INCREASED MYOEPITHELIAL CELLS OF BRONCHIAL SUBMUCOSAL GLANDS IN FATAL ASTHMA

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Key words: asthma, bronchi, exocrine gland, mucus, myoepithelial cell
ABSTRACT

Background: Fatal asthma is characterized by enlargement of bronchial mucous glands and tenacious mucus plugs in the airway lumen. Myoepithelial cells, located within the mucous glands, contain contractile proteins, which provide structural support to mucous cells and actively facilitate glandular secretion.

Objectives: To determine if myoepithelial cells are increased in the bronchial submucosal glands of fatal asthma.

Design: Autopsied lungs from 12 cases of fatal asthma (FA), 12 cases of persons with asthma dying of non-respiratory causes (NFA), and 12 non-asthma control (NAC) cases were obtained through the Prairie Provinces Asthma Study. Transverse sections of segmental bronchi from three lobes were stained for mucus and smooth muscle actin and the area fractions of mucous plugs, mucous glands and myoepithelial cells determined by point counting. The fine structure of the myoepithelial cells was examined by electron microscopy.

Results: FA was characterized by significant increases in mucous gland (p=0.003), mucous plug (p=0.004), and myoepithelial cell areas (p=0.017) compared with NAC. When the ratio of myoepithelial cell area to total gland area was examined, there was a disproportionate and significant increase in FA compared with NAC (p=0.014). Electron microscopy of FA cases revealed hypertrophy of the myoepithelial cells with increased intracellular myofilaments. The NFA group showed changes in these features that were intermediate between the FA and NAC groups; however the differences were not significant.

Conclusions: Bronchial mucous glands and mucous gland myoepithelial cell smooth muscle actin are increased in fatal asthma and may contribute to asphyxia due to mucous plugging.
INTRODUCTION
The WHO estimates that 255,000 people died of asthma in 2005, and that 300 million people are currently affected by asthma.[1] Death occurs by asphyxiation due to airway closure through bronchoconstriction and mucous plugging on a background of inflammation and airway wall remodelling[2-4]. Research has focused more on the changes in airway smooth muscle and less on the enlarged mucous glands and tenacious mucous plugs.[3;5-7] Overproduction of mucus in patients with asthma stems from a combination of hyperplasia of goblet cells, and enlargement of the bronchial submucosal glands.[5] The mucus in asthma has altered visco-elastic and biochemical properties that contribute to adhesivity and impaired clearance.[8;9] In asthma there is an increase in the ratio of mucous to serous cells within the mucous glands.[5] Mucus produced in the glands is secreted into the mucous gland ducts and from there expelled into the airway lumen.

Myoepithelial cells are ubiquitous components of exocrine glands. They lie between the basement membrane and the basal surface of the acinar cells and have “octopus-like” branching processes, which extend between the secretory epithelial cells.[10] They have been studied in the salivary, mammary, prostate, lachrymal, and sweat glands in several species.[10-12] Meyrick and Reid[13] described the anatomical features of myoepithelial cells beneath the serous, mucous and collecting duct cells of human bronchial submucosal glands. However, the mechanism of mucus expulsion from the glandular acini has not been studied in human bronchial mucous glands.

Myoepithelial cells are contractile in nature, possessing myofilaments composed of actin and myosin.[10;13;14] Their contraction contributes to glandular secretion.[14] The mechanism of stimulation varies by gland type and species. Contraction may be caused by cholinergic, adrenergic, or non-adrenergic /non-cholinergic mechanisms.[15]

As the area of the submucosal glands is increased in fatal asthma,[3;5-7] it is reasonable to assume that the myoepithelial cell network is also increased in relation to the increase in gland size. Given the striking amount of mucus found at autopsy in the airways in status asthmaticus and the marked increase in airway smooth muscle,[4;7] we hypothesized that contractile elements are disproportionately increased within the mucous glands in fatal asthma. We report that this is the case; a finding that has important implications for understanding and preventing death from asthma.
METHODS
Subjects and study design
The study was based on autopsy materials collected for the Prairie Provinces Asthma Study (PPAS); a multi-centred study of asthma fatalities occurring in Alberta, Saskatchewan, and Manitoba from November 1992 to October 1995 [16]. Ethics approval was obtained from the institutional review boards at the Universities of Calgary, Alberta, Saskatchewan, and Manitoba, Canada. Cases were defined as subjects dying of asthma (fatal asthma = FA). There were two control groups: a nonfatal asthma (NFA) group comprising subjects with a history of asthma but who had died of non-respiratory causes and a non-asthma control (NAC) group with no history of asthma or other respiratory disease at death. Deaths occurring in individuals with a history of asthma were reported to the study team through the medical examiners or coroner’s offices, hospitals and provincial vital statistics departments. Provincial departments of vital statistics were contacted every three months to ensure that no deaths classified as asthma in this age range were missed. Non-asthma control cases were obtained from the Alberta Medical Examiner’s Office and local hospitals. The criteria used by the pathologists and clinicians to classify the cases and control subjects, as well as the inclusion/exclusion criteria are available in the online supplement.

After notification of death, the study team contacted the next-of-kin to obtain consent for autopsy. Next-of-kin were asked to complete a questionnaire that sought information on asthma severity, age of onset, duration of asthma, asthma medications and smoking history. Those with a history of asthma were assigned a category for asthma severity, unrelated to the cause of death, based on 2006 guidelines from the Global Initiative for Asthma.[17] Subjects were classified as having severe asthma if they were using oral corticosteroids, reported hospitalizations for asthma (ever), or had daily symptoms. Subjects were classified as having moderate asthma if they had none of the above, but had symptoms on most days or nights (more than 3 days per week), used regular inhaled corticosteroids or used reliever medications on most days or nights. All other cases were classified as mild.

