

Abbreviations

ADM, Adrenomedullin; $AM^{+/-}$, heterozygous ADM gene deficient mice; $AM^{+/+}$, wild type mice; α -SM, alpha-smooth muscle, CT, calcitonin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin-receptor-like receptor; RAMP, receptor-activity-modifying protein; PAMP, proadrenomedullin N-terminal 20 peptide; FEV_{1.0}, forced expiratory volume in one second; BAL, bronchoalveolar lavage; R_L, lung resistance; E_L, lung elastance; MCh, methacholine; EC₂₀₀R_L, the concentration of MCh required to increase lung resistance to 200% of baseline values; BALF, BAL fluid; SAL; saline, OVA; ovalbumin, SA_v-HRP, horseradish peroxidase-conjugated streptavidin; PAS, Periodic acid Schiff; TNF- β , tumor necrosis factor β ; IFN- γ , interferon γ ; LTs, leukotrienes; LTC₄, LTD₄ and LTE₄, leukotriene C₄, D₄, and E₄

Introduction

Adrenomedullin (ADM) is a newly identified vasodilating peptide initially isolated from the extracts of human pheochromocytoma tissue (14). This peptide, which consists of 52 amino acids in human, belongs to the CGRP/CT superfamily of peptides including calcitonin (CT), amylin and calcitonin gene-related peptide (CGRP). ADM mRNA is demonstrated in a number of tissues, abundant in adrenal medulla, atrium, and lung (7), while ADM also circulates in the plasma (13). McLatchie et al. demonstrated that the calcitonin-receptor-like receptor (CRLR) functions as an ADM receptor in the presence of receptor-activity-modifying protein (RAMP) 2 (17). They also demonstrated that the expression of RAMP2 component was strongly recognized in the lung. Although it is speculated that ADM plays an important role in the lung tissue, the exact roles of ADM gene function on airway inflammation and airway remodeling remain to be little known.

In animals, Kanazawa and colleagues have reported that ADM inhibits histamine- or acetylcholine-induced bronchoconstriction in anesthetized guinea pigs (12) and that its bronchodilating effect is as potent as isoproterenol. They also demonstrated that the precursor of ADM, i.e., proadrenomedullin N-terminal 20 peptide (PAMP), has the same properties to induce bronchodilation (11). Furthermore, it has been demonstrated that ADM has the inhibitory effect on antigen-induced microvascular leakage and bronchoconstriction in guinea-pigs (20). In humans, it was also reported that human plasma ADM levels correlated negatively with the degree of airway obstruction as indicated by forced expiratory volume in one second; the plasma ADM concentration was associated with the severity of human asthma. This suggests that the level of ADM excretion may affect the degree of asthma severity, or that asthma-related bronchoconstriction and/or hypoxia increases the ADM levels as a compensative mechanism (3). Thus, it is reasonably assumed that ADM, PAMP, or their derivatives could be the new-generation bronchodilators used in the clinical settings. Meanwhile, the biological roles of innate ADM peptides based on ADM gene function in vivo remain unclear.

Because the familial or the genetic background is potentially associated with the etiology of asthma, a number of genes have been explored for the association of asthma (4). However, the exact molecular mechanisms underlying bronchial asthma still remain to be elucidated. We therefore hypothesized whether ADM gene could be involved in the pathogenesis of asthma.

Since the airway hyperresponsiveness (AHR) and inflammation including eosinophil infiltration are major characteristics of asthma (5, 6, 16), we examined the role of ADM in airway responsiveness using murine model of asthma. Although we recognized that homozygous ADM deficient mice ($AM^{-/-}$) are best suitable for the current experiments, they are unfortunately embryonic lethal (23). We, therefore performed following experiments using heterozygous ADM deficient mice ($AM^{+/-}$) and their littermate wild type control ($AM^{+/+}$).

Then, we examined the relationship between the AHR and the lung inflammation including eosinophil infiltration and other inflammatory mediators in mutant mice and control mice. In murine lung model of allergen-induced hyperresponsiveness, OVA challenge induces an eosinophilic inflammation, bronchial hyperresponsiveness and production of specific IgE (15). However, the precise role of various types of inflammatory cells and mediators involved in the pathophysiology of AHR remains to be fully determined. Thus we further examined the other inflammatory mediators including immunoglobulins (OVA-specific IgE and IgG1), Th1 and Th2 cytokines, and leukotrienes.

