

ORIGINAL ARTICLE

Time to death and mast cell degranulation in fatal asthma

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ABSTRACT

Background and objective: Attacks of fatal asthma have been shown to be either of short duration or long duration and associated with differing degrees of smooth muscle contraction, luminal mucus deposition and ratios of eosinophils to neutrophils in the airway wall. We hypothesized that this bimodal distribution might be related to airway mast cell degranulation.

Methods: Airway sections from cases of fatal asthma in the second Victorian asthma mortality study and from cases coming to coronial autopsy in Perth were examined. Tryptase-positive mast cells, numbers of intact and degranulated mast cells, post-mortem blood salbutamol levels and airway dimensions were measured.

Results: Although the total number of mast cells were similar, the ratio of degranulated to intact mast cells (D/I) was significantly increased in the smooth muscle ($P < 0.05$) and outer airway wall ($P < 0.001$), in short-duration cases compared with long-duration cases. Proportional muscle shortening was significantly increased ($P < 0.05$) in short-duration cases ($17 \pm 7\%$) compared with long-duration cases ($11 \pm 7\%$). Blood salbutamol levels were related to the total airway wall mast cell D/I ratio for all cases combined ($r = 0.57$, $P = 0.01$).

Conclusions: The duration of a fatal attack of asthma may be partly determined by the degranulation of mast cells.

Key words: airway wall, degranulation, fatal asthma, mast cell, smooth muscle.

SUMMARY AT A GLANCE

This study assessed whether the strength of an exacerbating stimulus might explain the consistent dichotomy in duration of fatal asthma attacks. Although total mast cell numbers were similar, the ratio of degranulated to intact mast cells and proportional muscle shortening were increased in short-duration compared with long-duration cases of fatal asthma. This is consistent with a stronger exacerbating stimulus.

INTRODUCTION

Patients with asthma have variable symptoms, including breathlessness, chest tightness and wheeze, which result from excessive airway narrowing. Fatal asthma is characterized pathologically by accumulation of mucus in the lumen, inflammatory cell infiltration of the airway wall and surrounding parenchyma, variable smooth muscle shortening and remodelling of various components of the airway wall.¹⁻⁴ Mast cells reside within the airway wall,⁵ and are activated when allergen-specific IgE cross-links IgE Fc receptors on their surface.^{6,7} Mast cells release tryptase, histamine and cytokines when they degranulate, and these pre-formed mediators, together with newly formed mediators such as leucotrienes and prostaglandins, induce airway smooth muscle shortening, mucous gland secretion, and promote inflammation.⁶ Our previous studies have shown that airway mast cell degranulation in asthma is related to disease severity and secretion of mucus into the lumen.^{5,8}

Fatal asthma attacks can be separated into two non-overlapping time-courses: short duration, where death occurs within a few hours of the onset of symptoms, and long duration, where death occurs 8 h or more after the onset of symptoms.^{3,4,9,10} In all these studies, the short-duration cases were associated with

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a reduced ratio of eosinophils to neutrophils in the airway wall, compared with long-duration cases. The reasons for this reproducible dichotomy are not clear; however, our previous studies have shown that in short-duration cases there is increased airway smooth muscle shortening, which is presumably an acute event, whereas in long-duration cases there tends to be increased mucus secretion, likely reflecting a more cumulative change.⁴ Degranulation of mast cells is associated with both smooth muscle contraction and mucous gland secretion.^{5,7,8} Factors that influence the intensity of mast cell degranulation,¹¹ as well as the site of mast cell degranulation within the airway, may affect the duration of a fatal asthma attack. The aim of this study was to examine the proportion of degranulated mast cells that were present in the airway wall, and particularly in the airway smooth muscle and associated with the submucosal mucous glands, in both short-duration and long-duration cases of fatal asthma. We hypothesized that there would be more degranulation of mast cells in the smooth muscle in short-duration cases compared with long-duration cases.

METHODS

Cases of fatal asthma were identified at two centres within Australia. The Melbourne cases formed part of the second Victorian asthma mortality study,¹² and the Perth cases were those coming to coronial autopsy within the metropolitan area.² At both centres, data regarding the history of asthma, asthma symptoms, use of asthma medications, smoking history, time off work or school due to asthma, hospitalization due to asthma and measurements of lung function (where available) were obtained from next-of-kin, hospital files, coronial files and the subjects' usual medical practitioners. The duration of the fatal attack of asthma was determined from these sources, and cases were categorized as: short duration, where the time of death from the onset of asthma symptoms was <3 h, or long duration, where the time of death from the onset of asthma symptoms was >8 h. The study was approved by the ethics committees of the Victorian Institute of Forensic Medicine and Sir Charles Gairdner Hospital.

