Increased Mast Cell Density and Airway Responses to Allergic and Non-Allergic Stimuli in a Sheep Model of Chronic Asthma

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Abstract

Background: Increased mast cell (MC) density and changes in their distribution in airway tissues is thought to contribute significantly to the pathophysiology of asthma. However, the time sequence for these changes and how they impact small airway function in asthma is not fully understood. The aim of the current study was to characterise temporal changes in airway MC density and correlate these changes with functional airway responses in sheep chronically challenged with house dust mite (HDM) allergen.

Methodology/Principal Findings: MC density was examined on lung tissue from four spatially separate lung segments of allergic sheep which received weekly challenges with HDM allergen for 0, 8, 16 or 24 weeks. Lung tissue was collected from each segment 7 days following the final challenge. The density of tryptase-positive and chymase-positive MCs (MCT and MCTC respectively) was assessed by morphometric analysis of airway sections immunohistochemically stained with antibodies against MC tryptase and chymase. MCT and MCTC density was increased in small bronchi following 24 weeks of HDM challenges compared with controls (P<0.05). The MCTC/MCT ratio was significantly increased in HDM challenged sheep compared to controls (P<0.05). MCT and MCTC density was inversely correlated with allergen-induced increases in peripheral airway resistance after 24 weeks of allergen exposure (P<0.05). MCT density was also negatively correlated with airway responsiveness after 24 challenges (P<0.01).

Conclusions: MCT and MCTC density in the small airways correlates with better lung function in this sheep model of chronic asthma. Whether this finding indicates that under some conditions mast cells have protective activities in asthma, or that other explanations are to be considered requires further investigation.

Introduction

Asthma is a chronic inflammatory disease of the airways, which is characterised by airway inflammation, reversible airflow limitation and hyperresponsive airways. Mast cells (MCs) are important mediator-secretor cells in allergic reactions and are thought to play a major role in the pathophysiology of asthma. Human MCs can be divided into two major groups based on their protease content. There are those that express tryptase but not chymase (MCT) and those that express tryptase and chymase (MCTC). Although these two MC phenotypes are known to differ in their tissue distribution and functional characteristics in healthy lungs, their relative contribution to the asthmatic phenotype is not fully understood.

It is widely established that MCs play a key role in the pathogenesis of asthma. In particular, tryptase released by MCs has been shown to induce airway inflammation and airway hyperresponsiveness (AHR) [1,2,3], while MC chymase promotes the recruitment of eosinophils and neutrophils to airway tissues [4]. Furthermore, increased MC numbers in the airway smooth muscle (ASM) of asthmatic patients correlates strongly with AHR [5].

Investigations of airway changes in asthmatic patients predominantly rely on airway tissue from bronchial biopsies collected from the large proximal airways, which offers little or no information on changes in the small peripheral airways. For mast cell-linked research, large animal models such as primates and sheep have been identified as being good models for chronic asthma [6,7,8,9] because, in contrast to rodent models, they have MCT and MCTC phenotypes distributed throughout the tracheobronchial tree [10]. In particular, sheep have a similar density of mast cells in the small airways to that reported for humans [11] making the ovine model relevant for studying the human disease.
In a recent study of small airway function in sheep, we characterized progressive changes in peripheral airway responses to chronic house dust mite (HDM) challenges using a segmental challenge technique [12]. We found that there was a decline in peripheral airway function with HDM exposure and that airway responses to both allergic and non-allergic stimuli were localized to specific treated areas of the lung. We now present an analysis of progressive changes in mast cell density with increasing exposure to HDM in these sheep. We also investigate the relationship between mast cell numbers and peripheral airway function in this large animal model of asthma.

Methods

Ethics statement

All experimental animal procedures and the collection of tissues/cells were approved by the Animal Experimentation Ethics Committee of the University of Melbourne (approval no. 06128).

Challenge Protocol

To investigate progressive changes in MC density we studied archived lung tissue collected from a segmental challenge protocol as previously described [12]. Briefly fourteen female Merino-cross sheep (6 months) were sensitized to HDM (Dermatophagoides pteronyssinus; CLS, Melbourne, Australia) via 3 subcutaneous injections of HDM-alum, delivered 2 weeks apart. Pre- and post-immunisation HDM-specific serum IgE was assessed by enzyme-linked immunosorbent assay (ELISA) and sheep with at least a 2-fold increase were considered atopic for HDM-challenges (n = 7) [13]. Four spatially separate lung segments were identified and challenged weekly with HDM for different durations. A fiberoptic bronchoscope was used for the delivery of solubilized HDM (1 mg in 5 mL phosphate buffered saline [PBS]). The commencement of challenges in each segment was staggered so that at the end of the challenge regime the right apical, right medial, right caudal and left caudal segments received 0, 8, 16 or 24 weeks of allergen challenges respectively, with the last challenge in each segment occurring 7 days prior to euthanasia. To ensure that there were no natural differences in mast cell density between segments, a separate group of control sheep that did not receive any HDM treatment was used (n = 7).

