

ORIGINAL ARTICLE

Distribution of airway smooth muscle remodelling in asthma: Relation to airway inflammation

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ABSTRACT

Background and objective: Pathological phenotypes of asthma have been based predominantly on inflammation, rather than airway wall remodelling. Differences in the distribution of airway smooth muscle (ASM) remodelling between large and small airways may affect clinical outcomes in asthma. The aim of this study was to examine the distribution of ASM remodelling and its relation to airway inflammation.

Methods: Post-mortem cases of asthma ($n = 68$) were categorized by the distribution of increased thickness of the ASM layer (relative to nonasthmatic controls, $n = 37$), into 'large only' (LO, $n = 15$), 'small only' (SO, $n = 4$) 'large/small' (LS, $n = 24$) or no increase (NI, $n = 25$). Subject characteristics, ASM and airway wall dimensions and inflammatory cell numbers were compared between groups.

Results: Apart from reduced clinical severity of asthma in NI cases ($P = 0.002$), subject characteristics did not distinguish asthma groups. Compared with control subjects, ASM cell number, reticular basement membrane thickness, airway wall thickness, percent muscle shortening and eosinophil number were increased ($P < 0.05$) in both large and small airways in LS cases and only the large airways in LO cases. Increased numbers of neutrophils were observed only in the small airways of LO cases.

Conclusions: Distinct distributions of ASM remodelling are seen in asthma. Pathology limited to the small airways was uncommon. Increased thickness of the ASM layer was associated with airway remodelling and eosinophilia, but not neutrophilia. These data support the presence of distinct pathological phenotypes based on the site of increased ASM.

SUMMARY AT A GLANCE

In a large number of asthma cases, we have shown that airway smooth muscle remodelling varies between individuals, and is associated with eosinophilic but not neutrophilic inflammation but is uncommonly confined solely to the small airways.

Key words: airway smooth muscle, asthma, inflammation, remodelling, small airway.

Abbreviations: ANOVA, analysis of variance; ASM, airway smooth muscle; CT, computed tomography; ECM, extracellular matrix; LO, large only; LS, large/small; MRI, magnetic resonance imaging; NI, no increase; N_L , airway smooth muscle cell number per millimetre length of airway; Pbm, perimeter of the basement membrane; PMS, percent of smooth muscle shortening; RBMT, reticular basement membrane thickness; SO, small only; VC, airway smooth muscle cell volume; WAI, inner airway wall; WAO, outer airway walls.

INTRODUCTION

The most striking and consistent aspect of airway wall remodelling in asthma is the increased thickness of the airway smooth muscle (ASM) layer throughout the bronchial tree,¹ which is associated with the clinical severity, but not duration of asthma.² Remodelling and shortening of ASM are most likely the cause of the variable and excessive airway narrowing and variable respiratory symptoms that characterize asthma.³ In asthma, airway responsiveness to bronchoconstrictors is usually assessed using spirometry, which provides little information regarding the site of airway pathology within the bronchial tree.

Functional studies in asthma have shown considerable regional heterogeneity of airway narrowing in

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asthma^{4,5} which is reproducible⁶ and which responds to bronchodilator in a regionally specific manner.^{7,8} Computed tomography (CT) scans have been used to investigate the structure–function relationships that exist between spirometry, airways inflammation and disease severity,⁹ but are limited in their ability to visualize airways with diameters less than 2 mm. A recent study by Svenningsen *et al.*,¹⁰ using hyperpolarized ³He magnetic resonance imaging (MRI), showed that 65% of asthma patients studied had regional ventilation defects. Compared with asthmatics without ventilation defects, this group had worse airway hyperresponsiveness, increased inflammation and airway remodelling. While the aetiology of ventilation abnormalities is not fully understood, it is suspected to be the result of regional airway narrowing which may be due to local airway remodelling.¹¹

Very little is known about the variability of distribution of ASM remodelling within and between asthma cases. The aim of the present study was to examine the distribution (large and/or small airways) and type (hyperplasia and/or hypertrophy) of ASM remodelling within the bronchial tree. In addition, how ASM remodelling is related to subject characteristics, asthma severity and airway inflammation was also assessed.