Tissue sampling for light microscopy and morphometry
Left lungs taken at autopsy were fixed in inflation via the blood vessels and airways with glutaraldehyde fixative (2.5% in 0.1 M phosphate buffer, pH 7.3). The airway pressure was 20 cm H₂O. The dual fixation method was developed to circumvent the poor airway perfusion due to mucous plugs in the FA group. Transverse sections from segmental bronchi from three lobes (LUL, LAB, and LPB) were used in this study. Segmental bronchi were selected because they contained the greatest proportion of mucous glands relative to airway wall size. Tissue blocks were embedded in paraffin wax and sections stained with Hematoxylin and Eosin (H&E) and Alcian blue/PAS (AB/PAS) at pH 2.5 for characterization of mucous plugs and mucous cells. Sections from the posterior segmental bronchus (LPB) were immunostained for smooth muscle actin (DAKO immunostain®) for identification of myoepithelial cells. More detail of the fixation techniques and sampling sites are available in the online supplement.

Morphometry
The area fractions of components of the airway wall and mucous glands were determined by point counting using a Zeiss-Axioplan light microscope, drawing tube and square lattice grid containing 240 points.[18] Structures identified and quantified by area on the H&E stained sections of the whole bronchus included bronchial mucous glands and the bronchial airway lumen (subdivided into free lumen and mucus/cells). The perimeter of the airway (Pbm) was determined on the same grid by counting the number of intersections between the grid lines and the luminal aspect of the epithelial basement membrane (laminar reticularis).[4] To normalize the mucous gland area to airway size, the mucous gland area was divided by the airway perimeter (Pbm). The area of the airway lumen occupied by mucous plugs was calculated by dividing the mucous plug area by the measured total airway luminal area and expressed as a percentage. Mucous gland duct ectasia was graded on the H&E stained sections on a five point (0-5) scale of increasing severity. Subcomponents of the mucous glands were measured on the sections stained for alpha smooth muscle actin. These included myoepithelial cells, acinar cells (nuclei and cytoplasm), acinar lumen, interstitium and blood vessels. These area fractions were estimated as the number of points falling on the feature of interest divided by the total number of points falling on the mucous gland. Further details of the morphometric methods are given in the online supplement.

**Electron microscopy**

Tissue for ultrastructural analysis was obtained from four subjects who had died of asthma (FA) and underwent autopsy at the Department of Pathology of São Paulo University between 2005 and 2007. All had a history of asthma and no other lung disease. Clinical data including treatment, smoking habits, duration of disease and previous hospitalizations were obtained by administering a questionnaire to the relatives.[19] Three control subjects were studied, all non-smokers with no history of asthma, wheeze, use of asthma medications or other lung disease and no gross or microscopic evidence of asthma at autopsy. Small (2x2x2mm) fragments of lobar bronchial wall were fixed in 2% glutaraldehyde dissolved in 0.15 M phosphate buffer at pH 7.2 for 1 h, followed by post-fixation in 1% osmium tetroxide dissolved in 0.9% sodium chloride for 1 hr and embedded in Araldite resin. Ultrathin sections were studied in a transmission electron microscope.

**Statistical Analysis**

A total of 108 cases and controls had been accessioned to the PPAS. For the study reported here we used a subset of 36 cases (12 FA, 12 NFA, 12 NAC). This sample size was based on preliminary data for airway smooth muscle which revealed that 12 cases per group would provide sufficient power (77%) to detect a significant effect. Further information regarding the power calculation is available in the online supplement. From the total (108) subjects we randomly selected 12 subjects per group, such that each group had equal numbers of males and females, and smokers and non-smokers. For example, the FA group had six males (3 smokers and 3 non-smokers) as well as six females (3 smokers and 3 non-smokers). This selection was done to provide balanced groups to test the relationship between the primary variable of interest, myoepithelial cell size and asthma group (severity). A comparison of the subgroup of 36 cases in this study with the total population (n= 108) showed no significant differences.
between the two groups for the demographic variables of age, time from death to autopsy, age at onset of asthma, asthma duration and (for smokers) pack years. For group comparisons (FA, NFA, and NAC), normality assumptions were tested and the appropriate parametric, non parametric tests were used accordingly. For comparison between groups, one way ANOVA with post hoc Tukey-Kramer multiple comparison or Student’s t-tests were used for continuous variables. Pearson Chi-squared and Fisher exact tests were used for categorical data. A p value of 0.05 or less was considered significant.
RESULTS

Patient characterization

The demographic characteristics and the causes of death of the PPAS study groups are shown in Table 1. No significant differences were observed between the FA and NFA groups for age at death, duration of asthma or age at onset of asthma. Asthma severity was significantly greater in the FA group compared with the NFA group ($p=0.032$ Pearson Chi-squared). This was reflected in the increased use of oral corticosteroids or short-acting bronchodilators in the FA group compared with the NFA group (Table 1). Inhaled corticosteroid use was similar for the FA and NFA groups. For those subjects who smoked, the NFA group had significantly ($p=0.04$) greater pack years than the FA group. The mean time between death and autopsy for the three groups was not significantly different (Table 1).
Table 1. Demographic characteristics of the study population on which lung tissue was used for morphometric analysis: Fatal asthma (FA), non-fatal asthma (NFA) and non-asthma control (NAC) patients.