In addition, the association of morphological changes with AHR in the murine model was examined. Because ADM is known to have anti-proliferative effect on smooth muscle cells and fibroblasts, altered ADM function may affect cell kinetics on airway wall, which may contribute to induction of AHR in the mice.

Materials and Methods

Animals

Heterozygous ADM deficient mice ($AM^{+/-}$) were established as previously described (23). Briefly, a targeting vector was constructed to replace the 2.4-kb fragment encompassing the 1.3-kb 5'-flanking region, exons 1 to 3, and part of exon 4 of proadrenomedullin with the neomycin resistance gene. The plasmid was linearized and then introduced into 129/Sv-derived SM-1 embryonic stem cells by electroporation. Homologous recombinants were identified by Southern blot analysis, and 2 independently targeted clones were injected into C57/BL6 blastocysts to generate chimeric mice. Male Chimeras were crossbred with C57/BL6 females, and germ-line transmission was verified by Southern blot analysis. All experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments. For genotyping, genomic DNAs were isolated from biopsied tail and subjected to PCR amplification. The animals were maintained on a

light-dark cycle with light from 7:00 to 20:00 at 23°C. Mice were fed with a standard laboratory diet and water ad libitum. Mutant mice and their littermate controls ($AM^{+/+}$), between 4 to 12 weeks of age, were used in the current study. There was no difference in the body weight between the wild type group and the mutant one ($AM^{+/+}$ Saline (n=11), 30.18 ± 0.76 g; $AM^{+/-}$ Saline (n=11), 30.36 ± 0.82 g; $AM^{+/+}$ OVA (n=14), 29.00 ± 1.28 g; $AM^{+/-}$ OVA (n=14), 29.79 ± 0.79 g).

Sensitization and antigen challenge

To develop allergen-induced asthma model mice, we performed allergen sensitization and inhalational antigen challenge as previously described (2). Briefly, on day 1, $AM^{+/+}$ or $AM^{+/-}$ mice were randomly selected and sensitized with intraperitoneal (i.p.) injection of 0.5 ml solution containing 0.1 mg OVA mixed with aluminum hydroxide (2 mg/ml). On day 8, the mice were subsequently boosted with the same mixture. On days 13 and 14, these sensitized mice were placed in an 18×11×11 cm plastic chamber and were challenged for 60 minutes with aerosolized 1 % OVA dissolved in saline, generated with an ultrasonic nebulizer (NE-U17, Omron, Japan). Others received intraperitoneal injection of saline and saline aerosols in the same manner. On day 15, we performed measurement of bronchial responsiveness or bronchoalveolar lavage (BAL).

Animal preparation

Animals were anesthetized with pentobarbital sodium (25mg/kg, i.p.) and ketamine hydrochloride (25mg/kg, i.p.) in combination and then paralyzed with pancuronium bromide (0.3mg/kg, i.p.). After tracheostomy, an endotracheal metal tube (inside diameter of 1 mm, length of 8 mm) was inserted in the trachea. Animals were mechanically ventilated (model 683, Harvard Apparatus, South Natick, MA) with tidal volumes of 10 ml/kg and frequencies of 2.5 Hz. An incision was made on the abdominal wall, then the diaphragm of the bilateral chest was incised and the chest was widely opened. Positive end expiratory pressure of 2cmH₂O was applied by placing the expired line underwater. During the experiments, oxygen gas was continuously supplied to the ventilatory system. A heating pad was used to maintain the body temperature of animals. Tracheal pressure was measured with a piezoresistive micro transducer (Endevco 8510B-2, San Juan Capistrano, CA) placed in the lateral port of the tracheal cannula. Tracheal flow was measured by means of a Fleisch pneumotachograph (model no. 00000, Metabo SA,

Lausanne, Switzerland). All signals were amplified, filtered at a cutoff frequency of 100Hz and converted from analog to digital with a converter (DT2801-A, Data Translation Inc., Marlborough, MA). The signals were sampled at a rate of 200 Hz and stored on an IBM-AT compatible computer. Lung resistance (R_L) and elastance (E_L) were measured as previously described (19).

