Airway basement membrane perimeter distensibility and airway smooth muscle area in asthma

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THE PERIMETER OF THE BASEMENT membrane (Pbm) of an airway viewed in cross section has been shown to be relatively constant despite variations in airway smooth muscle (ASM) shortening, lung inflation, and airway collapse (16–18). Subsequently, it has become established as a marker of airway size for the comparison of airway dimensions and inflammatory cell numbers between subjects with and without various airway diseases (3, 5, 6, 8, 11, 19, 22–25, 29, 30, 32). Most notably, comparisons of airways of similar size using the Pbm have been undertaken in a number of studies that compared the area of the ASM (ASMarea) layer between asthmatic and nonasthmatic individuals (4, 8, 19, 22).

Two studies (26, 27) have raised the possibility that the observed difference in the thickness of the airway smooth muscle layer between asthmatic and nonasthmatic individuals, based on comparisons that use the Pbm, may be partly due to artifact if the Pbm is stretched at higher inflation pressures. Both studies showed that when airway segments were taken from similar anatomic sites in the lung from humans (26) or pigs (27), the Pbm was greater in airways that were fixed at transmural pressures of >25 cmH2O, compared with the mean Pbm from airway segments fixed at ≤5 cmH2O. The authors of both studies suggested that if airway distensibility was reduced in cases of asthma (33, 35), then lungs fixed in inflation might distend the Pbm of airways from normal (nonasthmatic) lungs but not those from asthmatic lungs. This would result in a comparison of Pbm of smaller airways from control subjects with those of larger airways from asthma cases and an overestimation of the thickness of the airway smooth muscle layer in asthma. In contrast to the validation studies undertaken in whole lungs from humans and guinea pigs (16,17), the studies that showed distension of the Pbm (26, 27) were undertaken in segments from different subjects, without the surrounding lung parenchyma.

The symptoms of asthma are due to excessive airway narrowing, and the quantity of the ASM has been suggested as the most significant factor affecting the degree to which airways might narrow (28). Therefore, under- or overestimation of ASM thickness, based on the use of Pbm to compare airways, would have important implications for functional studies of the role of ASM in asthma. We hypothesized that if the Pbm stretched more in nonasthmatic cases than in asthmatic cases, then the linear plot of the square root of ASMarea against the Pbm of airways would be shifted to the right in inflated lungs from nonasthmatic cases compared with uninflated lungs from nonasthmatic subjects but would be similar in both inflated and uninflated lungs from cases of asthma. To test this hypothesis, we examined these relationships in 116 cases of fatal asthma and 83 control subjects where airway dimensions had been measured in uninflated or inflated lungs.
METHODS

Airway dimensions were measured in lungs from six postmortem studies that included cases of fatal asthma, where asthma had been the cause of death, and/or control cases where death had occurred without involvement of the lungs and where asthma had been excluded retrospectively (Table 1). The six collaborative centers were the following: a prospective study of fatal asthma from Perth, Western Australia (3, 5, 6); a 3-yr epidemiological study of asthma management and mortality from Melbourne, Australia (1); the New Zealand study of fatal asthma (2); the Canadian Prairies study of fatal asthma based in Calgary (10); a study of airway development in asthmatic and nonasthmatic children and adults from Sydney (25); and a study of fatal asthma in São Paulo, Brazil (23). This provided totals of 61 and 22 control subjects with inflated and uninflated lungs, respectively, and 31 and 85 fatal asthma cases with inflated and uninflated lungs, respectively.

Table 2 shows the various methods used at the centers to fix lung tissues and to sample and prepare airway sections. Lungs were fixed in inflation via both the airways and the vasculature at the Perth (25 cmH₂O) and Calgary (20 cmH₂O) centers. In the remaining four centers, lungs or tissue samples were fixed by immersion, without inflation. In these cases, whole lung lobes or dissected airways and parenchymal samples were immersed in fixative before histological preparation.

Airway measurements. Transverse sections (long-to-short axis ratio of < 1:3 and even thickness of airway epithelium and inner airway wall) of airways were cut at 4 or 5 μm and stained with hematoxylin and eosin or using the Masson’s trichrome or Gomori techniques. The length of the Pbm was measured using planimetry, and the ASM area was measured using point counts or planimetry. Airway dimensions were measured independently by different observers at each of the centers, and results from each of the centers have previously been published independently (1, 2, 6, 10, 23, 25). To examine systematic differences between centers, mean ASM area was compared between centers for cases grouped by asthma and inflation categories into four groups: control, uninflated (Cu); control, inflated (Ci); fatal, uninflated (Fu) and fatal, inflated (Fi). The total number of airways available for each group were the following: Ci = 1,341; Fi = 993; Cu = 143; Fu = 309. Initial analyses were undertaken using all available airways for each case. However, because all subjects in the Cu group had only airways with Pbm of 15 mm or less, analyses were repeated using only airways from each group with Pbm of 15 mm or less. The relationships between square root ASM area and Pbm for all airways (pooled) within each case group were compared and tested using Student’s t-tests. Second, to exclude an effect of weighting of results by uneven sampling or data distribution from individual cases, the mean regression line was derived from the regression lines of square root of ASM area vs. Pbm for all cases with three or more airways available. The mean slopes and intercepts of individual regression analyses from each case were then compared between groups using one-way ANOVA. Dunn’s post hoc test for differences between individual case groups was undertaken where ANOVA showed a group effect.