<table>
<thead>
<tr>
<th></th>
<th>FA (n=12)</th>
<th>NFA (n=12)</th>
<th>NAC (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>**Sex (M/F)**¹</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>**Age (years)**²</td>
<td>36 (21 - 51)</td>
<td>35 (18 – 55)</td>
<td>37 (19 – 58)</td>
</tr>
<tr>
<td>**Smoking (S/NS)**³</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td><strong>Pack years</strong>³</td>
<td>2.5 - 14</td>
<td>1.25 – 47.5</td>
<td>9 – 41.25</td>
</tr>
<tr>
<td>**Age at asthma onset (years)**²</td>
<td>11 (1 – 44)</td>
<td>17 (2 - 30)</td>
<td>N/A</td>
</tr>
<tr>
<td>**Duration of asthma (years)**²</td>
<td>25 (2 - 43)</td>
<td>19 (2 - 31)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Asthma severity score</strong>⁴</td>
<td>0/1/11</td>
<td>1/6/4 (1 unknown)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Oral corticosteroid</strong>⁵</td>
<td>2/4/5 (1unknown)</td>
<td>10/1/0 (1unknown)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Inhaled corticosteroid</strong>⁵</td>
<td>3/5/3 (1unknown)</td>
<td>7/2/3</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Short-acting bronchodilator</strong>³</td>
<td>0/1/11</td>
<td>3/5/4</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Time from Death to Autopsy</strong>⁶</td>
<td>26.3 (5.0 – 67.5)</td>
<td>26.6 (3.0 – 58.0)</td>
<td>30.7 (7.0 - 115.5)</td>
</tr>
<tr>
<td><strong>Cause of death</strong></td>
<td>Asthma</td>
<td>Heart Disease: 5</td>
<td>Drug Overdose: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asphyxia: 1</td>
<td>Heart Disease: 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Substance abuse: 3</td>
<td>Brain Tumor: 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Morbid Obesity: 2</td>
<td>Cerebral Aneurysm: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetes: 1</td>
<td>Musculoskeletal: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Seizure disorder: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Suicide: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Meningitis: 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Malignant Melanoma: 1</td>
</tr>
</tbody>
</table>

Abbreviations: F = female; M = male; NS = non-smoker; S = smoker; N/A = Not Applicable

¹ Data expressed as number of patients
² Data expressed as median and range
³ Data expressed as range
⁴ Number of patients; Mild/Moderate/Severe
⁵ Number of patients; None/Occasional/Everyday use
⁶ Data expressed in hours as mean and range
The demographic information for the subjects used for electron microscopy from Sao Paulo, Brazil is summarized in Table 2.

Table 2. Demographic characteristics of Sao Paulo subjects, on which lung tissue was used for ultrastructural analysis: Fatal asthma (FA) and non-asthma controls (NAC).

<table>
<thead>
<tr>
<th></th>
<th>FA (n=4)</th>
<th>NAC (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)¹</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Age (years)²</td>
<td>48 (20 - 51)</td>
<td>50 (46 – 51)</td>
</tr>
<tr>
<td>Smoking (S/NS)¹</td>
<td>2/2</td>
<td>0/3</td>
</tr>
<tr>
<td>Age of asthma onset (years)²</td>
<td>4(1-10), unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>Duration of asthma (years)²</td>
<td>40(10-47)</td>
<td>N/A</td>
</tr>
<tr>
<td>Time from Death to Autopsy³</td>
<td>12 (6 - 15)</td>
<td>9.3 (5 - 14)</td>
</tr>
<tr>
<td>Oral corticosteroid⁴</td>
<td>0/4/0</td>
<td>N/A</td>
</tr>
<tr>
<td>Inhaled corticosteroid⁴</td>
<td>0/2/2</td>
<td>N/A</td>
</tr>
<tr>
<td>Short-acting bronchodilator⁴</td>
<td>0/1/3</td>
<td>N/A</td>
</tr>
<tr>
<td>Cause of death</td>
<td>Asthma</td>
<td>Heart Disease</td>
</tr>
</tbody>
</table>

Abbreviations: F = female; M = male; NS = non-smoker; S = smoker; N/A = Not Applicable

¹ Data expressed as number of patients
² Data expressed as median and range
³ Data expressed in hours as mean and range
⁴ Number of patients; None/Occasional/Everyday use
**Histology**
The subjects with asthma showed increased airway smooth muscle, mucous gland enlargement, mucous duct ectasia, goblet cell hyperplasia of the lining epithelium, thickening of the subepithelial collagen layer and infiltration of eosinophils and lymphocytes in the bronchial wall. In addition, most of the FA cases showed extensive mucous plugs and bronchoconstriction. NAC cases showed no features of asthma. Sections stained for smooth muscle actin showed staining of bronchial smooth muscle, vessels, and myoepithelial cells of the bronchial mucous glands (Figure 1). The latter were located between the basal surface of the glandular epithelial cells (mucous and serous) and the adjacent basement membrane. Fine cytoplasmic processes were seen to extend between the epithelial cells. Myoepithelial cells were only seen in the acini and proximal collecting ducts. They were absent from larger ducts that communicated with the bronchial lumen. In the NAC group, the myoepithelial cells appeared to be discontinuous (Figure 1B) whereas in the FA group they appeared continuous, thicker, and showed patchy layering (Figure 1D). The changes in myoepithelial cells from the NFA group were similar to those seen in the FA group but less pronounced.