Airway responsiveness to methacholine (MCh) administration

At the start of the protocol, two deep inhalations (3 times tidal volume) were delivered to standardize volume history. All animals were then challenged with saline aerosol for 2 min. Aerosols were generated by an ultrasonic nebulizer (Ultra-Neb100, DeVilbiss, Somerset, PA) and delivered through the inspiratory line into the trachea. Measurements of 10 sec. duration were sampled during tidal ventilation one minute after administration of saline aerosol. This represented the baseline measurement. Then, each dose of MCh aerosol was administered for 2 minutes and measurements were performed one minute after each MCh inhalation in a dose response manner (0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 mg/ml). Airway responsiveness was assessed using the concentration of MCh required to increase lung resistance to 200% of baseline values (EC_{200R_L}) (18).

BAL fluid (BALF)

Bronchoalveolar lavage (BAL) was performed using 1 ml of phosphate-buffered saline (PBS) 5 times in each group. In each animal, approximately 90% (4.5ml) of the total injected volume was consistently recovered. After BALF was centrifuged at 450g for 10 min, the total and differential cell counts of the BALF were determined from the cell fraction. The supernatant was stored at -80°C until assays were performed. The concentration of protein was measured by Bradford's method using bovine serum albumin as a standard.

Assay of total immunoglobulin E (IgE) in BALF

Total amount of IgE was assayed followed by the PharMingen protocol (http://www.bdbiosciences.com/pharmingen/protocols/Mouse_IgE_ELISA.shtml). The purified anti-mouse IgE capture monoclonal antibody (mAb) (BD PharMingen, Cat. 02111D, clone R35-72) was used for the assay. The antibody titers were calculated by comparison with standard samples using serum of an OVA-immunized mouse, and

analyzed with the Microplate Manager software for the Macintosh computer (Bio Rad Inc., CA). The detection limit of the ELISA assays for IgE was 4.59 ng/ml.

Assay of OVA specific IgE in serum

OVA specific IgE was assayed in a manner similar to the PharMingen protocol of IgE. Briefly, a 96-well flat-bottomed ELISA plates were coated with the purified anti-mouse IgE capture mAb (BD PharMingen, Cat. 553413, clone R35-72, CA), and conjugated with biotinylated OVA and SA_v-HRP. We measured the absorbance on the microplate reader set at 450 nm. The detection limit of the ELISA assays for OVA-specific IgE was 20U/ml.

Assay of OVA specific immunoglobulin G1 (IgG1) in serum

An enhanced protein-binding ELISA plate was coated with the purified OVA. The plate was then blocked with 200 μ l blocking buffer per well. Samples or standards were put in each well at various dilutions in blocking buffer. Horseradish peroxidase-conjugated anti-mouse IgG1 (BD PharMingen, Cat. 559626, clone X56, CA) was diluted to 2 μ g/ml in blocking buffer and was added respectively. The plate was incubated at room temperature for 30 min. Substrate buffer was added to develop color reaction at room temperature for 20-30 min. We measured the absorbance on the microplate reader set at 450 nm. The antibody titers were calculated by comparison with standard samples using serum of an OVA-immunized mouse, and analyzed with the Microplate Manager software for the Macintosh computer (Bio Rad Inc., CA). The detection limit of the ELISA assays for IgG1 was 142U/ml.

Assays of leukotrienes, interleukin (IL) -4, 5, IL-13, and interferon (IFN)- γ in BALF

LTC₄/D₄/E₄ was measured using enzyme immunoassay (EIA) kit (GE Healthcare Bio-Sciences Corp., Cat. RPN 224, NJ). Detection limit of LTC₄/D₄/E₄ was 10 pg/ml. IL-4, 5, and IFN- γ in BALF were measured using ELISA kits (Pierce Biotechnology, Inc., Cat. EMIL42 (IL-4), EMIL52 (IL-5), EM1001 (IFN- γ)). IL-13 in BALF was measured using mouse IL-13 immunoassay kit (Genzyme TECHNE, Cat. 10003, MN). Detection limits of the ELISA assays for IL-4, IL-5, IL-13 and IFN- γ were 15, 20, 7.8, 37 pg/ml, respectively.

Assay of tissue ADM

Using lung specimens before and after MCh challenge, immunoreactive ADM (ir-ADM) levels in the lung tissues were measured using Adrenomedullin[1-50](Mouse) RIA kit(Phoenix Pharmaceuticals Inc. Cat. RK-010-31, CA). Before the assay, tissue sections were soaked in the 1.0ml of 0.1M acetic acid solution and were bathed in the 100°C water bath for 10 minutes. Then they were cooled down with ice, homogenized, and centrifuged at 13,000g for 5 minutes at 4°C. The supernatant was used for the assay. The values of the ir-ADM were expressed as the quantity per gram wet tissue samples.