RESULTS

Comparisons between centers but within asthma groups showed no systematic differences in mean thickness of the smooth muscle layer in medium and large airways (Pbm > 4 mm) (data not shown). However, for airways with Pbm < 4 mm, there were significant differences between the Perth (and São Paulo) and Calgary centers (Fig. 1). This was thought to be due to sampling strategies. Although both centers used inflated lungs, in Perth and São Paulo all small airways from parenchymal blocks were included, whereas only small airways along three axial paths were sampled at Calgary. This led to a bias to more small airways (with reduced ASM thickness relative to Pbm) in the Perth and São Paulo centers. The distributions of values of Pbm for airways with Pbm < 4 mm for the Perth and Calgary centers are shown in Fig. 2. Clearly, more very small airways were sampled in the Perth center, however, this had no effect on the relation between Pbm and ASM for both centers (Fig. 3).

The scatterplots and regression lines for the square root of ASM area against Pbm for all airways are shown in Fig. 4A. As expected, regression lines passed through or close to the origin, and there were few and no consistent differences in the intercepts between groups. Therefore, only the results for slopes of the regression lines are shown. The increased slope of Fi (0.065 mm/Pbm) relative to Ci (0.051 mm/Pbm) (P < 0.001) may be due to either an effect of asthma or an effect of inflation. Examination of the other regression lines suggests that both may apply. The increased slope of Cu (0.063 mm/Pbm) relative to Fi (P < 0.001) and of Fu (0.078 mm/Pbm) relative to Fi (P < 0.001) suggests an effect of asthma. The mean regression lines for each case group, derived from the individual regression lines of each case within that group, are shown in Fig. 4B. They show the same pattern as the regression lines derived from using all airways (Fig. 4A);

Table 1. Characteristics of asthma cases and control subjects from six collaborative centers

<table>
<thead>
<tr>
<th>Inflation</th>
<th>Perth</th>
<th>Melbourne</th>
<th>Sydney</th>
<th>São Paulo</th>
<th>New Zealand</th>
<th>Calgary</th>
<th>All Inflated</th>
<th>All Uninflated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cases</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>40</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>31</td>
<td>71</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>27/13</td>
<td>11/3</td>
<td>0/2</td>
<td>1/1</td>
<td>19/12</td>
<td>46/25</td>
<td>12/6</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>44±24</td>
<td>15±2</td>
<td>52±4</td>
<td>36±14</td>
<td>39±10</td>
<td>42±19</td>
<td>19±10</td>
<td></td>
</tr>
<tr>
<td>Ever smoked, yes/no/unknown</td>
<td>24/10/6</td>
<td>2/0/12</td>
<td>0/2/0</td>
<td>0/0/2</td>
<td>20/11/0</td>
<td>44/21/6</td>
<td>2/2/14</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fatal asthma cases</th>
<th>Yes</th>
<th>No</th>
<th>No</th>
<th>No</th>
<th>Yes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>19</td>
<td>18</td>
<td>3</td>
<td>19</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>11/8</td>
<td>12/6</td>
<td>3/0</td>
<td>7/12</td>
<td>13/27</td>
<td>15/10</td>
</tr>
<tr>
<td>Age, yr</td>
<td>43±18</td>
<td>19±13</td>
<td>17±2</td>
<td>48±13</td>
<td>24±11</td>
<td>33±14</td>
</tr>
<tr>
<td>Ever smoked, yes/no/unknown</td>
<td>4/15/0</td>
<td>4/6/8</td>
<td>0/0/3</td>
<td>8/11/0</td>
<td>0/0/40</td>
<td>12/13/0</td>
</tr>
</tbody>
</table>

Values for age are means ± SD; n, no. of subjects, M, male; F, female.
however, only Fu and Ci had significantly different slopes (P < 0.05). This difference could be due to the effects of remodeling of ASM and/or to stretch of the Pbm.

