Inhibition of mucin secretion with MARCKS-related peptide improves airway obstruction in a mouse model of asthma

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Patel Chest Institute, Delhi, India; 3Molecular Immunogenetics Laboratory, Institute of Genomic and Integrative Biology, Delhi, India; 4Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, North Carolina; and 5Department of Pulmonary Medicine, MD Anderson Cancer Center, Houston, Texas

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Methods

Agrawal A, Rengarajan S, Adler KB, Ram A, Ghosh B, Fahim M, Dickey BF. Inhibition of mucin secretion with MARCKS-related peptide improves airway obstruction in a mouse model of asthma. J Appl Physiol 102: 399–405, 2007. First published August 31, 2006; doi:10.1152/japplphysiol.00630.2006.—Allergic asthma is associated with airway epithelial cell mucous metaplasia and mucin hyperssecretion, but the consequences of mucin hypersecretion on airway function are unclear. Recently, a peptide derived from the myristoylated alanine-rich C kinase substrate protein NH2-terminal sequence (MANS) was shown to inhibit methacholine (MCh)-induced mucin secretion from airway mucous cells by >90%. We studied the effect of intranasal pretreatment with this peptide on specific airway conductance (sGaw) during challenge with Mch in mice with allergen-induced mucous cell metaplasia. sGaw was noninvasively measured in spontaneously breathing restrained mice, using a double-chamber plethysmograph. Pretreatment with MANS peptide, but not a control peptide [random NH2-terminal sequence (RNS)], resulted in partial inhibition of the fall in sGaw induced by 60 mM MCh (mean ± SE; baseline 1.15 ± 0.06; MANS/MCh 0.82 ± 0.05; RNS/MCh 0.55 ± 0.05 cmH2O/s). The protective effect of MANS was also seen in mice challenged with allergen for 3 consecutive days to increase airway hyperresponsiveness, although the degree of protection was less (baseline 1.1 ± 0.08; MANS/MCh, 0.65 ± 0.06; RNS/MCh 0.47 ± 0.03 cmH2O/s). Because routine sGaw measurement in mice includes nasal airways, the effectiveness of MANS was also confirmed in mice breathing through their mouths after nasal occlusion (baseline 0.92 ± 0.05; MANS/MCh 0.83 ± 0.06; RNS/MCh 0.61 ± 0.03 cmH2O/s). In all instances, sGaw in the MANS-pretreated group was ~35% higher than in RNS-treated controls, and mucous obstruction accounted for ~50% of the MCh-induced fall in sGaw. In summary, mucin secretion has a significant role in airway obstruction in a mouse model of allergic asthma, and strategies to inhibit mucin secretion merit further investigation.

Mucins, the major glycoconjugates of mucus, are produced by airway epithelium in mammals. Mucus entraps inhaled pathogens and particles, and the mucous layer serves as a first line of defense of the lungs against foreign materials (16). In many chronic inflammatory diseases of the airways, including asthma, cystic fibrosis, and chronic obstructive pulmonary disease, excessive mucus is produced that may lead to anatomic airway obstruction (14, 17). Expectoration of viscous mucous plugs and bronchial casts is commonly seen in patients with asthma, and extensive mucous plugging has been reported in cases of fatal asthma that failed to respond to conventional therapy (10, 13, 17). While the pathophysiological role of mucous obstruction seems intuitively apparent, neither has this hypothesis been formally tested, nor has the contribution of airway luminal mucus to pulmonary airflow obstruction been quantified. Previous studies have been limited to reversing bronchoconstriction in the presence of mucin discharge (9), but this approach is inexact and does not separate the effects of airway wall edema and other factors besides mucin release that lead to luminal narrowing.

An alternative approach to dissecting the pathophysiological role of mucus in airflow obstruction is selective inhibition of mucin secretion. This was not possible until recently, when a 24-amino acid peptide [myristoylated alanine-rich C kinase substrate (MARCKS) NH2-terminus sequence, (MANS)] corresponding to the NH2-terminal domain of the MARCKS protein, was found to inhibit mucin release from airway mucous cells by 90% (18). We used the MANS peptide to selectively block methacholine (MCh)-induced mucin hypersecretion in mice with allergen-induced airway epithelial cell mucous metaplasia. This allowed us to quantitatively resolve the contribution of mucin hypersecretion to pulmonary airflow obstruction and test the possible efficacy of specific inhibition of mucin secretion by the MANS peptide as a therapeutic approach in allergic asthma.