Semi-quantitative assessment of eosinophilia and airway mucus hypersecretion

Whole samples of tissue were fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and cut into 4- μ m sections. The sections were stained with hematoxylin and eosin (H&E), Luna, or Periodic acid Schiff (PAS) and Alcian Blue co-staining. Each slide was provided for the count of cells. Eosinophil infiltration of the peribronchial tissue was estimated by using Luna stained slides. Goblet cell hyperplasia and airway mucus hypersecretion were assessed by inspecting PAS and Alcian Blue stained slides. All slides were observed by the two uncensored persons and assessed by their subjective scoring from 0 (none) to 3 (abundant) under the microscope (Nikon Co. Japan). In terms of the reproducibility of this assessment, inter- or intraobserver variances were less than 5%.

Morphometric analysis of airway walls

Each section was also stained with Masson's trichrome for morphometric analysis and observed by systemic manner. The whole slide was scanned thoroughly and membranous bronchioles were carefully selected for the analysis. All selected regions are digitally photographed by the Nikon microscope. Measurements were performed by Image J software (version 1.37v, National Institutes of Health, USA), using an IBM-compatible computer equipped with a digitizing tablet. Measurements are as follows: 1) Abm means the area surrounded by the airway basement membrane, whose perimeter is Pbm; 2) Amo means the area surrounded by the airway smooth muscle outline, whose perimeter is Psm; 3) Ai means the internal airway lumen area, whose perimeter is Pi; 4) the area of epithelial cells (Ae) is calculated by $Abm - Ai$, the area of smooth muscles (Asm) is also calculated by

Amo-Abm; 5) airway longer diameter is DL, shorter one is Ds. Airway size was standardized by comparing Abm, i.e. Ai/Abm or Ae/Abm or Asm/Abm. 78 of 144 selected airway sections appeared in the slides were excluded by the following criteria; the short-to-long luminal diameter ratio (Ds/DL) was under 0.6 or the borders ill-defined. When we deal with the smooth muscle bundles, we carefully outlined the edge of the smooth muscle layer, and if we encountered the interruption of the locus, we followed it smoothly to the basement membrane to maximally exclude extra cellular matrix. If the interruption was longer than 10% of Pbm, we discarded the sections like this. All the other components were applied for the analysis. In the same way, analysis was performed for immunohistochemically stained sections.

We assessed the number of cells in the airway smooth muscle layer by counting the number of nuclei in the layer. To standardize by airway size, the nuclear cell count (NCC) was divided by Pbm^2 ; i.e. NCC/Pbm^2 . The standardized number of nuclear cells in the airway smooth muscle layer was compared among 4 groups, i.e., saline-treated wild type, saline-treated ADM mutant, OVA-treated wild type, OVA-treated ADM mutant mice.

Immunohistochemical staining for alpha-smooth muscle actin

Immunohistochemistry was performed using monoclonal antibodies against alpha-smooth muscle actin (α -SM actin) (DAKO Cytomation, Code M0851, clone 1A4, CA). After tissue sections were deparaffinized and rehydrated, antigen retrieval was accomplished by 15 minute incubation at 121°C in 0.01M citrate buffered solution (pH6.0). Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 . Anti- α -SM actin antibody of 1:50 dilution was prepared together with ENVISION+TM/HRP kit for Mouse (DAKO Cytomation, Cat. K4000, CA), then incubated with normal mouse serum for 60 minutes. This conjugate was added on each section and visualized by DAB. Labeling controls were performed under the same conditions other than the usage of mouse IgG2a negative control (DAKO Cytomation, Cat. X0943, CA) instead of anti- α -SM actin antibody at the same immunoglobulin concentration.

Materials and chemicals

Materials and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Data analysis

Comparisons of data among the experimental groups were carried out with one-way ANOVA (Scheffe's test), or the nonparametric Kruskal-Wallis test). Statistical significance was determined by P values under 0.05, and errors are given in SE (standard error) unless otherwise stated. The program Microsoft Excel 2003 was used for data management, and all statistics were performed using Dr. SPSS II (SPSS Japan Inc., version 14.0, Japan).