METHODS

Subjects

Cases of asthma ($n = 68$) were fatal cases who had died from asthma or nonfatal cases who had asthma, but died from nonrespiratory causes. Control subjects ($n = 37$) had no history of asthma and died of nonrespiratory causes. Subjects were included in the study if both large and small airways were available for examination. Post-mortem lung tissue and subjects' clinical data were collected and processed as previously described.² Ethics committee approval was granted from all participating centres and from the Sir Charles Gairdner Hospital Human Research Ethics Committee for the study.

Stereological measurements

Tissue samples were fixed in formalin or glutaraldehyde and paraffin-embedded blocks were prepared as previously described.² Consecutive sections of 0.5, 5 and 30 μm thickness were cut from each block and stained using the Masson's trichrome technique, HE and haematoxylin respectively. Airway dimensions and ASM cell parameters were estimated using stereological software (newCAST version 4.2.1, Visiopharm A/S, Hørsholm, Denmark) on airways cut in cross-section, as previously described.² On 30 μm sections, the area of the ASM layer was measured by planimetry: the numerical density of ASM cell nuclei was estimated using the optical disector¹² and the total number of ASM cells per mm of airway was calculated. The volume fractions of smooth muscle and extracellular matrix (ECM) were estimated using point counts on 0.5 μm sections and the mean cell

volume was calculated, with a correction for the volume fraction of smooth muscle within the ASM layer.

Airway dimensions and inflammatory cell counts

On 5 μm sections stained with HE, the inner and outer airway wall area (inside and outside the ASM layer, respectively) and the reticular basement membrane thickness were measured. On the same slide, eosinophil and neutrophil numbers were counted in the inner airway wall, around the entire airway section, based on staining and morphology criteria, and the area cell densities were calculated.¹³ The perimeter of the basement membrane (Pbm) was used to define airway size as large (Pbm > 6 mm) or small (Pbm < 6 mm).

Analysis

Cases of asthma were grouped based on the mean thickness of their ASM layer (in large and/or small airways) compared with control subjects. Increased mean thickness of the ASM in asthma cases was defined as more than one standard deviation above the mean value for control subjects. Asthma cases were therefore defined by the site(s) of increased thickness of the ASM layer: large airways only (LO); large/small airways (LS); small airways only (SO); or cases with no increase (NI) in ASM thickness in either the large or the small airways. Case means were compared between case groups and airway size groups using one-way analysis of variance (ANOVA) and appropriate post-hoc tests. Subject characteristics, asthma history and asthma severity were compared between groups using Student's *t*-tests or chi-square tests. Due to the small number of SO cases ($n = 4$), these were excluded from the ANOVA, but were compared separately with the control group. Statistical analyses were undertaken using SigmaStat version 12.5 (Systat Software Inc., San Jose, CA, USA).

RESULTS

Of the 68 cases of asthma, 24 LS, 15 LO, 4 SO and 25 NI cases could be distinguished (Table 1). An average of six large and four small airways were available per case. No differences in subject characteristics were observed between asthma groups except that in the NI group, a smaller proportion were cases of fatal asthma and a larger proportion had clinically mild to moderate asthma ($P \leq 0.002$). Data on corticosteroid use were available in only one of the SO cases resulting in 0% use within that group. In the LS, LO and NI cases, corticosteroid use was 71%, 100% and 63% respectively (Table 1). No significant differences in airway size were observed between groups (Table 2). The volume fractions of ASM and ECM in either small or large airways were similar in all groups, although the ECM fraction was increased in large airways in the SO cases (univariate analysis) which may be a result of the variance within the small number of cases in the