Where analyses were confined only to airways with Pbm < 15 mm (Fig. 5A), there was a significant and predominant effect of asthma with Fi (0.089 mm/Pbm) greater than Ci (0.054 mm/Pbm) (P < 0.001) and Fu (0.087 mm/Pbm) greater than Cu (0.059 mm/Pbm) (P < 0.001). In addition, the slope of the line for Cu was greater than the slope of Ci (P < 0.05), suggesting a small effect of inflation. The mean regression lines of each case group, derived from the individual case regression lines for airways with Pbm < 15 mm (Fig. 5B), showed a similar pattern. The slope of the line for Fi was significantly greater than Ci (P < 0.05). The slopes of Fu and Cu were not significantly different. There were only small, nonsignificant differences between the slopes of Fi and Fu, and between the slopes of Cu and Ci.

**DISCUSSION**

The present study used a collaborative collection of airway tissue from a number of study centers to compare the effects of disease (fatal asthma) and the effects of tissue preparation (lung inflation) on the relationship between a marker of airway size (Pbm) and the ASM area seen on transverse airway sections. The results show that the observed differences in the thickness of the smooth muscle layer between cases of control and fatal asthma are largely due to remodeling related to the disease with only a small effect due to stretch of the basement membrane.

This study included cases from six study centers. A variety of methods were used at the study centers to collect, fix and sample lung tissue, and stain and measure airway sections. It is therefore possible that the relationship between ASM area/Pbm could be systematically different between centers. This was suggested by the difference in mean ASM area/Pbm seen between the Calgary and Perth centers for airways with Pbm < 4 mm (Fig. 1). This was not observed for airways grouped as 4 –10 mm or >10 mm (data not shown). However, the differences between the Perth and Calgary centers were shown to be due to different sampling strategies, resulting in the collection of relatively more very small airways in the Perth center and therefore a lower ASM area/Pbm. This did not, however, affect the slope of ASM area/Pbm (Fig. 3). Different staining techniques were used in the different centers, and these included hematoxylin and eosin and either the Masson’s trichrome or Gomori trichrome techniques. All of these methods allow the unambiguous identification of both the length of the basement membrane at the base of the epithelial layer and of the total area of the smooth muscle layer at low-power (×4 or ×10) magnification. Planimetry was used to measure the basement membrane length in all centers and with calibration will give the same results. Both point counts and planimetry were used

![Fig. 1. Case means of airway smooth muscle area per millimeter of basement membrane (Pbm) for airways with Pbm < 4 mm for study centers: 1 = Perth, 2 = Melbourne, 3 = Sydney, 4 = São Paulo, 5 = New Zealand, 6 = Calgary. *P < 0.05 for Calgary vs. Perth and Calgary vs. São Paulo in the fatal asthma group.](image1.png)

![Fig. 2. Individual values of Pbm for all airways with Pbm < 4 mm for controls and cases of fatal asthma from Perth and Calgary study centers. *P < 0.05 for Calgary controls vs. Perth controls, Calgary controls vs. Perth fatal asthma, Calgary fatal asthma vs. Perth fatal asthma and Calgary fatal asthma vs. Perth controls.](image2.png)
to measure the area of smooth muscle in the airway wall. Again, both methods will give the same result if calibrated and the same object is measured. At high magnification, the ASM might be overestimated by planimetry because outlining the muscle layer will include areas of matrix that will not be included in points falling only on smooth muscle. However, this is less of a problem in the present study because point counts were used at low power to outline the smooth muscle layer, not strands of muscle within it.

To compare “similar” airways in different parts of the lung from the same subject or from different subjects raised a number of problems. Because the number of divisions of the bronchial tree from central bronchus to terminal bronchiole varies throughout the human lung (12, 13, 34), decisions about how to compare airways are based on the aims of the particular study. For example, if the aim were to compare structure in airways of the same generation (34) or order (13), then sampling must be undertaken using anatomic criteria to establish where each airway fits in the branching pathway. However, because the effect of disease is very different on airways of different generation (11, 21), it is important to use a marker of airway size that is independent of the branching structure. To this end, the Pbm has been shown to be ideal since it appears to be free from the effects of inflation, lung collapse or bronchoconstriction (16, 17).

The studies of McParland et al. (26) and Noble et al. (27) have suggested that the basement membrane may be stretched at higher transmural pressures. Because the airways of patients with asthma seem less distensible (33, 35), the possibility arises that stretch of airways fixed at inflation pressures of 20–25 cmH2O may affect the Pbm of control, nonasthmatic subjects and cases of fatal asthma differently. Airways compared by Pbm would lead to an overestimation of the thickness of the smooth muscle in cases of asthma because smaller airways with a stretched Pbm (controls) would be matched with larger airways (fatal asthma cases).

In our first analysis, we used all airways available in cases where there were three or more airways and where the data were spread over a range of Pbm values. This was done to avoid negative regression values. Use of ranges of values less than this resulted in a number of negative correlations between ASMarea and Pbm and large variability of the values for regression slopes and intercepts (data not shown). The participating centers in this study obtained tissue either at the discretion of the pathologist as part of the postmortem examination for diagnostic purposes or systematically where the whole lung or lung lobes were available for study. In routine postmortem examination, samples of lung parenchyma and some identifiable central airways were obtained, which resulted in a reduced number of large airways, relative to the number of small airways, and possible bias in the selection of more diseased airways macroscopically. In the centers where the large and small airways were sampled systematically, different sampling strategies resulted in relatively more small airways being examined in some centers than in others. These considerations account for the very different ranges in values...
for airway Pbm observed in the four case groups analyzed (see Fig. 2).