Results

Airway responsiveness to MCh administration

There were no significant differences in baseline R_L and E_L among each group. MCh dose response curves for R_L are demonstrated in **Fig. 1**. $AM^{+/-}$ mice showed the elevation of R_L to MCh dose of 2.5 to 5 mg/ml, while the other groups exhibited no significant responses.

Airway responsiveness was also assessed using $EC_{200}R_L$ (**Fig. 2**: saline-treated $AM^{+/+}$, 16.81 ± 2.01 mg/ml; saline-treated $AM^{+/-}$, 16.73 ± 2.34 mg/ml; OVA-treated $AM^{+/+}$, $7.95 \pm 0.98^*$ mg/ml; OVA-treated $AM^{+/-}$, 2.41 ± 0.63 mg/ml, respectively, * $P < 0.05$ vs. the other groups). While bronchial hyperresponsiveness to MCh was observed in the OVA-challenged wild type mice, responses in the OVA-challenged $AM^{+/-}$ mice were significantly enhanced compared to the OVA-treated wild type mice.

Assessment of the bronchoalveolar lavage (BAL) fluid

Cell fractions in BALF are shown in **Table 1**, indicating the increases in the eosinophil fractions in the OVA-sensitized groups. No differences in the fraction and the number of BALF eosinophil were observed between the OVA-treated wild type and the mutant. Measurements of total IgE levels (saline-treated $AM^{+/+}$, 5.30 ± 0.85 ng/ml (n=5); saline-treated $AM^{+/-}$, 4.44 ± 0.92 ng/ml (n=5); OVA-treated $AM^{+/+}$, 4.19 ± 2.33 ng/ml (n=9); OVA-treated $AM^{+/-}$, 4.40 ± 1.69 ng/ml (n=7), respectively) showed no significant differences among each group. Total protein amount in BALF was also assessed (saline-treated $AM^{+/+}$, 45.05 ± 12.40 μ g/ml (n=6); saline-treated $AM^{+/-}$, 43.35 ± 7.00 μ g/ml (n=5); OVA-treated $AM^{+/+}$, 82.47 ± 23.60 μ g/ml (n=9); OVA-treated $AM^{+/-}$, 69.64 ± 14.70 μ g/ml (n=7), respectively), but no differences were found among each group. IL-4, 5,

IL-13, and IFN- γ were measured but no difference was found between OVA-treated mutant mice and OVA-treated wild type mice. The level of LTC₄/D₄/E₄ in BALF obtained from each group was also measured, but no differences were found either (**Table 2**).

Assessment of the serum

Antigen exposure increased the OVA-specific IgG1 levels (OVA-treated wild type; 5578.40 ± 4513.11 U/ml (n=7), OVA-treated mutant; 1879.62 ± 1389.27 U/ml (n=7), saline groups not detectable), whereas no difference was observed between OVA-treated mutant mice and OVA-treated wild type mice. Meanwhile, the OVA-specific IgE in serum was not detectable (**Table 3**).

Immunoreactive (ir-) adrenomedullin

Ir-ADM levels in the lung tissue were demonstrated in **Fig.3**. Ir-ADM levels of OVA-treated groups did not significantly differ from those of OVA-untreated groups. Ir-ADM levels of the mutant groups before MCh challenge were also lower than those of the wild type groups. MCh inhalation enhanced these immunoreactivities of each group ($P < 0.01$). Ir-ADM levels after MCh inhalation were significantly different between OVA-untreated wild type mice and OVA-untreated mutant mice ($P < 0.01$). Similarly, ir-ADM level of the OVA-treated mutant mice after MCh challenge was lower than that of the OVA-treated wild type mice ($P < 0.01$).

Semi-quantitative assessment of eosinophilia and airway hypersecretion

As shown in **Fig. 4.**, mononuclear cells or eosinophils infiltrated markedly along the airways of the OVA-treated mice, while no difference was found between the wild and the mutant. On the Luna stained tissue, eosinophils were markedly found around the airways of the OVA-challenged groups, but the degree of eosinophil infiltration showed no significant difference between the $AM^{+/+}$ and the $AM^{+/-}$ mice (**Fig. 5**). OVA-treated groups apparently showed very intense staining of PAS/Alcian Blue, suggesting goblet cell hyperplasia and airway mucus hypersecretion. Although the OVA challenge caused the significant change in both animal groups, the magnitude of goblet cell hyperplasia and airway mucus hypersecretion was not different between the wild type mice and the mutant mice (**Fig. 6**).