Table 1 Subject characteristics

	Control	Asthma			
	(n = 37)	LS (n = 24)	LO (n = 15)	SO (n = 4)	NI (n = 25)
Gender, M/F	24/13	14/10	9/6	1/3	16/9
Age, years median (IQR)	29 (17–43)	27 (18–51)	30 (21–50)	37 (32–41)	28 (18–43)
Height [†] , cm	171 ± 13.5	168 ± 12.4	170 ± 15.1	165 ± 0.6	168 ± 14.6
Weight [†] , kg	70 ± 17.3	67 ± 15.3	82 ± 24.4	75 ± 8.4	84 ± 37.2
Ever smoked [†] , % (n = number of cases with available data)	57% (21)	47% (15)	64% (14)	75% (4)	63% (19)
Fatal asthma, %	NA	71%	73%	50%	16% [‡]
Age at onset of asthma [†] , years median (IQR)	NA	4 (2–12)	7 (1–18)	14 (12–15)	17 (6–30)
Duration of asthma [†] , years median (IQR)	NA	20 (16–27)	12 (6–36)	24 (20–28)	12 (6–18)
Corticosteroid—any use [†] , % (n = number of cases with available data)	NA	83% (6)	100% (6)	0% (1)	64% (11)
Asthma severity ^{†,§} , % (n = number of cases with available data)					
Mild/moderate	NA	27% (3)	33% (4)	0% (0)	83% (15) [‡]
Severe	NA	73% (8)	67% (8)	100% (3)	17% (3)

[†]Data not available for all cases. Median (interquartile range), mean ± SD.

[‡]P < 0.002 compared with other asthma groups.

[§]Asthma severity was based on medication use, frequency of symptoms, use of health care and time from work or school.²

NA, not applicable; F, female; LO, cases of asthma with increased thickness of the airway smooth muscle in large airways only; LS, cases of asthma with increased thickness of the airway smooth muscle in large and small airways; M, male; NI, cases of asthma with no increased thickness of the airway smooth muscle; SO, cases of asthma with increased thickness of the airway smooth muscle layer in small airways only.

Table 2 Structure of the airway smooth muscle layer in control subjects and cases of asthma

Control		Asthma			
(n = 37)		LS (n = 24)	LO (n = 15)	SO (n = 4)	NI (n = 25)
Airway size, perimeter of the basement membrane (Pbm), mm					
Small	3.8 ± 1.6	4.1 ± 1.4	3.6 ± 1.0	3.3 ± 1.4	3.3 ± 1.1
Large	13.6 ± 4.8	14.0 ± 6.1	15.9 ± 3.0	15.4 ± 3.7	12.3 ± 4.7
Thickness of the airway smooth muscle layer (ASMarea/Pbm), mm					
Small	0.019 (0.012–0.022)	0.040 ^{*,†,‡} (0.031–0.066)	0.020 (0.016–0.022)	0.033* (0.028–0.087)	0.017 (0.012–0.021)
Large	0.030 (0.022–0.038)	0.087 ^{*,‡} (0.066–0.11)	0.062 ^{*,‡} (0.052–0.091)	0.030 (0.028–0.034)	0.034 (0.028–0.042)
Volume fraction of airway smooth muscle within the smooth muscle layer (V _{ASML})					
Small	0.70 ± 0.07	0.68 ± 0.08	0.71 ± 0.10	0.66 ± 0.10	0.70 ± 0.07
Large	0.63 ± 0.07	0.65 ± 0.08	0.65 ± 0.07	0.61 ± 0.06	0.65 ± 0.06
Volume fraction of extracellular matrix within the smooth muscle layer (V _{ECM})					
Small	0.18 ± 0.04	0.18 ± 0.06	0.18 ± 0.07	0.18 ± 0.11	0.18 ± 0.04
Large	0.18 ± 0.04	0.16 ± 0.05	0.17 ± 0.04	0.24 ± 0.06*	0.17 ± 0.04
Airway smooth muscle cell volume (VC), µm ³ , ×10 ³					
Small	2.5 ± 0.9	3.0 ± 1.0	2.7 ± 0.7	2.6 ± 1.1	2.6 ± 0.9
Large	2.9 ± 0.8	3.7 ± 0.9*	3.5 ± 1.1	2.8 ± 1.3	3.2 ± 1.0
Airway smooth muscle cell number per millimetre length of airway (N _L) cell/mm, ×10 ⁵					
Small	0.19 (0.12–0.27)	0.32 ^{*,†,‡} (0.19–0.61)	0.15 (0.12–0.23)	0.45* (0.35–0.82)	0.14 (0.12–0.21)
Large	0.97 (0.60–1.34)	2.31 ^{*,‡} (1.09–3.34)	1.88 ^{*,‡} (1.64–2.44)	0.85 (0.58–1.31)	1.03 (0.76–1.41)

Median (interquartile range), mean ± SD.

