 13.0, Systat Software Inc., Erkrath, Germany). Cases of asthma were categorized initially according to the mean area densities of granulocytes within the inner airway wall of large and small airways, with cut-off values of 5 cells/mm², established from biopsy and sputum analyses.¹³ Based on these criteria, cases were initially classified into paucigranulocytic, eosinophilic, neutrophilic or mixed categories. As the number of cases categorized as 'eosinophilic only' or 'neutrophilic only' were small, and because we found no differences in participant characteristics, airway structure, PMS or MOR between the eosinophilic and neutrophilic asthma groups, all cases of asthma with granulocytic inflammation were grouped and labelled as granulocytic

asthma (GA). The remainder of the asthma subjects were labelled as paucigranulocytic asthma (PGA). In addition, as fatal asthma represents an exacerbation likely to be associated with inflammation including eosinophils and neutrophils,¹⁴ the relationship between remodelling and inflammation was also examined after excluding cases of fatal asthma.

Preliminary analyses were undertaken to examine the distribution of each outcome and the mean (or median) values of the various outcomes for each subject were calculated for airways grouped by size, small (Pbm < 6 mm) or large (Pbm > 6 mm) airways. All variables (transformed if necessary) were analysed as means using analysis of variance (ANOVA) with post hoc tests as appropriate.

RESULTS

Subject characteristics

There were 80 cases of asthma, categorized as PGA (*n* = 29) or GA (*n* = 51), and 48 controls (Table 1). There were no significant differences in duration of asthma, age of onset of asthma, use of corticosteroids or smoking history between nonfatal and fatal asthma cases or between inflammatory asthma groups. The PGA group had a smaller proportion of fatal asthma cases (*P* < 0.001) compared with the GA group. For cases where clinical data were available, there was an increase in severity in fatal asthma compared with nonfatal asthma (*P* = 0.02); however, there was no significant difference between GA and PGA cases. The area density of granulocytes is shown in Table 2.

ASM layer

No significant differences in the Pbm (airway sizes) were observed between groups (Table 3). The thickness

Table 2 Eosinophil and neutrophil densities within the inner airway wall

	Control <i>n</i> = 48	PGA <i>n</i> = 29	Granulocytic asthma <i>n</i> = 49
Eosinophil density within the inner airway wall (cells/mm ²)			
≥6 mm	1.56 (0.58–3.05)	1.29 (0.72–2.23)	10.11 ^{†‡} (2.36–41.69)
<6 mm	1.45 (0.00–6.72)	0.00 (0.00–2.80)	17.59 ^{†‡} (2.16–45.65)
Neutrophil area density within the inner airway wall (cells/mm ²)			
≥6 mm	2.79 (1.64–4.20)	1.81 (1.14–3.37)	5.32 ^{†‡} (2.63–9.26)
<6 mm	3.88 (0.00–8.52)	2.44 (0.00–4.53)	13.84 ^{†‡} (6.95–25.27)

Median (interquartile range); values shown are of non-transformed data.

[†]*P* < 0.05 vs control.

[‡]*P* < 0.05 vs PGA.

PGA, paucigranulocytic asthma.

of the ASM layer was significantly increased in large and small airways in both asthma groups, compared with controls. In large airways of cases of asthma, the thickness of the ASM layer was increased in the GA group compared with the PGA group. The volume fractions of ASM and ECM within the ASM layer in either small or large airways were similar in the control and asthma groups. Mean ASM cell volume was significantly increased in the large airways of the GA group

Table 3 ASM layer components

	Control <i>n</i> = 48	PGA <i>n</i> = 29	Granulocytic asthma <i>n</i> = 49
Basement membrane perimeter (mm)			
≥6 mm	13.9 ± 4.9	14.2 ± 5.1	13.9 ± 5.7
<6 mm	3.8 ± 1.54	4.0 ± 1.2	3.4 ± 1.2
ASM layer thickness (mm)			
≥6 mm	0.033 ± 0.015	0.049 ± 0.019 [†]	0.070 ± 0.036 ^{†‡}
<6 mm	0.019 ± 0.010	0.034 ± 0.025 [†]	0.028 ± 0.017 [†]
Volume fraction of ASM			
≥6 mm	0.63 ± 0.07	0.64 ± 0.09	0.65 ± 0.08
<6 mm	0.70 ± 0.07	0.69 ± 0.08	0.70 ± 0.08
Volume fraction of extracellular matrix			
≥6 mm	0.19 ± 0.04	0.18 ± 0.06	0.17 ± 0.05
<6 mm	0.18 ± 0.04	0.18 ± 0.06	0.18 ± 0.06
Mean ASM cell volume (μm ³ × 10 ³)			
≥6 mm	2.84 ± 0.72	3.35 ± 1.14	3.42 ± 1.19 [†]
<6 mm	2.47 ± 0.89	2.90 ± 0.97	2.74 ± 0.99
ASM cells per airway length (cell/mm × 10 ⁵)			
≥6 mm	1.03 (0.68–1.43)	1.32 (0.98–1.81)	1.90 [†] (0.99–2.61)
<6 mm	0.19 (0.12–0.27)	0.22 (0.16–0.40)	0.18 (0.13–0.29)

Mean ± SD or median and interquartile range.