Tissue preparation

For the Melbourne cases, tissue blocks were taken at random from various sites in the lung, at post-mortem examination. The tissue was fixed by immersion in 10% buffered formalin solution for >24 h and then passed through a series of graded alcohols and embedded in paraffin wax. Salbutamol assays were performed by gas chromatography-mass spectrometry, using post-mortem samples of whole blood or plasma, taken when the body arrived at the Victorian Institute of Forensic Medicine. A solid phase extraction procedure was used, with a limit of quantitation of 1 ng/mL for each analyte, in 1 mL of blood or plasma.¹³ For the Perth cases, whole lungs were

obtained at post-mortem examination and fixed by inflation in 10% buffered formalin solution for >24 h. Airways were systematically sampled using a stratified design and then passed through a series of graded alcohols and embedded in paraffin. For all tissues, immunohistochemical staining was performed on 4- μ m sections, using a mAb to mast cell tryptase (clone AA1, DakoCytomation, Sydney, Australia), as previously described.⁵

Morphological measurements

Using a light microscope, camera lucida and digitizing tablet connected to a personal computer, the following measurements were made on airways cut in transverse section, defined by an even thickness of mucosa and submucosa: the areas and perimeters defined by the luminal surface of the epithelium (Ai, Pi), basement membrane (Abm, Pbm), the outer border of smooth muscle (Ao, Po) and the outer adventitial border (At, Pt). Pbm was used to define airway size. The areas of smooth muscle, submucosal mucous gland and luminal mucus (including cellular debris) in airways were measured directly, using the digitizer.

Calculations

The following areas were calculated: total wall area ($W_{at} = At - Abm$), outer wall area ($W_{ao} = At - Ao$), inner wall area ($W_{ai} = Ao - Abm$) and epithelial thickness ($E_{pi} = Abm - Ai$). The per cent of smooth muscle shortening (PMS) was calculated by comparing the measured outer perimeter of the airway smooth muscle (Ao) with its calculated, relaxed perimeter.¹⁴ The amount of luminal mucus was expressed as a proportion of the calculated relaxed, fully dilated airway lumen—the mucus occupying ratio (MOR).¹⁵

Mast cell counts

Tryptase-positive mast cells were counted in the inner and outer airway wall, in airway smooth muscle and in the mucous glands and epithelium, when present. Tryptase-positive nucleated cells were identified as intact mast cells if they showed dense, uniform staining of the cytoplasm, or degranulated mast cells if they showed a non-uniform cytoplasm with extracellular granules and a disrupted cell membrane. Cells within blood vessels in the airway wall were not counted. Mast cell counts were expressed as the number of cells per mm² of airway wall, smooth muscle, submucosal mucous gland or epithelium and as the degranulated/intact (D/I) ratio within these airway wall compartments.

Analysis

Cases were categorized by time to death as either short or long duration. Statistical analysis included

tests of normality and homogeneity of variance. Differences between groups were compared using Mann–Whitney *U*-tests for variables that were not normally distributed and Student's *t*-tests for normally distributed variables. Results are expressed as mean $\pm$ SD or median and interquartile range (IQR). Blood salbutamol values were log transformed prior to analysis. All measurements were made by the one observer (J.E.) who was blinded to the case classification. Statistical analyses were performed using SigmaStat for Windows version 3.0 (SPSS Inc., Sydney, Australia).

RESULTS

Tissue from a total of 29 cases of fatal asthma with known time to death from the onset of symptoms was examined. Airways from the short-duration cases ($n = 20$) were larger ($P < 0.05$) than those from the long-duration cases ($n = 9$) (Pbm ranges 0.9–16.9 and 0.7–13.5 mm, respectively, Table 1). PMS was significantly increased ($P < 0.05$) in the short-duration cases ($17 \pm 7\%$) compared with the long-duration cases ($11 \pm 7\%$); however, there were no differences in the MOR (Table 1).