Bronchoalveolar lavage collection

Bronchoalveolar lavage (BAL) fluid was collected as previously described [12]. The total number of cells collected was determined with a haemocytometer. Differential leukocyte counts were performed on cytospots of BAL cells stained with Haem Kwik Differential Stains (HD Scientific, Wetherill Park, Australia) to identify leucocytes.

Lung tissue collection, immunohistochemistry and morphometric analysis

Sheep were euthanized by intravenous barbiturate overdose. Lung segments were inflated with optimal cutting temperature compound (ProSciTech, Thuringoura, Australia) to 3 subcutaneous injections of HDM-alum, delivered 2 weeks apart. Pre- and post-immunisation HDM-specific serum IgE was assessed by enzyme-linked immunosorbent assay (ELISA) and sheep with at least a 2-fold increase were considered atopic for HDM-challenges (n = 7) [13]. Four spatially separate lung segments were identified and challenged weekly with HDM for different durations. A fiberoptic bronchoscope was used for the delivery of solubilized HDM (1 mg in 5 mL phosphate buffered saline [PBS]). The commencement of challenges in each segment was staggered so that at the end of the challenge regime the right apical, right medial, right caudal and left caudal segments received 0, 8, 16 or 24 weeks of allergen challenges respectively, with the last challenge in each segment occurring 7 days prior to euthanasia. To ensure that there were no natural differences in mast cell density between segments, a separate group of control sheep that did not receive any HDM treatment was used (n = 7).

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Lung tissue collection, immunohistochemistry and morphometric analysis

Sheep were euthanized by intravenous barbiturate overdose. Lung segments were inflated with optimal cutting temperature compound (ProSciTech, Thuringoura, Australia) diluted in PBS (1:1). Mast cells were identified in a serial set of airway sections stained with mouse anti-ovine galectin-14 (clone EL1.2; Mast Cells and Peripheral Airway Function 0.784, P < 0.05; Fig. 1). The mast cells were predominantly located in the outer walls of small bronchi as illustrated in Figure 2. There was no difference in MC T density in segments from HDM-challenged sheep that received 8 challenges compared to control sheep (Fig. 1). A correlation analysis revealed that MC T density in bronchi was significantly positively correlated to the duration in weeks of HDM challenges (r = 0.510, P < 0.01; data not shown).

MC T density in the left caudal lung segment of the HDM-challenged group was significantly increased following 24 weeks of challenges compared to the control group (32 ± 8 vs. 8 ± 4 cells/mm², P < 0.01; Fig. 3a). The MC T/MC T ratio was significantly increased in the HDM-challenged group compared to the control group (0.784 ± 0.132 vs. 0.289±0.105, P < 0.05; Fig. 3b).
from baseline following a HDM challenge ($r_s = -0.96$, $P < 0.05$; Fig. 4a). There was no correlation between MC density and EAR at any of the other time-points (data not shown). MC'TC density after 24 weeks of HDM challenges was also negatively correlated with early-phase responses ($r_s = -0.82$, $P < 0.05$; Fig. 4b).

The percent dose of methacholine required for a 100% increase in $R_p$ (PC100) was positively correlated with MC'T ($r_s = 0.92$, $P < 0.01$; Fig. 5a). There was also a strong trend for a positive correlation between MC'TC density and the dose of methacholine for PC100 ($r_s = 0.74$, $P = 0.066$; Fig. 5b). There was no correlation between MC density and airway responsiveness to methacholine at any of the other time-points. Nor was there any correlation between MC'T or MC'TC density and methacholine for PC100 in the untreated control sheep ($r_s = -0.197$, $P = 0.662$ and $r_s = 0.060$, $P = 0.903$ respectively).

**Airway remodeling**

There was no significant difference in the amount of ASM in the lung segment that received 24 weeks of HDM compared to the corresponding segment in controls (0.029±0.005 vs. 0.025±0.004 mm²/BM; Fig. 6a). However, there was a significant increase in the amount of collagen (0.17±0.02 vs. 0.11±0.02 mm²/BM, $P < 0.05$ Fig. 6b). There was a trend for the increase in collagen to be associated with increasing MC'TC density ($r_s = 0.71$, $P = 0.088$). The amount of airway collagen also tended to be negatively associated with EAR ($r_s = -0.75$, $P = 0.066$).