Methods

This study was conducted in conformity with the American Physiological Society’s Guiding Principles in the Care and Use of Animals. Experimental protocols and study design were approved by the designated institutional review boards at the Vallabhba Patel Chest Institute and Institute of Genomic and Integrative Biology. Induction of airway mucous metaplasia. Six- to eight-week-old BALB/c mice between 16 and 20 g were purchased from the Center of Cellular and Molecular Biology, India, and housed in accordance with institutional guidelines. To induce mucous metaplasia, mice were sensitized to ovalbumin (20 µg ovalbumin grade V, 2.25 mg alum in...
saline, pH 7.4; Sigma, St. Louis, MO) administered by weekly intraperitoneal injection for 4 wk. Sensitized mice were then challenged by exposure for 30 min to an aerosol of either 0.9% (wt/vol) saline (Ova/Sal) or 2.5% (wt/vol) ovalbumin in 0.9% saline (Ova/Ova), as described (4). Twenty mice were subjected to daily ovalbumin challenge for 3 days (Ova/Ova3) to increase airway inflammation and airway hyperresponsiveness (AHR). Experiments were conducted 7 days after challenge because intracellular mucin reaches a maximum at that time (4).

Quantification of airflow obstruction. Specific airway conductance (sGaw) was measured as the time lag between flows recorded from the head and thoracoabdominal chambers in consciously breathing, restrained mice, using the two-chambered body plethysmograph system devised by Buxco (PLY-3351, Biosystem XA software) (5, 15). Briefly, this equipment consists of two plastic cylinders used as the thoracoabdominal and head compartments that can be attached to one another with an orifice, such that the head and neck of a mouse can pass through but not its shoulders. Mice were acclimatized to being restrained in the plethysmograph during the sensitization and challenge phase. To measure sGaw, mice were placed in the thoracoabdominal chamber, maneuvered to pass their head through the orifice, and gently wedged between the orifice and a piston posteriorly. Before attaching the head chamber, a suitably cut latex sheet was passed snugly over the neck and interposed between the two chambers to ensure that the chambers would be airtight. Phase lag between the airflow signals recorded from the two chambers was approximated to ensure that the chambers would be airtight. Phase lag between the airflow signals recorded from the two chambers was approximated to ensure that the chambers would be airtight. Phase lag between the airflow signals recorded from the two chambers was approximated to ensure that the chambers would be airtight.

Experimental protocol. Following the sensitization, challenge, and an additional 7 days to allow for epithelial cell mucus metaplasia, baseline sGaw was recorded for 10 min. The head chamber was then removed, and each mouse was administered 50 μL of either 0.1% or 0.1% (vol/vol) saline (Ova/Sal) or a control random NH2-terminal sequence (RNS) peptide as the thoracoabdominal and head compartments that can be attached to one another with an orifice, such that the head and neck of a mouse can pass through but not its shoulders. Mice were acclimatized to being restrained in the plethysmograph during the sensitization and challenge phases. To measure sGaw, mice were placed in the thoracoabdominal chamber, maneuvered to pass their head through the orifice, and gently wedged between the orifice and a piston posteriorly. Before attaching the head chamber, a suitably cut latex sheet was passed snugly over the neck and interposed between the two chambers to ensure that the chambers would be airtight. Phase lag between the airflow signals recorded from the two chambers was approximated to ensure that the chambers would be airtight.

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MANS therapy inhibits mucus secretion from the tracheobronchial airways. To determine whether intranasal MANS is effective in inhibiting tracheobronchial mucin secretion induced by MCh and/or ATP, retained intracellular mucin in axial and penetrating airways of MANS or RNS-pretreated mice was compared. Representative sections showing mucin secretion in response to MCh and/or ATP challenge after MANS or RNS treatment are shown in Fig. 1.

Histological analysis of mucous secretion. At the end of the experiment, mice were killed, and intracellular mucin remaining in the airways was assessed by routine and fluorescent periodic acid Schiff (PAS) staining (4). Serial sections of the main axial bronchus along with minor daughter branches that we refer to as penetrating bronchi were used for semiquantitative scoring. We scored each airway from 1 to 4 using a retained mucin score as follows: 1, near complete mucin secretion with many ghost cells (almost completely depleted of mucin granules), creating an empty appearance with faint outlines, see Fig. 1C) and most cells partially empty; 2, semicomplete mucin secretion with many cells partially empty, few ghost cells, and few cells densely populated with mucin granules (full cells); 3, limited mucin secretion with full cells and partially empty cells being equally predominant; 4, minimal mucin secretion with mostly full cells. To eliminate bias, scoring was done while blinded to the treatment. Two to three sections were scored per lung, and specimens from at least three different experiments were used per group.