Although the curvilinear relationship between ASM\(_\text{area}\) and Pbm was corrected using the square root ASM\(_\text{area}\), the slope of the regression line of ASM\(_\text{area}\) vs. Pbm may still differ between samples containing different numbers of large and small airways. Therefore, it may not be valid to extrapolate beyond the slopes and intercepts obtained from airways with Pbm < 15 mm. This was subsequently confirmed by the change in slope for the Fi and Ci groups from Fig. 4A to Fig. 5A. To overcome this problem, we repeated the analyses selecting a priori airways with Pbm < 15 mm, because airways of this dimension were available in all groups. This of course led to censorship of 28 and 26% of data points in the Ci and Fi groups, respectively, and Pbm is one of our outcomes of interest. If inflation does stretch the Pbm, then we would expect that some of the airways in the Ci subjects might have been stretched beyond 15 mm and to be censored from the analysis. This censorship could lead to an artifactual decrease of the regression slope of ASM\(_\text{area}\) on Pbm. Therefore, censorship of data may have led to an underestimation of the difference between Cu and Ci, that is, the effect of inflation. To assess this, we recalculated the slope for the Ci group, including airways with Pbm up to 16, 17, or 18 mm. This had no effect on the slope of the regression line for the Ci data or the difference between Ci and Cu (data not shown). Therefore, our final analyses and conclusions are based on Fig. 5a.

The small difference between the slopes of Cu and Ci in Fig. 5A suggests an effect of inflation. From the slopes of regression equations, we can calculate that this would result in a mean (±SE) difference in calculated ASM\(_\text{area}/\text{Pbm}\) of ~12 ± 1% between Cu and Ci, leaving 48% between Cu and Fi to make the total mean difference of 60 ± 2% between Fi and Ci. This corresponds well with the 44% difference observed between Fu and Cu (Fig. 5A). In other words, for inflated lungs, 20% of the total difference between controls and fatal asthma may be due to inflation, whereas 80% is due to the effect of remodeling of the airway smooth muscle. As hypothesized by McParland and colleagues (26), the lack of an effect of inflation in the fatal cases of asthma is consistent with reduced distensibility in the fatal cases. The study by McParland et al. suggested that the effects of inflation would be >14% (the increase in Pbm was from 9 to 14 mm). The reasons for the discrepancies between that study and the present study may be related to the use of a smaller number of isolated airway segments. Removal of the parenchyma from isolated segments may have altered the distensibility of airway wall components, although this has not been studied systematically to our knowledge.

There have been a number of studies in the literature that have compared the thickness of ASM or ASM\(_\text{area}\) in cases of fatal asthma with nonasthmatic controls. Despite the variety of methods used to sample, fix, and measure the airways and the smooth muscle and match it with control subjects, the results are quite consistent (20). Hossain and Heard (14) matched airways anatomically, using those from the same site in the bronchial tree of inflated lungs in all subjects. Dunnill et al. (7) used lungs that were inflated via the vasculature and by negative air pressure with steamed formalin fixation. Airways were matched in size by point counts falling on cartilage. In the study of Sobonya (31), lungs of asthmatic and nonasthmatic individuals who had died of nonrespiratory causes were all inflation fixed and attained the same volume. In that study, no differences were observed in ASM\(_\text{area}\) between groups. James et al. (19) studied airways from cases of fatal asthma where the lungs were generally not inflated and from control subjects where the lungs were mostly inflated. Carroll et al. (6) obtained similar findings where the lungs of all cases of asthma and the control subjects were inflated. In that study, identical recognizable and named central airways were sampled, and a random sample of all intraparenchymal airways down to the smallest membranous bronchioles was included, as above. Therefore, the airways were well matched anatomically. Other studies (9, 15, 29), which used different fixation techniques, with or without inflation, also showed significant differences between asthmatic and nonasthmatic groups, in large and small airways. Ebina et al. (9) demonstrated that the differences between case groups were not uniform in relation to airway...
size. Therefore, many studies in the literature, each with its own control group, and using sampling methods that are independent of the effects of inflation (6, 7, 14), strongly support our conclusion that inflation has little or no systematic effect on the estimated area of ASM, relative to the basement membrane length.

In summary, we conclude that inflation pressures may increase the length of the Pbm to a small degree in inflated lungs. However, this effect is small and does not account for the observed differences in the area of the ASM between cases of asthma and control subjects.

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REFERENCES