Morphometric analysis

Ai/Abm of the OVA-treated mutant mice was significantly smaller than that of the OVA-untreated mutant type mice ($P=0.0007$), but it was not different from that of the OVA-treated wild type mice ($P=0.20$) (**Fig. 7**). Ae/Abm of the OVA-treated mutant mice was greater than that of the OVA-untreated mutant mice ($P=0.00007$), but it was not different from that of the OVA-treated wild type mice ($P=0.20$) (**Fig. 8**). Similarly, Asm/Abm of the OVA-treated mutant mice was also larger than that of the OVA-untreated mutant mice ($P=0.00007$). However, Asm/Abm of the OVA-treated mutant mice was significantly greater than that of the OVA-treated wild type mice ($P=0.00010$) (**Fig. 9**). The number of cells in the airway smooth muscle layer of the OVA-treated mutant mice was significantly greater than that of the OVA-untreated mutant mice ($P=0.0019$), or that of the OVA-treated wild type mice ($P=0.012$) (**Fig. 10**).

Assessment of airway smooth muscle mass of the tissue sections immunohistochemically stained with α -SM actin antibody (**Fig. 11**) was also performed. Asm/Abm of the OVA-treated mutant mice was significantly greater than that of the OVA-treated wild type mice ($P=0.0004$) (**Fig. 12**).

Discussion

The current study clearly revealed greater AHR of the heterozygous ADM mutant mice in the allergen-induced asthma model. To our knowledge, this is the first report to study whether ADM gene function could be involved in the AHR using mutant mice. In addition, the MCh dose, which causes significant elevation of R_L in $AM^{+/-}$ mice, was very lower than that of usual model of AHR in other mice. Thus, the enhanced AHR suggests that innate ADM might play a pivotal role in AHR in allergen-induced asthma.

We observed that ir-ADM level after MCh challenge was significantly greater in the wild than in the mutant. This finding may indicate that the tissue ADM expression could be enhanced by MCh challenge to protect against the airway contraction while ADM secretion of the mutant is so restricted that the mice could not react fully against MCh inhalation challenge. As a result, ADM-induced bronchodilation after MCh challenge could not occur thoroughly in the mutant. Since we found that altered ADM gene function is associated with AHR in allergen-induced asthma model, we then examined possible mechanisms of enhanced AHR in the mice.

Firstly, we examined the roles of eosinophilic inflammation on AHR in the

mice. Current experiments showed that eosinophils infiltrated markedly along the airways of the OVA-treated mice irrespective to ADM gene modulation. In asthma, a chronic inflammatory process of the airways leads to the development of airflow limitation and AHR. Extensive cellular infiltration is seen around the asthmatic airway. Particularly, airway eosinophilia is one of the most common features in asthmatic subjects and could be involved in bronchial hyperresponsiveness (5, 16). Although vasoactive peptides including ADM might affect airway inflammation after antigen challenge, our results indicate that the disruption of ADM gene has greater effects on AHR, but little effects on antigen-induced airway eosinophilia in mice.

Secondly, we examined the potential pro-inflammatory mediators including immunoglobulins (OVA-specific IgE and IgG1), Th1 and Th2 cytokines, and leukotrienes. In the current study, OVA-specific IgE in serum was not detected in each group, and the total IgE in BALF was not significantly different between the OVA-treated wild type mice and the OVA-treated mutant mice. Th1 subtype preferentially produces IL-2, stimulating T lymphocyte proliferation, tumor necrosis factor β (TNF- β), and interferon γ (IFN- γ) that inhibit B lymphocyte activation and IgE synthesis. We examined IFN- γ as a Th1 cytokine, but no evidence was found suggesting its participation. Meanwhile, the OVA-specific IgG1, which is synthesized through Th1 and Th2 pathway, was not significantly suppressed in the serum of OVA-treated mutant mice. These findings indicate that the modulation of ADM gene might unaffected IgE production mechanism through Th1 pathway. We also measured IL-4 and IL-5 that could evoke Th2 immune system and no difference was found in the levels of IL-4 or IL-5 between the wild and the mutant animals, suggesting that the activation of Th2 pathway cannot be evoked by ADM insufficiency. Furthermore, cysteinyl LTs (LTC₄, LTD₄ and LTE₄) levels were not different between mutant mice and wild mice. Although cysteinyl LTs are reported to be one of the most important targets to treat bronchial asthma, they may not contribute to the AHR that is associated with ADM gene modulation.