**Inflammatory cells**

There was a significant increase in the density of eosinophils in the airway walls following 24 weeks of HDM challenges compared to the corresponding segment in controls (64.9±19.4 vs. 10.9±3.1, $P < 0.01$; Figure 6c). There was no correlation between eosinophil density and MC'T or MC'TC concentration in HDM-challenged or unchallenged airways ($r_s = 0.17$, $P = 0.713$ and $r_s = 0.57$, $P = 0.200$; data not shown). Nor was there a correlation between eosinophil numbers and functional indices; i.e. EAR and PC100(MCh) in the HDM challenged segment ($r_s = -0.071$, $P = 0.906$ and $r_s = 0.20$, $P = 0.662$ respectively, data not shown). Table 1 shows that the number of eosinophils and other inflammatory cells was increased in the BAL 48 hours post-HDM at week 20 compared to the level prior to challenge. However, there was no correlation between any of the cell types examine and MC numbers, or functional indices (data not shown).

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**Figure 1. Tryptase-positive mast cell (MC'T) density following challenge with house dust mite (HDM) allergen.** Bars show the number of MC'T per mm² in the airway wall of bronchi in four spatially separate lung segments from control and HDM challenged groups. The number in italics indicates the number of HDM challenges each lung segment received. Line shows the mean, boxes show 25th and 75th percentile, whiskers represent 5th to 95th percentile. $n = 7$, *P < 0.05. R – right; L – left. doi:10.1371/journal.pone.0037161.g001

**Figure 2. Airway sections stained with antibodies against mast cell tryptase and chymase.** (a) Tryptase-positive mast cells and (b) chymase-positive mast cells. The house dust mite (HDM) treated segment received 24 weekly challenges and the control segment was untreated. Negative control shows omission of the primary antibody. Arrows indicate positive cells. doi:10.1371/journal.pone.0037161.g002
Discussion

While mast cells have long been implicated in the pathophysiology of asthma, very few studies have systematically examined the effects of mast cells on small airway function. To our knowledge this is the first time that the temporal dynamics of the mast cell population has been examined in the lungs in a large animal model of asthma. The key findings from this study are that MCT and MCTC density increased significantly following chronic allergen challenge and that MC density is positively correlated with the length allergen exposure and improved airway responses to allergic and non-specific stimuli.

It is noteworthy that the proportion of MCs with the chymase phenotype increases markedly with allergic challenge. Our result confirms a previous study in sheep where airways exposed to allergen have increased density of MCTC [15] and is consistent the finding of increased MCTC in the small airways of asthmatics [16]. It cannot be deduced from our results whether the MCTC were converted from existing MCT phenotype or were differentiated from newly infiltrating mast progenitor cells which originated in the bone marrow. Early studies examining mast cells developed in vitro from human fetal liver cells, and/or cord blood, suggest that commitment to develop as an MCT or MCTC phenotype may occur in MC precursor cells and that there is no evidence for conversion of MCT to MCTC in mature mast cells [17]. However, other in vitro studies provide evidence for phenotype conversion in that MCTC co-cultured with human airway epithelial cells are known to convert to the MC phenotype [18,19], suggesting that this may also be a possibility in the ovine in vivo experiments presented here.

Interestingly, in the present study we found a positive correlation between increased MC density and small airway function. This finding was surprising at first, given the well acknowledged central role of airway mast cells in orchestrating allergic inflammatory responses in asthma [20]. However, a close examination of the literature reveals that a positive relationship between mast cell density and lung function has been previously reported in two separate clinical studies of asthma [16,21] and also

Figure 3. Chymase-positive mast cell (MCTC) density increases following chronic allergen challenge. (a) MCTC per mm² of airway wall (b) ratio of MCTC and tryptase-positive mast cells (MCT). The house dust mite (HDM) treated segment received 24 weekly challenges and the control segment was untreated. Line shows the mean, boxes show 25th and 75th percentile, whiskers represent 5th to 95th percentile. n = 7, *P<0.05, **P<0.01 compared to controls. doi:10.1371/journal.pone.0037161.g003

Figure 4. Relationship between mast cell density and peak early bronchoconstriction response. Correlation between the percentage increase in peripheral resistance (R₂) 30 minutes post-HDM challenge and (a) MCT (rₛ = −0.89, P<0.05) and (b) MCTC (rₛ = −0.82, P<0.05). doi:10.1371/journal.pone.0037161.g004
in studies of chronic obstructive pulmonary disease (COPD) [22,23]. In asthma in particular, MC density in the small airways is correlated with improved lung function in mild-to-moderate and severe asthmatic subjects [16,21]. It is unknown whether the aforementioned studies indicate that under some conditions mast cells possess protective activities in allergic airways or if other possibilities are to be considered.