[†]*P* < 0.05 vs control.

[‡]*P* < 0.05 vs PGA.

ASM, airway smooth muscle; PGA, paucigranulocytic asthma.

compared with controls. A similar trend was observed in the PGA group, although this was not significant. The number of ASM cells per length of airway was significantly increased in the large airways of the GA group compared with the control group (Fig. 2).

Airway wall components, muscle shortening and luminal mucus

The RBMt was most evident in large airways and its thickness was increased in the both GA and PGA cases compared with controls (Table 4). The WAI (excluding ASM) and WAO were increased in the large airways in the GA group compared with the controls. In the GA group, the WAO was also increased compared with the PGA group. The submucosal gland area was increased in the GA group compared with the controls, although not significantly greater than the PGA group. ASM shortening was increased in the large and small airways in the GA group compared with both other groups and increased in the small airways compared with controls (Table 4). The MOR was significantly increased in the small airways in the GA group compared with controls.

Analysis excluding fatal asthma cases

When cases of fatal asthma were excluded, the thickness of the ASM layer remained increased in the large airways in both the GA and PGA groups, compared with controls, although the difference between the PGA and GA groups was no longer observed. In the small airways, the thickness of the ASM layer in the PGA group, but not the GA group, remained greater than the control group. Other aspects of airway remodelling, MOR and muscle shortening were not significantly different from controls when the fatal cases of asthma were excluded (data not shown).

DISCUSSION

We found that in cases of asthma with increased granulocytes, the RBMt, ASM layer, inner and outer airway wall compartments and submucosal mucous glands were all increased in volume. Apart from the increased thickness of the ASM layer, these structural changes were predominantly in large airways. In contrast, in PGA, the only components of airway remodelling that were increased were the thickness of the ASM layer and RBMt. These data suggest that most features of airway remodelling are to some extent dependent on inflammation while others, particularly remodelling of the ASM, are also partly independent of granulocytic airway inflammation.

The classification of inflammatory phenotypes of asthma has been largely based on analyses of sputum^{15–17} and has been shown to predict response to therapy,^{16,17} be related to severity of symptoms and possibly driven by allergic and non-allergic exposures.^{16–18} We used a cut-off value of 5 cells/mm² based on a large study¹³ in which both sputum and bronchial biopsies were obtained in the same patients and used to quantify inflammation. Cases with only increased eosinophils or only increased neutrophils were less common, and as

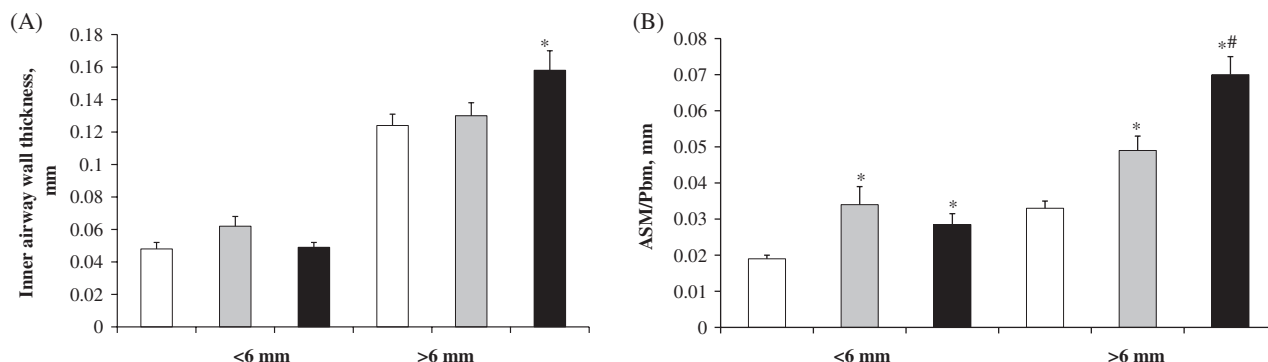


Figure 2 Graph (A) showing the thickness of the airway smooth muscle layer per mm of the basement membrane perimeter (ASM/Pbm) and (B) the inner airway wall thickness in control subjects (open bars) and paucigranulocytic (grey bars) and granulocytic (black bars) asthma cases in airways less than or greater than 6 mm of the Pbm (mean $\pm$ SE).

such all cases with increased granulocytes in either the large or small airways were grouped for analysis. Asthmatic subjects with inflammation in the present study therefore reflect a mixed-granulocytic phenotype.

