Mast cell numbers in airway smooth muscle and PC\textsubscript{20}AMP in asthma and COPD

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**KEYWORDS**
Mast cell; Adenosine; Asthma; COPD; Bronchial hyperresponsiveness; Airway smooth muscle

**Summary**

*Introduction:* Most patients with asthma and many patients with COPD show bronchial hyperresponsiveness to adenosine (BHR\textsubscript{AMP}). BHR\textsubscript{AMP} may be caused by release of mast cell histamine, which induces smooth muscle contraction.

*Aim of the study:* To evaluate whether mast cell numbers in airway smooth muscle are increased in patients with asthma and COPD compared to their age-matched controls, and whether mast cell numbers are correlated with BHR\textsubscript{AMP}.

*Patients:* Twenty-two non-smoking subjects with asthma (age 31 yr, FEV\textsubscript{1}: 89\% pred, PC\textsubscript{20}AMP: 2.7 mg/ml), 18 ex-smoking subjects with COPD (age 62 yr, FEV\textsubscript{1}: 58\% pred, PC\textsubscript{20}AMP: 52.4 mg/ml).

*Methods:* Snap-frozen bronchial biopsies were immunostained with anti-mast cell tryptase and anti-desmin antibodies. Mast cell number was expressed as the number of tryptase positive cells per area of smooth muscle.

*Results:* There were no significant differences in mast cell number between patients with asthma, COPD, and their respective age-matched healthy controls. Furthermore, there was no significant correlation between mast cell number and FEV\textsubscript{1} or PC\textsubscript{20}AMP in any of the groups. Surprisingly, the mast cell number was negatively correlated with reversibility to salbutamol in COPD patients (rho = -0.47, P < 0.05).

*Conclusion:* Mast cell numbers in central airway smooth muscle apparently do not contribute importantly to bronchial hyperresponsiveness to adenosine.

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**Introduction**

Most patients with asthma and many smoking patients with COPD show increased bronchial hyperresponsiveness to adenosine (BHR\textsubscript{AMP}).\textsuperscript{1,2} The underlying mechanism of this BHR\textsubscript{AMP} is not completely understood, partially because...
there are no selective adenosine receptor antagonists available in humans so far. Probably, adenosine acts indirectly through activation of specific (A2) receptors on intermediate inflammatory cells such as mast cells and on afferent nerve endings.1-5

A role of mast cells in the responsiveness to adenosine is supported by the observation that plasma histamine increases in atopic non-asthmatics after a challenge with AMP,4 next to histamine, prostaglandin D2 and tryptase increases in bronchoalveolar lavage fluid.5

Because BHRAMP may well depend on the number and activation state of bronchial mast cells, this test might be a useful marker of allergic airway inflammation in clinical practice.6 Indeed, atopic subjects respond relatively more to inhaled adenosine than to methacholine as compared to non-atopic controls.4 Also, the early asthmatic reaction after house dust mite is better reflected by an increase in hyperresponsiveness to AMP than to methacholine.7,8 Non-atopic patients with COPD who currently smoke show also more severe hyperresponsiveness to AMP than ex-smokers.9 Because the number of mast cells is larger in the peripheral10 as well as the central airways11 of smokers than of non-smokers, mast cells may also play a role in the increased hyperresponsiveness to AMP in smoking-related airway diseases.

Based on the above one would expect a close relationship between the severity of BHRAMP and mast cell number in bronchial biopsies from allergic patients with asthma and smokers with COPD. However, we could not demonstrate such a relationship in stable patients with asthma, nor in COPD.12,13 One explanation for this discrepancy may be that the number of mast cells was measured in the bronchial mucosa, and not in the smooth muscle area of the bronchus. After all, in vitro sensitisation with common aeroallergens increases mast cell numbers specifically within the smooth muscle area of human bronchi, in contrast to mast cells in the epithelium, lamina propia or adventitia.14 In addition, airway smooth muscle- and mast cells may interact closely as smooth muscle cells secrete stem cell factor which activates mast cells and prolong their survival.15 Moreover, mast cells secrete tryptase which may stimulate smooth muscles to produce TGF-/31,16,17 This chemokine and also RANTES18 have chemotactic activity to mast cells. Therefore, we set out to investigate whether the numbers of smooth muscle mast cells are higher in patients with asthma and COPD than in their respective non-hyperresponsive controls and if these numbers are related to the severity of BHRAMP.