One possible explanation for this correlation is that the higher concentration of MCs observed in individuals with the best airway function reflects a lower degree of degranulation and thus, a less deleterious effect. In support of this hypothesis are reports that patients who have died from asthma have a lower number of MCs in their airways compared to control cases [24] and that MC degranulation is positively associated with disease severity [25] and hence, poorer lung function.

As suggested by others, it is also conceivable that in addition to their harmful activities, MCs offer some protection against deteriorating lung function in states of disease [16]. In particular, the positive relationship between MCs and lung function could reflect an adaptive response of MCs to down-regulate some of their pro-inflammatory activities [26]. In support of this is the finding that mouse MC protease 4 (MCP-4), a chymase that is functionally homologous to human chymase, has a protective role in allergic airway inflammation [26]. Mice lacking MCP-4 were found to have significantly higher AHR and airway inflammation when sensitized and challenged with ovalbumin compared with their wild type counterparts [26].

It has been previously suggested that MCs may be able to exert protective effects by regulating ASM cells [26]. In particular, MC chymase has been shown to inhibit smooth muscle cell proliferation and thus, may prevent the associated increase in peripheral airway resistance in chronic disease [27]. It is possible that MC regulation of the ASM cells could explain the lack of an increase in ASM area in HDM challenged sheep relative to controls observed in the current study.

Another possibility is that the release of MC tryptase has some long-term protective effects for lung function by stabilising lung structure. For example, tryptase has been reported to repair and remodel damaged airways in asthmatic lungs [28]. The release of tryptase into the airways has been suggested to drive fibrosis and extracellular matrix turnover [29], particularly the synthesis and secretion of type I collagen [29], potentially contributing to a stiffening of the bronchial walls and the prevention of airway collapse on expiration, thereby serving a protective function in diseased airways [30,31]. Consistent with this hypothesis is the finding from our study that bronchial

Figure 5. Relationship between mast cell density and airway responsiveness. Correlation between the percent dose of methacholine required to increase $R_p$ by $100\%$ ($PC_{100}(MCh)$) from baseline and (a) $MC_T$ ($r = 0.92$, $P<0.01$) and (b) $MC_{TC}$.

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Figure 6. Airway remodeling and eosinophils following chronic allergen challenge. (a) Airway smooth muscle (ASM) per mm basement membrane (BM) length (b) collagen area per mm BM and (c) eosinophils per mm$^2$ airway wall, in untreated control sheep and sheep challenged with house dust mite (HDM) allergen weekly for 24 weeks. Line shows the mean, boxes show 25th and 75th percentile, whiskers represent 5th to 95th percentile. n = 7, *$P<0.05$, ***$P<0.001$ compared to controls.

doi:10.1371/journal.pone.0037161.g006
collagen deposition was significantly increased after 24 weeks of allergen challenges, and that amount airway wall collagen tended to be positively associated with MC_{TC} density. It is important to note that therapeutic interventions against mast cell activation are likely to be important when mast cells are active in coordinating the detrimental immune response at the onset of the allergic reaction. However, the findings from the present study and that of Balzar et al [16], Zanini et al [21] and Gosman et al [22], that increased numbers of mast cells are associated with relatively better lung function in diseased airways, suggest that any therapeutic interventions that are active against mast cells will also need to accommodate this aspect in long-term management of airway disease.

In the present study we show a correlation between mast cell density and better airway responses to allergic and non-allergic stimuli in a large animal model. Our results support the findings of several clinical studies that report positive correlations between increased mast cell concentrations and improved lung function indices. Whether this finding indicates that under some conditions MCs have protective activities in asthma, or that other explanations are to be considered, requires further investigation.

Author Contributions
Conceived and designed the experiments: KS JV. Performed the experiments: JV DB GB. Analyzed the data: JV EK. Wrote the paper: JV KS.

References

Table 1. Leukocyte composition of bronchoalveolar lavage cells.

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<td>36.5</td>
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Bronchoalveolar lavage (BAL) was collected from the left caudal lung segment of sheep prior to and 48 hours following HDM challenge. Differential cell counts were performed on cytospots of BAL cells. Values shown are cells (x10^6) per mL BAL retrieved. n = 6. doi:10.1371/journal.pone.0037161.t001
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