**Electron Microscopy**
In the FA and NAC cases, myoepithelial cells were positioned between the epithelial cells and the basement membrane of the submucosal glands; the latter was thickened in the acini of the asthmatic subjects. The myoepithelial cell cytoplasm contained dense bodies and myofilaments, which lay parallel to the long axis of the cell. The nuclei were oval or elongated. The myoepithelial cells were attached to the acinar gland cells with desmosomes. In FA the myoepithelial cells had more myofilaments, and more prominent cytoplasmic processes extending between the mucous cells (Figure 2 and 3). These changes were not quantified.

**Morphometry**
Airway size (Pbm) did not differ significantly between groups (NAC, NFA, and FA) (Table 3). Mucous gland area was increased (p=0.003) in the FA group compared with the NAC group when data from all three lobes were included in the analysis. The average normalized values of subcomponents of the mucous glands were increased in FA compared with NFA and NAC (Table 4). However, only myoepithelial actin in the FA group was significantly increased (p=0.017) compared with the NAC group. When the ratio of myoepithelial cell area to total gland area was examined, there was a disproportionate and significant (p=0.014) increase for FA compared with the NAC group (Table 3). The NFA group showed changes in mucous gland area and in the components of the gland that were intermediate between the FA and NAC group values. None of these differences were significant. No significant association between smoking status and myoepithelial actin was found among our groups. Both smoking and non-smoking asthmatics showed equivalent increases in myoepithelial cell area. Thirty-one percent of the airway lumen from the FA group was occupied by mucus which was significantly (p=0.004) greater than the area of airway occupied by mucous plugs in the NAC group (6.9%) but not the NFA group (15.3%; p=0.068) (Table 3). The grade of mucous gland duct ectasia was significantly greater (p=0.012) in the FA group compared with the NAC group. There was no significant affect of smoking status on mucous gland size or mucous plug area among our groups.
Table 3. Morphometry of selected airway features in fatal asthma (FA) and non-fatal asthma (NFA) cases and non-asthma control (NAC) subjects.

<table>
<thead>
<tr>
<th>Morphometric parameter</th>
<th>Study Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAC (n = 12)</td>
</tr>
<tr>
<td>Airway perimeter, Pbm (mm)</td>
<td>16.6 ± 1.2*</td>
</tr>
<tr>
<td>Total Mucous Gland Area&lt;sup&gt;2&lt;/sup&gt;</td>
<td>128.6 ± 17.3</td>
</tr>
<tr>
<td>Mucous Plug Area/total luminal area&lt;sup&gt;3&lt;/sup&gt;</td>
<td>6.9 ± 3.2</td>
</tr>
<tr>
<td>Myoepithelial Cell Area / Mucous Gland Area&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>Grade of Mucous Gland Duct Ectasia&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4 (33%)</td>
</tr>
</tbody>
</table>

* Values expressed as means ± standard error of the mean

1 P value for the FA group compared with the NAC group using ANOVA.
2 These areas were normalized to the basement membrane perimeter (A/Pbm)
3 Expressed as a percentage.
4 Data expressed as number of patients with a grade of 2 or greater (percentage of patients)
5 P value for the FA group compared with the NAC group using Pearson Chi-squared test.
Table 4. Morphometry of selected airway mucous gland subcomponents in fatal asthma (FA) and non-fatal asthma (NFA) cases and non-asthma control subjects.

<table>
<thead>
<tr>
<th>Mucous Gland Subcomponents</th>
<th>NAC (n = 12)</th>
<th>NFA (n = 12)</th>
<th>FA (n = 12)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin: myoepithelial</td>
<td>14.2 ± 2.3*</td>
<td>22.1 ± 2.7</td>
<td>32.7 ± 6.9</td>
<td>0.017²</td>
</tr>
<tr>
<td>Actin: non-myoepithelial³</td>
<td>2.8 ± 0.5</td>
<td>3.3 ± 0.4</td>
<td>3.3 ± 0.4</td>
<td>0.736²</td>
</tr>
<tr>
<td>Acinar cell cytoplasm</td>
<td>41.0 ± 5.4</td>
<td>63.8 ± 11.1</td>
<td>71.9 ± 15.4</td>
<td>0.151²</td>
</tr>
<tr>
<td>Acinar cell nuclei</td>
<td>4.5 ± 0.6</td>
<td>8.7 ± 1.5</td>
<td>9.0 ± 2.3</td>
<td>0.147²</td>
</tr>
<tr>
<td>Acinar Lumen</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>2.0 ± 0.8</td>
<td>0.118²</td>
</tr>
<tr>
<td>Interstitium⁴</td>
<td>66.1 ± 9.1</td>
<td>97.5 ± 14.2</td>
<td>109.8 ± 20.2</td>
<td>0.120²</td>
</tr>
</tbody>
</table>

* Values expressed as means ± standard error of the mean

1 Expressed as area/basement membrane perimeter (A/Pbm)

2 P value for the FA group compared with the NAC group using ANOVA.

3 Includes arteries, arterioles, veins and lymphatics.

4 Includes connective tissue, nerves and inflammatory cells.
DISCUSSION

In this study we show that the increased size of bronchial mucous glands in fatal asthma is accompanied by a disproportionate increase in smooth muscle actin in the myoepithelial cells of the glandular acini. These changes were associated with mucus plugs within the airway lumen and ectasia of the gland ducts. Our results suggest that myoepithelial cells contribute to the abundant mucus that is characteristic of fatal asthma.