Materials and methods

Subjects

Bronchial biopsies were obtained from two previously published studies.12,13 Subjects participating in these 2 studies were recruited from the pulmonary outpatient clinic of the University Medical Center Groningen and by advertisements in local newspapers. All subjects gave written informed consent. The medical ethics committee of the University Medical Center Groningen approved this investigation.

Asthmatic subjects aged between 18 and 45 years were selected based on a history consistent with asthma: a positive history of atopy, i.e. positive intracutaneous tests against house dust mite or two other common aero-allergens (ALK, Groningen, The Netherlands), FEV1 > 1.5 l and > 60% predicted, PC20 methacholinebromide 4 9.8 mg/ml and PC20 AMP 4 80 mg/ml. Age matched healthy volunteers were selected on: no history of lung disease, FEV1 > 1.5 l and > 85% predicted, no atopy and no airways responsiveness to methacholine or AMP.

COPD subjects aged between 45 and 75 years were selected based on criteria of the American Thoracic Society (ATS); a negative history of atopy, negative skin test to 18 common aero-allergens (ALK, Groningen, The Netherlands), i.e. negative specific serum IgE for 11 common aero-allergens (phadiatop). Healthy volunteers did not have a history of pulmonary disease, had a normal lung function, and were matched for age and packyears smoking.

Any subject who smoked during the past year, suffered from a respiratory infection during the past 4 weeks, or was treated with oral or inhaled corticosteroids or antibiotics during the past 4 weeks was excluded.

Lung function measurements

Forced expiratory volume in one second (FEV1) was performed according to the standardized guidelines of the European Respiratory Society.19 Reversibility was tested 30 min after inhalation with 400 mg budesonide. Subjects were not allowed to use short-acting bronchodilators within 12 h, or long-acting bronchodilators within 24 h.

Bronchial provocation test to AMP

Provocation tests were performed using a 2 min tidal breathing method.20 After an initial nebulised saline challenge, subjects inhaled doubling concentrations AMP, ranging from 0.04 to 80 (asthma) or 320 mg/ml (COPD) (Sigma, St Louis, USA) till FEV1 declined 20% compared to the baseline value. The arbitrary cut-off point for healthy subjects was set at 80 mg/ml AMP (asthma controls) and 320 mg/ml AMP (COPD controls).

Bronchoscopy and processing of the biopsies

Bronchoscopy was performed using an Olympus B1 IT10 flexible fiberoptic bronchoscope (Olympus Optical, Tokyo, Japan), according to the guidelines of the ATS.21 Biopsies were taken from the subcarinae of the left or right lower lobe using a fenestrated forceps (FB-21C, Olympus, Tokyo, Japan). At least five biopsies per patient were collected. Biopsies were mounted in Tissue Tek (optimal cutting temperature medium) embedding compound and snap-frozen by immersing in isopentane (–80°C). Every biopsy was stained with haematoxylin and eosin and qualitatively examined for the amount of smooth muscle present. Sections without smooth muscle were excluded. Frozen sections of 4 mm thickness were double immuno-stained with anti-mast cell tryptase (AA1 from DAKO, ITK, Denmark) and anti-desmin antibodies (anti-Desmin from DAKO, Glostrup, Denmark, Fig. 1). Anti-mast cell antibodies were labelled with alkaline phosphatase (resulting in blue colour precipitate), and the anti-desmin antibodies with peroxidase/AEC (red colour precipitate). The observer
was blinded for subject characteristics by coding of all sections. Counting was carried out using a light microscope at a magnification of 200×. Tryptase positive cells were counted in 2 random, but representative sections of one biopsy per patient with at least an 80μm interval. Counting was started at those locations that met best the above-mentioned criteria. The area of smooth muscle was determined by grid counting using an eyepiece graticule (cross-points each 100μm at 100×). In this way, we evaluated per subject a mean (SD) area of 0.59 (0.40) mm². Mast cell number was expressed as the number of tryptase positive cells per area smooth muscle.

Data analysis

All analyses were performed with the SPSS 10 software package (SPSS Inc., Chicago, IL). Values of \( P < 0.05 \) were considered statistically significant. The Student’s t-test was used to compare clinical variables between groups, on condition of a normal distribution. The Mann–Whitney U test was used to compare mast cell density between groups. Correlations between mast cell density and clinical variables were made using Spearman’s rank correlation tests.