Mucous glands were present within the airway wall in 29% of airways and fully intact epithelium along the basement membrane was present in 34% of airways, with no differences between the groups. The mast cell area density (cells/mm²) was similar for short-duration and long-duration cases for intact, degranulated and total mast cells in the inner and outer airway wall, in the airway smooth muscle, and in the epithelium and mucous glands (Table 2). The

D/I ratio was significantly increased in short-duration compared with long-duration cases in the outer airway wall (0.59 ± 0.12 vs 0.39 ± 0.14 , $P < 0.001$) and in the airway smooth muscle (0.65 ± 0.22 vs 0.47 ± 0.19 , $P < 0.05$), but not in the inner airway wall (0.62 ± 0.18 vs 0.48 ± 0.17 , $P = 0.06$) (Table 2, Fig. 1). There were no significant differences in the D/I ratio in the epithelial layer or in mucous glands (Table 2). There was no relationship between PMS or MOR and D/I ratio in the total airway wall for all cases combined or for short- and long-duration cases examined separately (data not shown). There were no differences for current smokers ($n = 6$) compared with non-smokers ($n = 13$) in total mast cell density (59 cells/mm² (IQR 52–83) vs 119 cells/mm² (IQR 69–141), $P = 0.27$) or D/I ratio (0.59 ± 0.17 vs 0.58 ± 0.18 , $P = 0.9$) in the total airway wall or within specific compartments.

Of the 29 cases, blood salbutamol levels were measured for 18 of the Melbourne cases. There was a positive relationship between D/I ratio in the total airway wall and log salbutamol levels for all cases combined ($r = 0.57$, $P = 0.01$), although there was no significant relationship for short- or long-duration cases analysed separately (Fig. 2). There was a positive relationship between PMS and log salbutamol levels for all cases combined ($r = 0.52$, $P = 0.03$), but no significant relationship for short- or long-duration cases analysed separately.

DISCUSSION

In this study of fatal asthma, cases were separated *a priori* into two groups, based on the duration of the fatal attack. In short-duration cases, death occurred < 3 h from the onset of symptoms, whereas in long-duration cases, death occurred > 8 h from the onset of symptoms. Although there were no differences in the overall mast cell density in any compartment of the airway wall, the short-duration cases showed a significant increase in the ratio of degranulated to intact mast cells in the outer airway wall and on the smooth muscle, compared with long-duration cases. In addition, there was a significant increase in the per cent airway smooth muscle shortening in short-duration cases compared with long-duration cases.

Four separate cohort studies of fatal asthma, including two of our previous studies,^{3,4,9,10} have shown that the duration of the fatal attack falls into two distinct, non-overlapping time frames. In each of these four studies of fatal asthma, the dichotomy in time to death was matched by a dichotomy in the ratio of eosinophils to neutrophils in the airway wall. As these observations may provide clues regarding the mechanisms associated with fatal asthma or exacerbations of asthma, aspects of airway remodelling and inflammation were examined in the present study. Muscle shortening was previously shown to be greater in short-duration cases, while secretion of mucus tended to be greater in long-duration cases.⁴ The results of the present study show that differences in mast cell degranulation are related to the duration of the attack. This suggests that increased mast cell degranulation,

Table 1 Characteristics of the subjects and airway size and number in relation to time to death in cases of fatal asthma

	Short-duration cases $n = 20$	Long-duration cases $n = 9$
Time to death (h)	1.3 ± 0.6	12 ± 5.6
Age (years)	28 ± 18	29 ± 15
Gender, male/female	13/7	5/4
Weight (kg)	59 ± 17	63 ± 23
Height (cm)	162 ± 18	160 ± 19
Smoking, yes/no/ex	3/9/5	3/4/1
Airways per case	3.9 ± 3.7	4.3 ± 3.3
Airway size (Pbm, mm)	$4.8 (3.3–7.7)$	$3.1 (2.2–3.8)^*$
Salbutamol (ng/mL)	$104 (23–257)$	$23 (9–41)$
(n)	(10)	(8)
Inhaled corticosteroids in the last month, % (n)	$40 (8)$	$44 (4)$
Muscle shortening (%)	17 ± 7	$11 \pm 7^*$
Mucus occupying ratio	18 ± 15	23 ± 25

* $P < 0.05$.

Data are mean $\pm$ SD or median (interquartile range).

Pbm, perimeter of basement membrane.