RESULTS

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MANS therapy inhibits airflow obstruction induced by escalating doses of MCh. To determine whether inhibition of mucin secretion by MANS can attenuate MCh-induced airflow obstruction, we studied the effect of MANS pretreatment on the MCh dose-response curve in mice. AHR to MCh was observed in both Ova/Ova and Ova/Ova3 mice compared with control (Fig. 2). As expected, repeated allergen challenge increased AHR, as seen by a leftward shift of the dose-response curve, i.e., lower doses of MCh were required for similar degree of fall in sGaw. In mice with mucous metaplasia, pretreatment with MANS significantly improved sGaw in the midrange of the dose-response curve and caused a rightward shift (Fig. 2).
No significant effect of MANS was found in control mice that lack mucous metaplasia, indicating that the mechanism of MANS action is by inhibiting mucus secretion. The MCh dose associated with a 35% fall in sGaw from baseline was >200 mM in Ova/Sal mice, 110 mM in Ova/Ova mice, and 75 mM in Ova/Ova3 mice. MANS pretreatment increased this to 165 mM in Ova/Ova mice and 100 mM in Ova/Ova3 mice. Thus MANS inhibits MCh-induced airway obstruction in mice with mucous metaplasia. However, it was not possible to precisely separate the effect of mucus and bronchoconstriction in an escalating MCh dose-response experiment, since the former is likely to get depleted, while the latter increases progressively. Thus, to precisely study the role of mucus in MCh-induced airflow obstruction, single-challenge experiments were performed.

MANS therapy inhibits mucus-related airflow obstruction induced by a single dose of MCh. Baseline sGaw of Ova/Ova and Ova/Ova3 mice that had mucous metaplasia (1.15 ± 0.06, 1.1 ± 0.08 cmH2O/s) was not different from control mice without mucous metaplasia (Ova/Sal, 1.3 ± 0.1 cmH2O/s). After a single challenge with 60 mM MCh that caused near complete mucus secretion, Ova/Ova mice pretreated with the
MANS peptide had a higher mean post-MCh sGaw than those given the control RNS peptide, [MANS/MCh, 0.82 ± 0.05 (−30% compared with baseline sGaw); RNS/MCh, 0.55 ± 0.05 (−51%); MANS vs. RNS, +0.27 ± 0.07 (49% MANS-induced improvement), P < 0.05] (Fig. 3). The protective effect of MANS peptide was also seen in Ova/Ova3 mice [baseline 1.1 ± 0.08; MANS/MCh, 0.65 ± 0.06 (−40%); RNS/MCh 0.47 ± 0.03 (−57%); MANS vs. RNS, +0.18 ± 0.07 (38%), P < 0.05], but the degree of protection was less than in Ova/Ova mice. However, MANS did not protect against the fall in sGaw in Ova/Sal mice that did not have mucous metaplasia, confirming that the mechanism by which MANS attenuates the MCh effect is by inhibiting mucin secretion [baseline, 1.3 ± 0.15; MANS/MCh, 1.12 ± 0.08 (−14%); RNS/MCh, 1.1 ± 0.1 (−15%); MANS vs. RNS, +0.02 ± 0.13 (+1%), P = not significant]. Thus, MANS inhibits mucus-related airway obstruction during MCh challenge.

MANS-induced improvement in airflow obstruction is not limited to the nasal airways. To determine whether the effect of MANS on airflow obstruction was limited to the nasal airways, we studied the effect of MANS therapy in Ova/Ova mice that had their nostrils occluded by tape before MCh challenge and were thus breathing from their mouths. In mice that had their nostrils occluded by tape, MANS pretreatment continued to cause significant improvement in sGaw [baseline 0.92 ± 0.05; MANS/MCh, 0.83 ± 0.06 (−10%); RNS/MCh 0.61 ± 0.03 (−32%); MANS vs. RNS, +0.22 ± 0.08 (+36%), P < 0.05] (Fig. 4). Nasal occlusion was generally well tolerated by mice for the duration of the experiment. However, such recordings contained intermittent short stretches of erratic flow waveforms from the head chamber. These were marked by a sudden isolated fall in the amplitude of the flow tracing from the head chamber that did not correspond to the simultaneous flow tracing from the thoracoabdominal chamber. These are likely to be related to temporary closing of the mouth and were discarded.