The cause(s) of the disproportionate increase in myoepithelial cell area in fatal asthma are unknown. However two plausible explanations may be postulated. Firstly, it could be due to an increased workload required to expel the highly viscous mucus characteristic of fatal asthma[6;8;20] into the airway lumen. Increased intra-duct pressures might also contribute to the mucous gland duct ectasia described in this and other studies.[21] Secondly, it may reflect an increased stimulus for mucus secretion in asthma in response to asthma triggers. In this regard, it would be one more component of the airway wall affected by remodelling. The area of the airway smooth muscle layer is also increased in asthma,[4;22] so that a generalised effect of inflammation, release of growth factors or mechanical influences could affect the amount of contractile tissue within the airway wall, including myoepithelial cells.

We assessed the area fraction of myoepithelial cells based on staining of smooth muscle actin. This gives an estimate of the overall area of myoepithelial cells and since the thickness of the section is less than 10 percent of the thickness of the myoepithelial cell, the area fraction provides a good estimate of the volume fraction of these cells. The increased area and volume fraction of actin staining suggests an increase in contractile tissue. The two-dimensional approach used in this study did not allow us to accurately estimate the number or size of individual myoepithelial cells and thus we were not able to determine if the changes to the myoepithelial cells resulted from cell hyperplasia and/or hypertrophy. This awaits a separate study.

Studies of myoepithelial cells in bronchial mucous glands in humans are scarce in the literature.[13] To our knowledge, this is the first study to examine myoepithelial cells in bronchial mucous glands of asthmatics. Myoepithelial cells are contractile in nature, possessing myofilaments composed of actin and myosin, dense bodies, caveolae and elongated mitochondria.[13;14;23] The mechanism of stimulation varies by gland type and species. For example, myoepithelial cells from human apocrine sweat glands respond to alpha-adrenergic stimulation,[24] whereas porcine bronchial submucosal glands contract in response to acetylcholine.[25] In dogs, secretion from mucous type epithelial cells occurs in response to both β-adrenergic and cholinergic stimulation.[15] In humans tachykinin mechanisms may also be important.[26;27] Thus, myoepithelial cell contraction may be caused by cholinergic, β-adrenergic or peptidergic mechanisms. Our findings may be relevant to studies of smooth muscle cell function in asthma. Mast cells and neutrophils are increased in the submucosal glands of asthmatics.[28] The differentiation and function of myofibroblasts is regulated by mast cell mediators.[29] Muscarinic receptors, especially the M3 receptor subtype, have been implicated in smooth muscle proliferation[30] and contractile protein expression in mesenchymal cells in asthma.[31] Whether similar pathogenetic pathways occur in the myoepithelial cells
of the bronchial submucosal glands of asthmatics is not known, but further research on these topics is warranted. An increase in bronchial submucosal gland area has been reported as a pathological marker of asthma. Our study supports this observation. We found a significant increase in mucous gland area as a proportion of airway size in fatal asthma compared with non-asthma controls. Deposition of mucus into the airway lumen is also a prominent feature of fatal asthma, where it is thought to contribute to increased airway resistance and is readily apparent at autopsy in cases of fatal asthma. Our study found that luminal mucus content was increased in both NFA and FA groups compared with the NAC group. The FA cases had, on average, 30% of their airway lumen occupied by mucus compared to approximately 7% in the NAC group. Similar findings have been reported by other investigators. Smoking is also associated with an increase in the size of mucous glands in the conducting airways. Smoking evokes a reflex increase in tracheal submucosal gland secretion in dogs. The effect of smoking on myoepithelial cells is largely unknown, but myoepithelial cell ultrastructure was reported to be unaffected by smoke exposure in rat tracheal submucosal glands. In the present study, cigarette smoking was not associated with an increase in myoepithelial cell smooth muscle actin. Our failure to detect an effect of cigarette smoking on myoepithelial cell actin may be related to small sample sizes, the relatively young age of the population and/or the low to moderate pack years of the cigarette smokers. In summary, we show that excess luminal mucus in fatal asthma is associated with enlarged submucosal glands, increased myoepithelial cell area and a disproportionate increase in myoepithelial smooth muscle actin. These findings add to our understanding of the complex mechanisms leading to death during an acute asthma attack. The results of this study indicate that new therapeutic strategies for asthma treatment might include medications designed to reduce myoepithelial cell contractility.
LEGENDS FOR FIGURES

Figure 1
A. Low power (X 100) photomicrograph of a bronchial mucous gland from an eighteen year old, non-smoking, non-asthmatic female. The section is stained for smooth muscle actin and shows staining of myoepithelial cells on the outer border of the mucous gland acini. More intense smooth muscle actin staining is seen of small blood vessels (BV) within and adjacent to the gland.

B. Higher power (X 400) view of bronchial mucous gland shown in Figure A. The myoepithelial cell network (arrows) in the normal acinus appears discontinuous.

C. Low power (X 100) photomicrograph of a mucous gland in the bronchial wall of a 42-year-old, non-smoking male, who died of asthma. The gland is larger than that seen in the non-asthma control (A above). The section is stained for smooth muscle actin. There is marked staining of myoepithelial cells within the mucous gland.

D. Higher power (X 400) view of center of gland shown in Figure C. There is increased staining of myoepithelial cells around the acini. The myoepithelial cells are thicker than in the control (B above) and are continuous around the circumference of the acini and in some areas appear multi-layered (arrows).