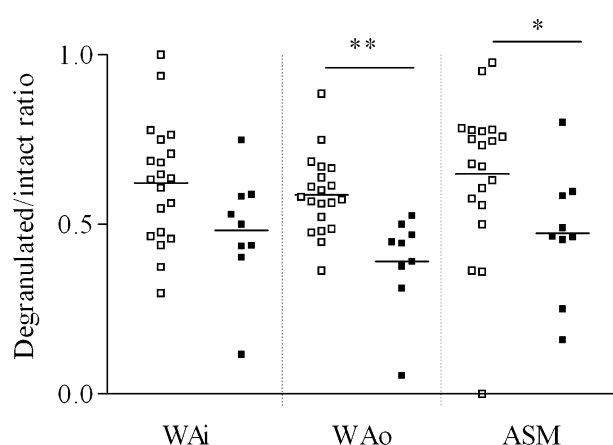
Table 2 Mast cell density and ratio of degranulated/intact mast cells in the airway wall in relation to time to death in cases of fatal asthma

	Inner wall	Outer wall	Smooth muscle	Epithelium	Submucosal mucous gland
Total mast cells (cells/mm ²)					
SD	59 (31–91)	100 ± 78	113 ± 93	61 ± 47	17 (11–33)
LD	55 (32–71)	105 ± 69	98 ± 70	40 ± 38	23 (9–60)
Degranulated mast cells (cells/mm ²)					
SD	30 (22–55)	38 (27–87)	62 (38–114)	26 ± 28	10 (7–20)
LD	28 (17–45)	28 (19–62)	39 (30–96)	14 ± 13	13 (6–36)
Ratio of degranulated/intact mast cells					
SD	0.62 ± 0.18	0.59 ± 0.12**	0.65 ± 0.22*	0.27 ± 0.27	0.57 ± 0.24
LD	0.48 ± 0.17	0.39 ± 0.14	0.47 ± 0.19	0.23 ± 0.30	0.68 ± 0.14

* $P < 0.05$, ** $P < 0.01$.

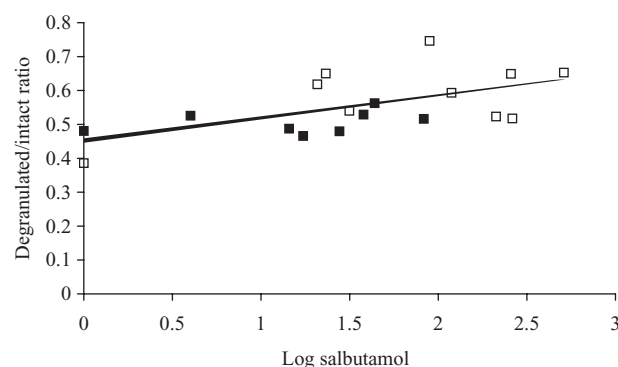
Data are median (interquartile range) or mean ± SD.

SD, short-duration cases; LD, long-duration cases.

**Figure 1** Scatter plots showing the ratios of degranulated/intact mast cells in the inner (WAI) and outer airway wall (WAO) and on the airway smooth muscle (ASM) in short-duration cases (open squares) and long-duration cases (solid squares). The horizontal lines indicate the group means. * $P < 0.05$, ** $P < 0.01$.

occurring concurrently with remodelling, airway inflammation or other factors may account for the two distinct time-courses of fatal attacks.

The mechanisms that underlie the two distinct non-overlapping time-courses (long and short) of fatal asthma attacks remain unclear. Previous suggestions have included a distinct inflammatory phenotype,⁹ differences in airway remodelling,³ or differences in mechanisms of airway narrowing, smooth muscle shortening or mucus secretion into the airway lumen.⁴ However, it might be expected that a continuum of abnormal airway remodelling or inflammatory processes would result in a continuum, rather than a dichotomy of duration of fatal asthma attacks. As hypothesized, we found that short-duration cases showed more degranulated (relative to intact) mast cells on the smooth muscle layer. However, there were no significant differences between short-duration and

**Figure 2** Correlation between log blood salbutamol concentration (ng/mL) at time of death and ratio of degranulated/intact mast cells in the total airway wall. The regression line for all cases combined is shown ($r = 0.57$, $P = 0.01$). Short-duration cases are indicated by open squares and long-duration cases by solid squares.

long-duration cases, in the total number of mast cells in any tissue compartment.