Results

Clinical characteristics

Twenty-two patients with asthma, 18 patients with COPD, and 2 control groups of 9 healthy subjects participated in this study (Table 1). The 37 male and 21 female subjects were equally distributed between the four groups \( (P = 0.19) \). Mean reversibility of the FEV₁% predicted after 400μg salbutamol of the asthmatic subjects was significantly larger than that of healthy controls (10.5 vs. 2.0%), whereas reversibility of patients with COPD and their healthy controls was similar (5.6 vs. 3.3, respectively). Geometric mean PC_{20AMP} of the COPD patients was 52.4 mg/ml (range 1 to >320) 9 individuals had a PC_{20AMP} >320 mg/ml. Geometric mean PC_{20AMP} of the asthmatic patients was 2.7 mg/ml (range 0.18–22.8). None of the participants used mast cell stabilizers, H1 blockers, 5-lipoxygenase inhibitors, theophyllines, or any other agents that may have influenced mast cells.

Differences in mast cell density in airway smooth muscle

There were no significant differences between asthmatics, patients with COPD and their controls, or between the two healthy control groups (see Table 1).

Correlations between mast cell density and clinical variables

In the group of 18 COPD patients, the correlation (rho) between mast cell density and FEV₁% predicted, reversibility % predicted FEV₁ and PC_{20AMP} is 0.42 (not significant),

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics and mast cell density.</th>
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<tbody>
<tr>
<td></td>
<td>Asthma</td>
</tr>
<tr>
<td>Male/female</td>
<td>13/9</td>
</tr>
<tr>
<td>Age, years</td>
<td>31.3 (8.9)</td>
</tr>
<tr>
<td>Pack years</td>
<td>0</td>
</tr>
<tr>
<td>Current smoking</td>
<td>0</td>
</tr>
<tr>
<td>Atopy</td>
<td>*</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>89.2 (15.8)</td>
</tr>
<tr>
<td>Reversibility, % predicted FEV₁</td>
<td>10.5 (9.9)*</td>
</tr>
<tr>
<td>PC_{20AMP}, mg/ml</td>
<td>2.7 (0.18–22.8)*</td>
</tr>
<tr>
<td>Tryptase+ cell number/mm²</td>
<td>96.6 (13–222)</td>
</tr>
</tbody>
</table>

Baseline characteristics presented as mean (so). PC_{20AMP} presented as geometric mean (range). Tryptase+ cell number/mm² presented as median (range). *\( P < 0.05 \) vs. COPD.
Mast cells in airway smooth muscle

**Figure 2** Significant correlation between the density of tryptase positive cells (mast cells) in the smooth muscle area (per mm²) of bronchial biopsies and the degree of reversibility of FEV₁% predicted in 18 subjects with stable COPD.

–0.47 (P<0.05) (see Fig. 2), and 0.35 (not significant), respectively.

In the group of 22 asthmatic patients, the correlation (rho) between mast cell density and FEV₁% predicted, reversibility % predicted FEV₁, and PC₂₀AMP is 0.17, –0.24, and 0.28, respectively (all not significant).

**Subgroup analyses**

Retrospectively we analysed the subgroup (n=9) of AMP positive COPD patients. No significant differences between COPD and their healthy controls were found regarding mast cell density in airway smooth muscle. Also, no significant correlation was found between mast cell density and PC₂₀AMP.

**Discussion**

Increased bronchial hyperresponsiveness to AMP can be demonstrated in all asthmatics and many patients with COPD. Increased BHRₐmₚ may be caused by a release of mast cell histamine, which induces smooth muscle contraction. Therefore, it was expected to find a close relationship between mast cell density in airway wall biopsies in the epithelial and subepithelial layer and PC₂₀AMP. In former studies no such relationship was found, neither in patients with asthma nor in patients with COPD. Therefore, we speculated that we might have to look more specifically to mast cells in the smooth muscle area of bronchial biopsies. Nevertheless, in the present study mast cell density in smooth muscle area was not increased in asthma and COPD compared to age matched controls, and mast cell density did not correlate with BHRₐmₚ. Interestingly, we demonstrated a negative correlation between mast cell density and reversibility of airway obstruction to salbutamol in COPD patients, despite the low level of reversibility present. We do not have a good explanation for the latter finding.