Figure 2
Ultrastructural aspect of a submucosal gland in a non-smoking, non-asthmatic control patient, female, 50 years old. A and B: Myoepithelial cells (mep) lie under the glandular cells (gc) inside the basal lamina (bl). C: The cytoplasm of the myoepithelial cell has a filamentous appearance (due to the presence of actin and myosin) with dense bodies (arrowheads). The myoepithelial cell is attached to an acinar glandular cell via desmosomes (arrow).

Figure 3
Ultrastructural aspect of a submucosal gland in a 45 year old male smoker with fatal asthma. A: Myoepithelial cell (mep) with large cytoplasmic branches around the acinar glandular cells (gc). The glandular basal lamina seems to be thickened (bl). B: Detail of a myoepithelial cell with prominent cytoplasm, rich in myofilaments and showing dense bodies (arrowheads).
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The authors have no conflict of interest to declare.

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Supplemental information on the methodology:

Increased Myoepithelial Cells of Bronchial Submucosal Glands in Fatal Asthma

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1. Protocol for lung fixation using glutaraldehyde (PPAS subjects)

Because subjects who died of asthma usually had extensive mucus plugs that airway fixatives would not penetrate we developed a dual perfusion/fixation procedure that involved simultaneous perfusion of airways and vasculature at different pressures. The method is illustrated in figure 1. We used glutaraldehyde for its superior preservation of ultrastructure and architecture.
Figure 1. Diagram of equipment used for lung fixation.

**Equipment and Procedures: (refer to figure1)**

a. **Perfusion of airways**

1. One 5-litre canister with bottom outlet.

2. The canister contains glutaraldehyde and is fitted with a tight-fitting rubber stopper and glass tubing, reaching to bottom of canister. This establishes a constant pressure head of 20cm H₂O.

3. Tubing connector is tied into main bronchus and the connection reinforced with tissue cement.
b. Perfusion of Pulmonary Vascular Bed

1. Two canisters are used. One canister contains 5L N saline, the other glutaraldehyde. Their bases are maintained 20cm and 40 cm respectively above the lung.

2. The canisters are interconnected by latex tubing and a Y connector. Below the Y connector, the latex tubing that extends to the lung is fitted to a plastic tube connector that can be firmly tied to the stump of the pulmonary artery.

c. Preparation of the lung

1. The lung is carefully inspected and the main bronchus, pulmonary artery and pulmonary veins are located. Extraneous connective tissue is removed from around the major structures, leaving long stumps.

2. All tears in the pleural surface are located and repaired by gluing tissue paper or gauze to them with cyan acrylic tissue cement (e.g. Vet Bond or Superglue).

d. Rinsing the pulmonary vascular bed

1. The tubing connector of perfusion line is tied into the stump of the pulmonary artery and reinforced with cyan acrylic tissue cement.

2. Perfusion lines are filled with saline and glutaraldehyde and the glutaraldehyde line is shut off.

3. The pulmonary vasculature is perfused with saline at 20cm H₂O pressure until the outflow from pulmonary veins runs clear. As the lung begins filling with saline, fixation is commenced with infusion of both the airways and the pulmonary artery at 20cm H₂O pressure.

4. Saline flow is shut off.

e. Fixation

1. The lung is placed in a 20L storage container, containing glutaraldehyde fixative.
2. After saline rinse begin flow with glutaraldehyde.

3. Glutaraldehyde perfusion flow is reduced to approximately 2L/hr and perfused for at least 2 hours, or until contents are exhausted, whichever is longer.

4. Perfusion pressures are maintained. The vascular perfusion canister should deliver a pressure of 40cm H₂O, and the bronchial instillation canister at 20cm H₂O. If the vasculature is very leaky, perfusion pressure in vasculature is reduced to approximately 20cm.

5. Fixation is completed with tubing still attached and pressure head intact, in ventilated storage box for 24 hours.

**f. Immunohistochemistry:**

In pilot studies, we fixed samples of left and right lungs from the same case both with either 10% formaldehyde or 2.5% glutaraldehyde and compared the immunostaining for a variety of proteins. Contrary to our expectations we found staining for smooth muscle actin was superior with the glutaraldehyde fixative compared with formaldehyde. Therefore, we did not have to use antigen retrieval methods to achieve excellent staining.

2. Sao Paulo Subjects

The Sao Paulo subjects were used for ultrastructural analysis only. After autopsy a domiciliary interview with the next-of-kin was performed to obtain data related to asthma history. The interviews assessed demographics, per capita income, living conditions, school education and smoking habits. Ethnicity was defined by the next of kin. Body mass index was obtained from the autopsy records. The history of asthma was characterized by age at onset of symptoms, parental history of asthma, use of regular medical care and medications received in the last 6 months. Relatives were asked if the
deceased had been hospitalized due to asthma in the last year and whether the patient had ever been admitted to an Intensive Care Unit for an asthma exacerbation. When patients had received regular medical care in a hospital, the institutions were contacted to obtain medical information.


As stated in the text of the manuscript, our patients were characterized as having poorly-controlled, under-treated and severe asthma. Indeed, most did not receive regular medical care or anti-inflammatory treatment with corticosteroids. Many had persistent symptoms and signs of severe asthma such as hospitalizations and intensive care admissions for asthma.