MANS-induced improvement in airflow obstruction is not dependent on bronchoconstriction. To determine whether bronchoconstriction was essential for mucus-related airflow obstruction, we administered ATP after MCh challenge. ATP has been previously reported to cause airway smooth muscle relaxation (6) and is also a mucus secretagogue, unlike other bronchodilators. ATP challenge caused modest bronchodilation in Ova/Ova mice with MCh-induced airway obstruction (baseline, 1.18 ± 0.09; MCh, 0.62 ± 0.04; MCh/ATP, 0.84 ± 0.06; MCh vs. MCh/ATP, −0.22 ± 0.07, P < 0.05). In mice given MCh and ATP, MANS pretreatment resulted in signifi-
Table 1. Mucus-related obstruction is an important component of the MCh response

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<th>Mucus-related Obstruction, % total obstruction</th>
<th>MANS-induced Improvement in sGaw, %</th>
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<tr>
<td>Ova/Sal, MCh</td>
<td>10</td>
<td>1</td>
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<tr>
<td>Ova/Ova, MCh</td>
<td>45</td>
<td>48</td>
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<td>Ova/Ova, MCh (nasal occlusion)</td>
<td>78</td>
<td>36</td>
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<tr>
<td>Ova/Ova, MCh/ATP</td>
<td>80</td>
<td>28</td>
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<td>Ova/Ova3, MCh</td>
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The percentage of total airflow obstruction after a single 60 mM methacholine (MCh) challenge attributable to mucus and the MANS-induced improvement in specific airway conductance (sGaw) are summarized. Ratios were derived from group means for each category, and, therefore, variance could not be calculated, and statistical tests of difference were not performed. Ova/Sal, allergen sensitized but not challenged; Ova/Ova, single-allergen challenge; Ova/Ova3, daily allergen challenge for 3 days.

DISCUSSION

In this study, we found that intranasal instillation of a MARCKS-related peptide, MANS, inhibited mucin secretion in the lung and improved airflow obstruction after MCh challenge. This extends the previous work by Singer et al. (18) describing the inhibitory effect of intratracheal MANS on mucin secretion in vivo and provides a functional basis for potential use of MARCKS-related peptides as therapy in diseases characterized by mucus-related obstruction.

While we histologically confirmed the inhibition of mucin secretion from bronchial mucus cells, we did not directly measure the amount of mucus secreted into the lumen. Singer et al. have previously found that MANS therapy caused near complete inhibition of secreted mucin (Muc5AC). Importantly, this was by ELISA-based measurements in bronchoalveolar lavage specimens and was different from histological bronchial mucin measurements. In their study, post-MCh intracellular bronchial mucin content was reduced by 20% in MANS-treated mice and 97% in RNS-treated mice, i.e., MANS treatment resulted in ~80% inhibition of secretion. That is comparable to the changes seen by us.

To determine the functional consequences of MANS treatment, we utilized restrained double-chamber plethysmography that has been shown to be more sensitive to MCh-induced airflow obstruction than classical invasive mechanics (5). However, noninvasive methods are unable to adequately resolve changes in peripheral airway resistance (Raw) and lung parenchymal mechanics. Additional investigations based on partitioning of respiratory system impedance may, therefore, be useful in future studies.

We found that mucus-related obstruction accounted for about one-half of the total airflow obstruction induced by an intermediate dose of MCh that induced maximal mucous secretion but not maximal bronchoconstriction (Fig. 2). Mucus-related obstruction was a significant component of MCh-induced obstruction seen in dose-response experiments used to quantify AHR (Fig. 3). It is, therefore, important that changes in AHR to MCh should not be directly equated with changes in airway smooth muscle contractility, especially when using noninvasive methods.

Since changes in nasal resistance can affect sGaw, it was necessary to eliminate the possibility that MANS was effecting changes by altering the nasal airway architecture. We occluded the nostrils of spontaneously breathing mice, forcing them to breathe from their mouths, thereby nullifying the effect of any changes in nasal resistance. We found that MANS-induced
improvement in sGaw was not significantly affected by nasal occlusion (open, 0.27 ± 0.07; occluded, 0.22 ± 0.08) and is thus unrelated to changes in nasal resistance. It was not possible to quantitatively isolate the nasal component of the MCh or MANS response in our experiments because oropharyngeal resistance in orally breathing mice was an unknown additional variable. The limited effect of MANS on the nasal airways is probably related to differences in mucin secretion between the lung and the nose. The nose contains mucin-secreting submucosal glands, connected to the lumen by ducts (2, 12). In contrast, the respiratory epithelium lining the tracheobronchial airways of mice lacks such glands or mucous cells, but is rich in Clara cells, similar to the peripheral small airways of humans that are of approximately the same size. During mucous metaplasia, Clara cells increase mucin production, resulting in the development of a mucous cell phenotype. Since the mechanism of action of MANS involves contact with the target cell membrane, it is much more likely to affect mucous cells lining the airway lumen than submucosal glands. Newer peptides related to MANS that are more soluble and suitable for aerosol delivery may be more effective and should be studied further.