*P < 0.05 versus control; [†]P < 0.05 versus LO; [‡]P < 0.05 versus NI. The P values shown in the SO column represent significance level between SO cases and control subjects only (t-test).

LO, cases of asthma with increased thickness of the airway smooth muscle in large airways only; LS, cases of asthma with increased thickness of the airway smooth muscle in large and small airways; NI, cases of asthma with no increased thickness of the airway smooth muscle; SO, cases of asthma with increased thickness of the airway smooth muscle layer in small airways only.

Table 3 Airway wall dimensions and area densities of eosinophil and neutrophils in control subjects and cases of asthma

		Asthma			
		LS (<i>n</i> = 24)	LO (<i>n</i> = 15)	SO (<i>n</i> = 4)	NI (<i>n</i> = 25)
Inner airway wall thickness (WAI/Pbm), mm					
Small	0.054 (0.05–0.08)	0.089 ^{*,†} (0.06–0.12)	0.063 (0.05–0.08)	0.074 (0.06–0.08)	0.05 (0.04–0.07)
Large	0.151 (0.11–0.18)	0.230 ^{*,†} (0.19–0.30)	0.202 [*] (0.18–0.24)	0.176 (0.14–0.25)	0.161 (0.11–0.19)
Outer airway wall thickness (WAO/Pbm), mm					
Small	0.13 ± 0.08	0.24 ± 0.16 ^{*,†,‡}	0.13 ± 0.06	0.11 ± 0.04	0.10 ± 0.05
Large	0.76 ± 0.29	1.06 ± 0.49 ^{*,‡}	1.03 ± 0.41 [†]	1.15 ± 0.33 [*]	0.70 ± 0.28
Reticular basement membrane thickness (RBMt), μm					
Small	3.6 ± 2.3	7.6 ± 3.2 ^{*,‡}	5.7 ± 3.0	5.2 ± 2.7	4.0 ± 1.9
Large	4.9 ± 1.9	7.9 ± 2.2 ^{*,‡}	6.7 ± 2.1 [*]	6.5 ± 2.3	5.8 ± 1.9
Mucous gland area (mm ² /Pbm), mm					
Large	0.06 (0.04–0.10)	0.10 (0.06–0.17)	0.10 [†] (0.07–0.20)	0.09 (0.05–0.16)	0.04 (0.01–0.10)
Percent of smooth muscle shortening (PMS)					
Small	5.1 (1.8–10.5)	17.7 ^{*,‡} (12.3–19.5)	12.3 (9.0–19.9)	8.2 (5.3–11.0)	6.7 (2.3–12.4)
Large	6.1 (2.5–10.2)	14.6 ^{*,‡} (9.3–20.7)	11.2 (5.8–15.2)	12.1 (2.4–19.0)	7.2 (4.1–11.9)
Eosinophil density in the inner airway wall (cells/WAi), mm ²					
Small	1.2 (0.0–6.5)	14.4 ^{*,‡} (1.5–44.0)	8.9 (2.3–34.2)	16.9 (8.8–25.0)	0.0 (0.0–8.2)
Large	1.5 (0.6–2.8)	4.8 [*] (1.4–20.2)	4.6 [*] (2.4–15.0)	3.7 (1.2–43.1)	2.0 (0.6–8.6)
Neutrophil density in the inner airway wall (cells/WAi), mm ²					
Small	3.7 (0.0–8.1)	6.7 (2.9–18.7)	13.6 [*] (6.9–24.2)	2.5 (0.6–4.4)	5.9 (0.0–25.6)
Large	2.8 (1.7–4.0)	3.2 (1.2–7.4)	4.8 (3.6–7.2)	5.2 (1.7–9.8)	4.1 (1.5–6.9)

Median (interquartile range), mean ± SD.

^{*}*P* < 0.05 versus control; [†]*P* < 0.05 versus LO; [‡]*P* < 0.05 versus NI. The *P* values shown in the SO column represent significance level between SO cases and control subjects only (*t*-test).

LO, cases of asthma with increased thickness of the airway smooth muscle in large airways only; LS, cases of asthma with increased thickness of the airway smooth muscle in large and small airways; NI, cases of asthma with no increased thickness of the airway smooth muscle; SO, cases of asthma with increased thickness of the airway smooth muscle layer in small airways only.