3. Study Design and Inclusion/Exclusion Criteria for the Prairie Provinces Asthma Study (PPAS)

The Prairie Provinces Asthma Study was a multi-centered case control study conducted in the Canadian Prairie Provinces over a three-year period from November 1991 to October 1995. Ethical approval for the study was obtained from the four major universities in each province: The University of Calgary, University of Alberta, University of Saskatchewan and University of Manitoba. Informed consent was obtained from the next of kin.

Suspected asthma deaths were reported to the study. The notification came through the medical examiners/coroners offices, emergency departments and vital statistic departments.
To insure participation in the study and notification of the asthmatic deaths, letters introducing the study were sent to hospital administrators, health record departments, emergency departments, admission departments (Manitoba only) and Vital Statistics departments in the three provinces.

Twenty-four centers were identified in the three provinces where the asthma kits would be stored, making the kits accessible to any pathologist in the event of an asthmatic death.[1]

The asthma kits were preassembled to insure the participation of the pathologist and to ensure the collection of specimens would be according to study protocol.

After notification of an asthma death, contact was made with the pathologist. The specimens were collected and couriered to the pathology study center in Calgary. The next of kin were asked to complete a questionnaire regarding the deceased’s asthma.

**Consent for Pathological Specimen Collection**

For medical legal autopsies, consent of the next of kin for the autopsy was not required. For these cases consent for the research component was obtained after the autopsy.

Pathological specimens specified by the study pathology protocol were obtained at time of autopsy to substantiate the diagnosis of asthma but the results were not used for study purposes unless the family consented. This approach allowed the family more time to consider whether they wanted to participate in the study. It was also more practical for collecting the pathology samples in the cases where contact with the next of kin prior to the autopsy was not possible.

When an autopsy was not required for medical legal reasons, the next of kin were contacted and consent for autopsy was sought. The contact with the family was established through the deceased’s personal physician.

**Procurement of Specimens**
Upon the notification of an asthma death, contact with the pathologist was made to ensure that an asthma kit was available and that the pathology protocol would be followed. The specimens were sent via courier to The University of Calgary where the distribution, processing and analysis occurred.

**Questionnaire to the Next of Kin**

A condolence letter was sent to the family of the asthma fatality after the notification of death. The family would then be contacted by telephone to solicit participation in the study. If the family agreed, a questionnaire accompanied by a letter was mailed to the family. Follow-up with the family by telephone or mailed correspondence continued until the questionnaire was returned. If the family did not feel comfortable completing the mailed out questionnaire, a telephone or in-person interview was arranged.

**Pathology Controls**

The procedure for obtaining consent for participation was the same as for the asthma fatalities. The next of kin agreed to donate pathology specimens and complete the questionnaire. Two types of controls were identified:

1) Non-Fatal Asthma; those with a history of asthma or asthma discovered at autopsy but asthma was not the cause of death and

2) Non-Asthma Controls; those with no history of asthma or evidence of asthma at autopsy and with no significant lung disease at autopsy

The latter group received a modified questionnaire in which most of the questions pertaining to asthma were removed.

**4. Classification of Cases and Inclusion/exclusion Criteria**

A classification form was developed to classify the cases and controls. The scene investigation report, medical information, the pathologist’s autopsy report, the study
histology report and toxicology results were reviewed in order to complete the
classification of the case. The classification form is shown below.

### Prairie Provinces Asthma Study

**FORM USED FOR CASE CLASSIFICATION**

<table>
<thead>
<tr>
<th>Name: ____________________________</th>
<th>Case #: __________</th>
<th>Lab #: __________</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOD: ____________________________</td>
<td>Location: __________</td>
<td>Gender: _________</td>
</tr>
</tbody>
</table>

All sections need to be filled out for each case.

#### A. Preliminary History

Were circumstances prior to death compatible with asthmatic death?

- __ definitely __ unlikely
- __ possibly __ not at all
- __ not known

#### B. Pathological Criteria

Was an autopsy performed?

- __ yes __ no

If yes, answer (1) and (2)

1. Asthma evident? (based on histology)
   - __ yes __ no

2. Acute asthma evident? (based on gross and microscopic examination including hyperinflation, atelectasis, mucus plugs and bronchial constriction)
   - __ yes __ no

If no to (1) or (2), what was the cause of death? ________________

EXPLANATION:
- (a) Yes to (1) and (2) classifies as an asthma case and involves the epidemiological component
- (b) Yes to (1), no to (2) is an asthma control and doesn’t involve the epidemiological component
- (c) No to (1) and (2) is a non-asthma control and has no epidemiological follow up

#### C. Identifiable Co-factors Contributing to Death or Exacerbating Asthma

1. External Factors
   - __ yes __ no
   - __ food inhalation
   - __ water inhalation
   - __ drugs / alcohol
   - __ medication
   - __ other: ________________

2. Natural Disease
   - __ yes __ no
   - __ obesity
   - __ epilepsy
   - __ cardiac disease
   - __ other: ________________

EXPLANATION:
- (a) For an asthmatic case, no to (1) and (2) constitutes a classic asthmatic death. The study epidemiology component is to be pursued
(b) Yes to either (1) or (2) puts the asthma case into a subgroup. The study epidemiology component is to be pursued.

D. Clinical Decision (used for inclusion if no autopsy)
(1) Clinical history of asthma before death? __ yes __ no

(2) Features of asthma found in review of clinical history and witness reports of final illness/event? __ definitely __ unlikely
__ possibly __ not at all __ not known

EXPLANATION: (a) Yes to (1) and the responses definitely or possibly to (2) classifies an asthma case and involves the epidemiological component
(b) The case is not enrolled in the study if the response to (1) is no or the response to (2) is either unlikely or not at all.