Both airway inflammation and remodelling are characteristic pathologic abnormalities of asthma (Fig. 3). There is current debate regarding the relationship between these processes. Some suggest inflammation precedes and drives remodelling, and may be related to primary abnormalities of epithelial repair and a background of atopy.¹⁹ Others suggest remodelling may be independent of inflammation and arise before the clinical manifestation of asthma.²⁰ Another view is that inflammatory signals from inherently abnormal ASM²¹ drive both remodelling and inflammation.²² It is likely that both inflammation and remodelling may vary over short periods of time due to viral infections, allergen

exposure or even due to ASM contraction,²³ perhaps on a background of 'steady-state' remodelling.¹⁰

The only features of airway remodelling present in subjects with PGA were increased thickness of the ASM and RBMt, suggesting these abnormalities are not dependent on current inflammation, or perhaps inflammation was not the primary driver producing remodelling. Studies in children are often cited as evidence to dispel the cause and effect relationship between inflammation and remodelling. The thickness of the ASM and RBMt is increased in biopsies from preschool wheezers⁵ and increased thickness of ASM is associated with asthma at school age.⁶ In a subsequent study on preschool wheezers, there was no relationship between inflammation in biopsies and airway wall structure, including ASM thickness and RBMt.²⁴ Another study²⁵ showed that children who develop asthma and have current symptoms as young adults had abnormal lung function as neonates, raising the possibility of very early (perhaps preexisting) structural change.²⁶ Together, these findings suggest that remodelling of the ASM and RBMt are present early in the clinical course of the disease and are not dependent on coexisting inflammation.

However, airway remodelling is clearly exacerbated by granulocytic inflammation. The GA group had greater ASM thickness compared with the PGA group, and an increase in the thickness of the inner and outer walls relative to the controls. The increase in ASM thickness in GA was accompanied by a significant increase in the number of ASM cells per length of airway and cell volume, indices of hyperplasia and hypertrophy, respectively. An association between inflammation and ASM proliferation has been demonstrated in horses with heaves, a naturally occurring animal model of allergic airway disease,²⁷ and recently in human subjects where activation of airway mast cells during an exacerbation of asthma was associated with increased proliferation of ASM cells in vitro.²⁸ Compared with proliferation, hypertrophy of ASM cells may follow a different time course, as demonstrated in mice where an acute proliferative phase was followed by hypertrophic growth after chronic allergen challenge.²⁹

The excessive airway narrowing in asthma is due to shortening of the ASM around the lumen,³⁰ increased thickness of the airway wall which exaggerates the

Table 4 Airway wall components, muscle shortening and MOR

	Control n = 48	Paucigranulocytic asthma n = 29	GA n = 49
Reticular basement membrane thickness (μ m)			
≥ 6 mm	5.0 $\pm$ 1.8	6.1 $\pm$ 2.3 [†]	6.9 $\pm$ 2.2 [†]
Inner airway wall thickness (mm)			
≥ 6 mm	0.124 $\pm$ 0.045	0.130 $\pm$ 0.042	0.158 $\pm$ 0.077 [†]
<6 mm	0.048 $\pm$ 0.025	0.062 $\pm$ 0.028	0.049 $\pm$ 0.017
Outer airway wall thickness (mm)			
≥ 6 mm	0.76 $\pm$ 0.32	0.74 $\pm$ 0.35	0.98 $\pm$ 0.46 ^{†‡}
<6 mm	0.13 $\pm$ 0.08	0.15 $\pm$ 0.10	0.17 $\pm$ 0.13
Mucus gland thickness (mm)			
≥ 6 mm	0.078 $\pm$ 0.045	0.114 $\pm$ 0.102	0.122 $\pm$ 0.063 [†]
Percent muscle shortening (%)			
≥ 6 mm	6.9 $\pm$ 5.3	10.2 $\pm$ 11.3	14.1 $\pm$ 15.3 ^{†‡}
<6 mm	7.0 $\pm$ 6.6	9.8 $\pm$ 9.1	15.3 $\pm$ 9.1 [†]
MOR (%)			
≥ 6 mm	4.9 $\pm$ 9.4	4.5 $\pm$ 6.5	11.0 $\pm$ 12.3
<6 mm	6.9 $\pm$ 12.4	12.7 $\pm$ 18.8	21.0 $\pm$ 18.1 [†]

Mean $\pm$ SD.

[†]P < 0.05 vs control.

[‡]P < 0.05 vs GA.

GA, granulocytic asthma; MOR, mucus occupying ratio.