SO group (Table 2). There was a significant increase in the mean ASM cell volume in large airways only in LS cases compared with control subjects. The number of ASM cells per airway length was increased in large and small airways in the LS cases, only in large airways in LO cases and only in the small airways in SO cases (univariate analysis) compared with control subjects (Table 2).

Airway dimensions and cell counts of control subjects and asthma case groups are shown in Table 3. The inner and outer airway wall areas and percent of smooth muscle shortening were increased in both large and small airways in LS cases compared with control subjects and NI cases, and the inner airway wall area was increased in only large airways in LO cases compared with control subjects, and the outer airway wall compared with NI cases (Table 3). The thickness of the reticular basement membrane was increased in large and small airways of the LS cases compared with control subjects and NI cases and large airways of the LO cases compared with control subjects.

Eosinophil area density was increased in large and small airways in LS cases and only in large airways in LO cases, compared with control subjects. Neutrophil area density showed a different pattern than that for eosinophils and was increased only in small airways in the LO cases, with no significant differences in either large or small airways in the LS cases, compared with controls (Table 3).

DISCUSSION

In the present study, cases of asthma could be distinguished where the ASM remodelling (increased thickness) was present only in the large airways, in both the large and small airways or, uncommonly, only in the small airways. Twenty-five cases (37%) had no increase in the thickness of the ASM layer compared with control subjects. These cases were less likely to be cases of fatal asthma and, in those where data were available, were more likely to have clinically mild or moderate, rather than severe asthma. Subject clinical characteristics did not otherwise distinguish case groups. Increased thickness of the ASM layer was associated with hyperplasia of ASM cells, increased thickness of the sub-basement membrane and the airway wall overall, and increased area density of eosinophils. Hypertrophy of the ASM cells was present only in the large airways in the LS cases. Paradoxically, the area densities of neutrophils were increased only in the small airways of LO cases. In the few SO cases, increased thickness of the ASM was associated with hyperplasia of ASM cells and there was a trend to more eosinophils in the small and large airways.

No differences were detected between the asthma categories with regard to subject characteristics including gender, age, height, weight, smoking history, duration of asthma or clinical severity of asthma. The power to detect small differences

between groups in the present study was low due to the reduced number of cases where complete histories were available. Although a total of 495 airways were examined in the present study with approximately five airways per case examined, a range of airways per subject (2–12) were available for examination. Two cases, one each from LO and SO groups, were represented by two airways only (one large and one small), but the removal of these two cases did not alter the results. Case means were calculated for each individual per airway size group, avoiding cases with larger numbers of airways being overrepresented. The extent of the variation of remodelling within groups of large or small airways is yet to be determined. As in previous studies, the present study samples a small proportion of the large or small airways, and the systematic examination of differences within lobes of the same subject is needed.

The present study strengthens the observed association between eosinophilic airway inflammation and airway wall remodelling, particularly increased thickness of the ASM layer, irrespective of whether it is due to hyperplasia or hypertrophy. Eosinophilic inflammation of the airways has been associated with increased thickness of the reticular basement membrane^{14,15} and of the airway wall, assessed by CT scan.¹⁶ In children with treatment-resistant asthma, airway pathology is characterized by increased eosinophils and increased mass of ASM.¹⁷ However, there are studies that suggest that eosinophil numbers and airway remodelling may be independent of each other.^{18,19} Among asthma patients in remission, remodelling persists despite the lack of activation or increased number of eosinophils, which suggests that eosinophil activation is necessary before the remodelled airway has a functional impact.²⁰ Of course, the possibility that eosinophilic inflammation drives airway remodelling is not excluded.²¹

The lack of correlation between ASM remodelling and increased numbers of neutrophils observed in the present study raises questions about the role of neutrophils in asthma. It has been hypothesized that inflammation is important for the manifestation of asthma symptoms,²² and that in some patients, neutrophils are the predominant cell, arising in response to innate immune mechanisms.^{23,24} Treatment of asthma with azithromycin, which has anti-neutrophilic actions, results in improved exacerbation rates in patients with asthma and neutrophilic airway inflammation.²⁵ However, the present study shows that while neutrophils are increased in LO cases (in the small airways only), they are not significantly increased in LS and SO cases despite the presence of ASM remodelling. This suggests a specific phenotype of asthma, although the mechanisms are unclear. We did not undertake a subgroup analysis within groups due to small numbers.