E. Classification of Case

__ Asthma Case – Classic: cause of death is asthma; age range is 5-50 years old, EPI component pursued
__ Asthma Case – Subgroup: cause of death is asthma but co-factors involved (C above), age range is 5-50 years old, EPI component pursued
__ Asthma Control: History of asthma but asthma not cause of death
__ Non-Asthma Control: No history of asthma
__ Excluded from study: State reason: __________________________________________________________

Reviewed by: ______________________
Name __________________________
Date __________________________

5. Stains and Morphometry

Stains for Mucous Glands and Glandular Components

The area of mucous glands was evaluated on Hematoxylin and Eosin stained sections. Mucous glands were also stained with Alcian-Blue/PAS (AB/PAS) and alpha smooth muscle actin.

The AB/PAS stain was used to differentiate between the different types of mucins produced by the mucous cells of the gland and between mucous and serous cells. The AB/PAS stain results in acidic mucins colouring blue, neutral mucins staining magenta,
and various other epithelial mucins staining purple. Serous cells do not stain using AB/PAS, resulting in clear cells which can be differentiated from the variously colored mucous cells. Some mucous cells which have secreted their mucins also may not stain with AB/PAS. This stain was primarily used to determine the relationship between myoepithelial cells and the different types of epithelial cells within the glands; mucous, serous and ductular. Attempts to quantify the mucous cells by mucin type were unsuccessful due to the large overlap of colors, making a determination of mucus type very subjective.

The alpha smooth muscle actin stain was used to visualize the myoepithelial cells surrounding the acini of the mucous gland. The protein actin was coloured brown. Myoepithelial cells, containing much actin, were easily identified. Structures counted using this stain were:

- Actin-stained myoepithelial cells: these were found around the acini within the gland
- Actin-stained non-myoeptihelial cells: this group was almost entirely composed of blood and lymphatic vessels.
- Acinar nuclei: stained blue to black using the actin stain
- Acinar lumen: included both free lumen and lumen containing mucin
- Acinar epithelial cytoplasm: included mucous and serous cells
- Interstitium/Other: this category included points not falling on any of the other counted structures and included connective tissue, nerves, inflammatory cells and fat cells.

**Morphometry of the Mucous Glands and Their Subcomponents**

We used a standardized nomenclature of airway compartments and features as previously described by Bai et al., 1994 and James et al 2009.[2,3] First, at low
magnification (20x), the area fraction of mucous glands in a horizontal and complete profile of the segmental bronchi was calculated; second, the individual components of the glands were calculated at higher magnifications (100-400x), dependent on the size of the gland.

All profiles of mucous glands in an airway section were evaluated on the H&E stain sections. The mucous gland capsule was defined as the outer boundary of the gland. Points falling on the capsule, or inside it, were counted as gland, those falling outside were ignored for purposes of this study. Point counting of the whole mucous gland was performed at a magnification of 25X using a square grid containing 240 points. Intersection counts of the grid lines with the basement membrane of the airway epithelium were used to calculate the length of the airway basement membrane. This unit was called ‘Pbm’ and used to normalize the mucous gland measurements for differences in airway size.

The individual subcomponents of the mucous glands were evaluated on the smooth muscle actin stain sections. All profiles of glands within a given section were evaluated.

**Formulas used in Morphometry**

Point counts can be expressed as areas using the formula:

\[
\text{Area} = (Z^2)(\text{Point count value})
\]

Where Z is a magnification constant, obtained by measuring the distance between the grid intersections at a specific magnification.

The same grid was used to determine the perimeter of the airway using the following formula:

\[
\text{Airway Basement Membrane Perimeter (Pbm)} = (\pi . l . Z) / 4
\]
Where \( I \) is the number of intersections of the grid lines with the luminal aspect of the airway basement membrane and \( Z \) is related to the distance between the lines, corrected for magnification.

**Normalization**

The area fractions of the mucous glands were standardized to airway size by dividing the area of a feature by the perimeter of the airway (\( A/P_{bm} \)).

The area fractions of the subcomponents of the mucous glands were normalized to the area of the gland and expressed as a percent of the total gland area.

**Power Calculation**

The statistical power of our study was calculated using the methods described by Rasch B. et al (2006) and one way analysis of variance (ANOVA).[4] Taking into account our study design, we calculated the effect size \( F \). The sample used for our power calculation consisted of 36 individuals that had been accessioned early in the study and evaluated for airway smooth muscle thickness. We considered that this would be an appropriate marker for a closely related cell type; the myoepithelial cell. There were 12 cases in each group (FA, NFA, and NAC), with equal numbers of males and females. The FA group had 5 smokers and 7 non-smokers. The NFA group had 7 smokers and 5 non-smokers, and the NAC group had 9 smokers and 3 non-smokers. The mean (range) ages by group were: FA; 35 (18-50), NFA : 29 (21-54), NAC; 41 (26-57) years. The mean ± SE pack years of smoking by group (FA, NFA, and NAC) were 16 ± 4.0, 37± 19.2, and 22 ± 4.3, respectively. Using the preliminary observed mean/standard deviation of airway smooth muscle area for the three groups, we obtained effect size \( F=0.53 \). Through the aforementioned method, an effect size \( F=0.53 \), an \( \alpha \) error probability=0.05, total sample size=36, and number of groups=3, we obtained a value of critical \( F=3.28 \) and a power of 0.77.
Literature Cited

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