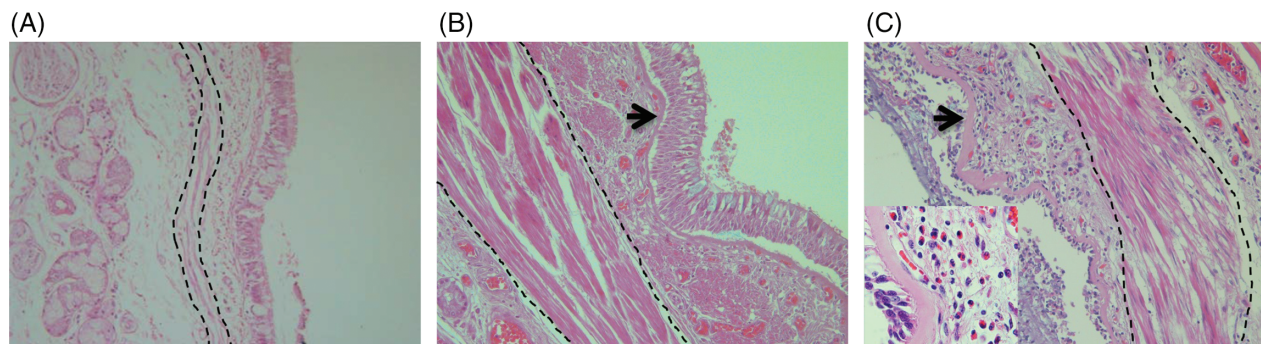


Figure 3 Representative images of the inner airway walls of large airways (>6 mm basement membrane perimeter) from a control subject (A) and cases of paucigranulocytic (B) and granulocytic (C) asthma ($\times 400$). The insert in (C) ($\times 1000$) shows granulocytic inflammation in the inner airway wall. The airway smooth muscle layer is highlighted by dashed lines and the black arrow head indicates the increased basement membrane thickness in the asthma cases.

narrowing effect of the ASM shortening³¹ and partial occlusion of the airway lumen with mucus.³² In our study, the percent shortening of the ASM layer was increased in both the large and small airways, and the percent of the airway lumen that was occupied by mucus was increased in small airways in GA. That is, airway narrowing was only increased relative to control in cases with granulocytic inflammation.

Given that granulocytic inflammation was associated with increased airway narrowing, it was expected that this would be associated with greater disease severity. While the limited information available on patient severity was not different between groups, there was greater incidence of fatal asthma in the PGA group. As severe and fatal asthma are associated with increased airway eosinophils^{33–35} and fatal asthma represents an exacerbation likely to be associated with inflammation including eosinophils and neutrophils,¹⁴ we also examined the relationship between remodelling and inflammation within the nonfatal asthma group. This analysis showed that the difference in the thickness of the layer of ASM between the PGA and GA groups was no longer observed, raising the possibility that the increase in ASM thickness, rather than the presence of granulocytes (mainly eosinophils) mainly accounts for the increased severity of asthma seen in the fatal cases. Other aspects of airway wall remodelling, mucous gland area, the MOR and percent muscle shortening were not significantly different from controls when the fatal cases of asthma were excluded. While we did not assess the number of lymphocytes, we have previously shown that lymphocyte numbers correlated with the number of eosinophils in fatal asthma and to a lesser extent in nonfatal asthma and controls,³⁶ suggesting an increase in lymphocytes numbers in GA.

Finally, treatment of airway inflammation with corticosteroids results in rapid improvements in symptoms and inflammatory cell numbers within sputum of the airway wall.^{37–40} However, airway responsiveness to nonspecific stimuli, a possible marker of airway wall remodelling, and RBMT, which is related to thickness of the ASM,⁴ return to optimal values only after much longer periods of treatment.^{37,39,40} These observations suggest that inflammation, on a background of remodelling, drives excessive airway narrowing and

perhaps over long periods, may also sustain remodelling or increase it intermittently.⁴¹

This study suggests RBMT and ASM remodelling has both inflammation-dependent and -independent elements and that other aspects of remodelling, ASM shortening and mucus secretion may be mainly inflammation-dependent. The combination of ASM remodelling which is independent of inflammation and the additional remodelling and airway narrowing effects of inflammation may contribute to asthma severity and the propensity to fatal attacks. Treatment of both components of remodelling may be required to most effectively treat asthma, particularly severe asthma. Currently, there are no studies which have examined the effects of treatment with corticosteroids or the newer monoclonal agents directed against inflammatory cytokines on remodelling of the ASM. This awaits development of techniques to accurately, non-invasively and longitudinally measure the thickness of the ASM in asthma. In this regard, optical coherence tomography assessment of birefringence of the airway wall shows promise.⁴² Our study suggests that more aggressive treatment of persisting airway inflammation (which can be assessed by sputum analysis) may help reduce the risk of fatal attacks of asthma.

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