The mechanisms of mucous-related airflow obstruction are likely to be primarily related to changes in airway caliber. This can be divided into two components. First, airway epithelial height increases by up to 5 μm during mucous metaplasia (4). This should slightly reduce the diameter of the proximal airways and increase Raw that varies to the fourth power of the radius during laminar flow. In our experiments, development of mucous metaplasia (Ova/Ova and Ova/Ova3 mice) was associated with a trend toward increased Raw (15% rise in Raw assuming constant lung volume) that failed to reach statistical significance (P = 0.065). Second, during triggered mucin secretion, intracellular mucin that is stored in a condensed phase is secreted into the lumen, where it gets hydrated and expands up to 500 times (20). Since this expansion is far greater than the associated loss of epithelial height during mucin secretion, it is likely to cause airway narrowing and consequent airway obstruction. From the differences in post-MCh sGaw in MANS- and RNS-treated mice that were breathing orally, it can be estimated that tracheobronchial Raw increased by about one-third (assuming constant lung volume) due to mucin secretion. This requires an airway narrowing of slightly less than 10% on average or ~15–20% in the proximal and upper airways that are the sites of mucous production and the major contributors to resistance to airflow (cross-sectional area increases with increasing generations). The potential mucous volume [conservatively assuming hundrefold expansion (20)] stored in the mucin granules of mucous cells is ~0.1 μl/mm² (~10 nl/mm² [mucin volume density (4)] × 100). Thus a 1-mm segment of a large proximal airway with mucous metaplasia (0.5–1 mm diameter) has potential mucous content of 0.18–0.35 μl and a volume of 0.2–0.8 μl. Even if one-half were secreted, the airway would narrow by >20%. These numbers are only representative of the potential for mucous-related narrowing, since the actual dynamics of mucous accumulation into the lumen are complex and include the function of the mucociliary escalator that clears mucus. However, it can be seen that the magnitude of changes in Raw reported by us are well within the potential for mucus-related airway narrowing.

The time course of mucous-related obstruction in our study was similar to the kinetics of mucin secretion. In recent studies, mucin exocytosis was 100% complete at 3 min when measured by capacitance changes during granule fusion (3); and 50% complete by 3.7 min, when measured as morphometric apical volume loss (8). Differences between MANS- and RNS-treated mice were seen within the first set of stable measurements (~6 min from start of MCh exposure) and lasted for ~10–15 min, by which time both recovered to baseline. This correlates well with the expected time frame of mucous secretion and implies that mucus is being effectively cleared, since no lasting effect is seen.

The mouse model of mucous hypersecretion we used was designed to mimic mucus-related obstruction in human asthma. While the model provides an excellent means of analyzing the secretory event, there are some differences between this model and human asthma. Unlike severe human asthma where extensive mucus plugging can be seen, the effect of mucous secretion on airway conductance in our model was modest and transient (13). The reasons for this could be manifold. First, the airways of mice constitute a large percentage of the lung and have a relatively large airway lumen compared with humans. This large airway caliber is speculated to reduce the flow-resistive load that would otherwise result from the rapid respiratory rate (250–350 breaths/min) required by the mouse to maintain body temperature (11). Second, occlusion of airways by mucus probably requires either excessive mucus and/or extremely narrow airways. Since murine airways have relatively large caliber and mucous metaplasia is limited to the first five generations of airways (5), it is likely that significant airway plugging is uncommon in murine models. While there were no plugged airways in our histological specimens, displacement during inflation with formalin cannot be excluded. Third, marked airway inflammation as in severe asthma is associated with compromised epithelial integrity and reduced mucociliary clearance. Exuded plasma proteins and cellular debris may promote the production of viscous mucus and the formation of luminal mucus plugs (7). These features may not be sufficiently recapitulated in our model, since our experiments were performed 1 wk after allergen challenge, by which time mucous metaplasia is maximal, but inflammation may have partially subsided. Airway closure has been detected in response to MCh challenge in allergically inflamed mice lungs shortly after allergen exposure (21). Thus further evaluation of the MANS peptide in alternative models of chronic or severe asthma would be helpful (19).

In summary, mucin secretion has a significant role in airway obstruction. Administration of a MARCKS-related peptide blocks mucin hypersecretion and improves mucus obstruction.

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