Degranulation of mast cells results from cross-linking of high-affinity IgE receptors on the cell surface, and a number of factors may determine the extent of mast cell degranulation.¹⁶ These include serum levels of specific IgE and allergen exposure,¹⁷ and the arrangement or density of epitopes on allergen molecules.¹¹ The differences in mast cell degranulation that were observed in the present study may have arisen from a combination of these factors. The release of various mediators from mast cells is time-dependent and may influence the duration of fatal asthma attacks. Histamine and proteoglycans are released immediately, followed by leucotrienes and prostaglandins (within minutes) and cytokines such as TNF- α and IL-4, IL-5, IL-6, IL-8 and IL-13 (minutes to hours).¹⁸

Mast cells have also been shown to express Toll-like receptors, which upon activation stimulate the

release of pro-inflammatory mediators, such as IL-1 β , TNF- α , IL-6 and IL-13. Mast cell degranulation may also occur, although this may be specific to the stimulus.¹⁹ It is interesting to speculate that the increase in mast cell degranulation in the short-duration cases may have been due to specific stimuli.

It is possible that short-duration cases of fatal asthma occur due to rapid shortening of airway smooth muscle, secondary to rapid mast cell degranulation. Long-duration cases of fatal asthma may result from the slower accumulation of airway mucus, accompanied by less airway smooth muscle shortening, resulting from less mast cell degranulation. There was no significant relationship between the D/I ratio and either muscle shortening or mucus accumulation in the lumen. This is not surprising because a number of factors, including post-mortem changes or artefacts due to tissue preparation, are likely to have altered muscle shortening or luminal mucus, and the absence of a correlation may also have been due to the small number of cases. However, the increased D/I ratio and increased muscle shortening in the short-duration cases suggest that mast cell degranulation may account for sudden death due, at least partly, to bronchospasm, in the short-duration cases. We were not aware of any artefactual post-mortem degranulation of mast cells.

The positive relationships between blood salbutamol levels at death and both the ratio of degranulated to intact mast cells and the percent muscle shortening were unexpected since the actions of salbutamol include stabilization of mast cells (Nightingale *et al.*,²⁰ #723; Russo *et al.*,²¹ #6724) as well as relaxation of smooth muscle (Johnson *et al.*,²² #3134). This suggests that an overwhelming stimulus results in death by degranulating mast cells, an irreversible event that occurs probably before sufficient concentrations of salbutamol can be distributed into the airway wall. The resultant acute muscle shortening and mucus release may therefore overwhelm the effects of salbutamol. Other possibilities include tachyphylaxis of mast cells to beta-agonist medication or increased gastrointestinal, rather than airway, deposition of salbutamol associated with severe airway narrowing.

Death from asthma may be thought of as an extreme exacerbation. In children,²³ and to a lesser extent in adults, exacerbations are related to viral respiratory infections.²⁴ Our previous data have shown increased eosinophil numbers, as well as increased degranulation of mast cells, in cases of fatal compared with non-fatal asthma.^{1,5} The findings from the present study suggest that degranulation of mast cells is an important process contributing not only to fatal attacks of asthma, but also to the duration of such attacks.

Antigen-triggered immune responses within the lung include the activation of mast cells and other cells, with early and late phases of mediator release. The timing of recruitment of specific inflammatory cells to the airway wall is dependent on this process. Neutrophils and mast cells may or may not interact in the airway wall. First, both mast cell degranulation and neutrophil recruitment may result from exposure to the same stimulus, independently of each other.

Second, neutrophil recruitment may occur as a consequence of mast cell degranulation and the release of mediators, such as IL-4, IL-5, IL-6, TNF- α , IL-8 and IL-13, and third, mast cell degranulation may result from the release of cytokines and chemokines from neutrophils.

The present study has shown that short-duration fatal asthma attacks are accompanied by increased mast cell degranulation compared with long-duration fatal asthma attacks. Death due to asthma results from hypoxia secondary to excessive airway narrowing/closure that can only result from airway smooth muscle shortening and/or accumulation of mucus in the airway lumen. Both of these processes are likely to be mediated by mast cell degranulation and the actions of preformed mediators. Factors that might determine the extent of degranulation of mast cells include strength/dose of the initial stimulus, levels of circulating or localized total or specific IgE, and the specificity of airway mast cells. This study suggests that although a variety of stimuli may provoke a fatal attack of asthma, they all act through the mast cell. Understanding the factors that determine the magnitude of the mast cell response will be important for understanding the causes of fatal attacks and exacerbations of asthma.

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