There are several explanations why mast cell numbers in airway smooth muscle are not increased in our patients with asthma or COPD, and why this is not related to severity of bronchial hyperresponsiveness to AMP. First, mast cell numbers increase upon allergen exposure and smoking. Our patients were very stable and not recently exposed to allergens or cigarette smoke. It may thus well be that the mast cells in the bronchial wall were not increased in number nor activated. Second, we took bronchial biopsies from the central, but not peripheral airways. It is possible that peripheral mast cell activation is more important than central mast cell activation with respect to increased hyperresponsiveness to AMP, since it has been shown that mast cell mediators (histamine, prostaglandin D₂, tryptase) are increased in (peripherally collected) BAL fluid after challenge with AMP. In line with this Carroll et al. showed in patients with fatal and non-fatal asthma that the density of mast cells in the complete bronchial wall, but also in airway smooth muscle, is much higher in the peripheral airways than in the central airways. Moreover, a significant part of these mast cells (66–75%) was found to be degranulated. Third, we took bronchial biopsies that represent only small and superficial parts of the bronchial wall. In contrast, the complete smooth muscle area of bronchial sections was examined in the study of Ammit et al., who showed increased numbers of mast cells in sensitised smooth muscle. Fourth, we applied specific immunostaining techniques directed at tryptase (AA1), using the light microscope to evaluate the number of tryptase positive cells. Although less likely in our stable study subjects, it cannot completely be ruled out that mast cells may have degranulated to a certain extent; these degranulated cells may not be visualized and this could contribute to underestimation of the real number of mast cells and their activation status.

Finally, there is evidence that other pathways than via the A2B-receptor on mast cells, namely involving the A₁-receptor, may be responsible for the AMP-induced bronchoconstriction. Although the A₁-receptor is not abundantly present in the human lung tissue in binding studies, it is associated with nerves and functional studies suggest a possible neural pathway. Furthermore, it has been shown that activation of adenosine A₁ receptors directly stimulates contraction of human bronchial smooth muscle cells by activation of Ca²⁺-channels.

One other study investigating the same research question had different results. This study demonstrated significantly more mast cells in airway smooth muscle of asthmatics than in healthy controls. Furthermore, a positive correlation was found between mast cell numbers in smooth muscle areas and bronchial hyperresponsiveness to methacholine in asthmatics. There are some differences between their and our study. First their asthmatics were on average 10 years younger and some of their asthmatics were non-atopic. They used a (semi)automated computerized method while we counted manually. Finally they used methacholine to stimulate airway smooth muscle; a challenge not acting via mast cells. In contrast in our study AMP was used to stimulate airway smooth muscle cells indirectly by releasing histamine from immunologically primed mast cells. Therefore, it is intriguing that they found a positive correlation between mast cell density and hyperresponsiveness, while we did not. Obviously more studies are needed using both AMP and methacholine to determine the relationship between bronchial hyperresponsiveness and mast cell infiltration in airway smooth muscle.
This study demonstrated a weak negative correlation between airway mast cell density in the smooth muscle area and reversibility of airway obstruction in COPD patients (ρ = −0.47, P = 0.048), which was not present in asthma. Interestingly, Grashoff and coworkers demonstrated a very similar correlation between mast cell density and reversibility of airway obstruction (ρ = −0.42, P = 0.03) in smoking and non-smoking individuals with and without COPD. They used resected lung specimens, demonstrating the above-described relationship in the non-epithelial part of the small airways. There are a few hypothetical explanations why an increased airway mast cell density may be related to a loss of airway reversibility with salbutamol. Mediators of airway inflammatory cells, including mast cells, may impair the function of β2-receptors. However, one would also expect to find a relationship between increased mast cell density and increased BHRamp if an increased bronchomotor tone due to desensitised β2-receptor function plays a role. Furthermore, mediators of inflammatory cells in the airway wall may lead to a decreased distensibility, which may be an expression of airway remodelling. Indeed mast cells have been suggested to contribute importantly to the development of airway fibrosis via proteolytic activation cascades and/or the release of heparin and subsequent effects on growth factor activity, but future studies have to confirm this.

We conclude that mast cell numbers in airway smooth muscle in central airways do not contribute importantly to bronchial hyperresponsiveness to adenosine neither in asthma nor in ex-smokers with COPD.

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