Previous observations on neutrophilic asthma are largely based on neutrophil counts in induced sputum.^{23,24,26} The source of neutrophils (large or small airways) in these studies has not been established. We used counts of cells within the wall of large airways which correlate with those seen in sputum.²⁷ Despite the use of post-mortem tissues, we found that when

we phenotyped our cases of asthma, based on the presence of neutrophils and/or eosinophils in the large airways, the following groups emerged: eosinophilic = 16%, neutrophilic = 9%, mixed = 24% and paucigranulocytic = 50%. These values fit within the large range previously reported.²⁴ We did not find a relation between these categories and case group categorized by remodelling. If our observations are generalizable, then the presence of high numbers of neutrophils in cases of asthma might suggest thickening of the ASM layer confined to the large airways only. The usefulness of this finding as a marker for specific therapies (such as thermoplasty) remains to be determined.

Twenty-five (37%) of the 68 cases of asthma were classified as having no ASM remodelling as has been observed in studies comparing groups of asthmatics and non-asthmatics.¹ The NI group was less clinically severe than the other asthma groups. Although case misclassification may account for some of this group, it is unlikely to be the sole cause as four of these cases had asthma shown as the primary cause of death on their death certificate. Interestingly, the study by Svenningsen and co-workers¹⁰ regional ventilation abnormalities were also absent in 35% of the asthma patients, and was associated with less airway hyper-responsiveness, inflammation and airway remodelling. It is possible that this may be related to sampling, or that in these cases, excessive airway narrowing may result from factors other than ASM remodelling, such as excessive production of mucus or altered contractile properties of the ASM.

The extent to which LS, LO and SO cases represent truly distinct phenotypes of asthma is unclear. Comparisons of ASM remodelling in cases of asthma and controls have necessarily compared airways of similar size. The average values of ASM thickness have shown increases in both the large and small airways in asthma.¹ Only one study has attempted to examine the distribution of ASM remodelling within cases. In 10 cases of fatal asthma, Ebina *et al.*²⁸ found that remodelling of the ASM layer was present only in the large airways in five cases and in both the large and small airways in five cases, and like the present study was associated with remodelling of the airway wall generally. The authors did not distinguish remodelling confined to the small airways in their small sample.

It has been suggested that patients with pathology affecting the small airways may have more severe asthma and/or be more resistant to treatment with inhaled corticosteroids.^{29,30} This is not supported by the present study that shows that it is uncommon for ASM remodelling and inflammation to be confined to the small airways and that cases with involvement of the small airways (LS and SO) were not distinguishable by subject or clinical characteristics. Since we found few cases of exclusive involvement of the small airways (SO), the challenge remains to find methods that reliably and easily detect small airway involvement in asthma in the presence of large airway involvement.^{29,31}

While we have focused upon the differences between small and large airways in the present study,

these comparisons may overlook the importance of variation within small and large airways—heterogeneity of remodelling. The detection of airway remodelling (increased wall area and reduced luminal area) has been shown in asthma by using CT and MRI.^{7,10,11} These studies, however, while showing evidence of regional remodelling are limited as they cannot determine structural differences that are present (e.g. increased ASM, mucous glands or reticular basement membrane thickness) and have difficulty measuring images beyond the 5th or 6th generation of airways. This is important as the pathology of the small airways may be more important than increased thickness *per se* in determining the severity of disease and the therapies that are most effective.

The present study shows that cases of asthma may be distinguished by the distribution of remodelling of the ASM layer between small and large airways. ASM remodelling parallels other aspects of airway wall remodelling as well as eosinophilic, but not neutrophilic, inflammation. It remains to be determined if an increase in the amount ASM at a specific site within the lung is the main cause of regional ventilation dysfunction.³² It is unknown if specific types of ASM remodelling arise from different causes, have different physiological phenotypes or respond to different therapeutic approaches, including differences in methods of delivery. Further work is needed to quantify the heterogeneity of ASM remodelling within individuals.

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