Thirdly, we investigated the morphological alteration of airways in the mice. Based on the fact the biochemical parameters do not primarily contribute to increase AHR in the mice, there may be another mechanism for AHR in the ADM-deficient mice. Histologically, OVA-treated airway specimen presents mucus hypersecretion involving hyperplasia of goblet cells and submucosal gland cells, which is compatible with the airways of asthmatic subjects. Therefore, goblet cell hyperplasia and associated mucus

hypersecretion might be one of the important factors of the airflow limitation and AHR in this animal model. To make this point clear, we stained each lung section by PAS and Alcian Blue co-staining (PAS/Alcian Blue). OVA-treated animals showed the goblet cell hyperplasia and hypersecretion as indicated by the considerable staining with PAS/Alcian Blue. This phenomenon was consistently observed in the animals with OVA challenge, but not related to the ADM gene disruption, suggesting that goblet cell hyperplasia itself do not contribute the AHR in this animal model. However, our morphometric analysis data on each histological section revealed that the internal airway lumen area (A_i/Abm) of the OVA-treated mutant mice was significantly smaller than that of the OVA-untreated mutant mice. However, it was not different from that of the wild type mice. These findings cannot endorse our physiological data that the OVA-treated mutant mice exhibit greater MCh-induced AHR. The area of airway epithelial cell layer (A_e/Abm) of the OVA-treated mutant mice was significantly larger than that of the OVA-untreated mutant mice. However, it was not different from that of the wild type mice. Therefore, the thickening of airway epithelial cell layer cannot clearly explain greater airway responsiveness of the mutant mice.

ADM gene is known to induce the vaso-muscle relaxation and inhibit muscle cell proliferation. The ADM gene insufficiency may be involved in the airway wall integrity in terms of cell kinetics and muscle tones. In fact, the area of the airway smooth muscle cell layer (A_{sm}/Abm) was larger in the sensitized mutant mice than that in the sensitized wild type mice. The peribronchial trophic changes of airway smooth muscle cells may be a primary mechanism of the current allergen-induced asthma model mice. We confirmed the adrenomedullin deficiency increases airway smooth muscle cell proliferation by using immunohistochemical staining of α -SM actin in the mouse airways. These results are consistent with the increase of nuclear cells around the basement membrane in the mice.

In this study, we did not perform in vitro experiment concerning the effects of extra-cellular adrenomedullin on the airway smooth muscle proliferation. However, there are plenty of data about the inhibitory effects of adrenomedullin on smooth muscle cell proliferation in vitro and in vivo. Rossi and coworkers have demonstrated that adrenomedullin exhibits a potent dose-dependent inhibiting effect on angiotensin II (AngII) induced- human aorta smooth muscle cell proliferation and a stimulatory effect on proliferation of quiescent cells (22). Rauma-Pinola T and coworkers have

demonstrated that AM overexpression inhibits neointimal smooth muscle cells growth and enhances apoptosis of the neointimal smooth muscle cells (21). It has been also reported that AM contributes to reduction of neointima formation by the inhibition of vascular smooth muscle cell proliferation via cGMP-dependent signaling pathway (1). Furthermore, ADM has an inhibitory effect on aldosterone-induced cell proliferation of adventitia in rats. This indicates that ADM may inhibit cell proliferation of both smooth muscle cells and fibroblasts in vivo (9). Consistently, it has been reported that ADM inhibits aldosterone-induced fibroblast proliferation and ERK activity in vitro (10). Thus, it may be reasonable to speculate that ADM inhibits bronchoconstrictive stress-induced cell proliferation of both smooth muscle cells and fibroblasts.

In addition, it has been reported that ADM causes the relaxation of vascular smooth muscle cells which is associated with the intracellular cyclic AMP formation via G protein coupled adenylate cyclase pathway(8, 14). Although the similar effects of ADM on the airway smooth muscles have not been proved yet, ADM deficiency may be associated with the less muscle relaxation through lower levels of intracellular cyclic AMP formation.

In conclusion, heterozygous ADM deficient mice showed greater MCh-induced airway responsiveness. This is the first study that allergen-induced AHR is augmented by ADM gene disruption. Impairment of ADM gene expression does not affect airway inflammation including eosinophilic infiltration. The airway mucus secretion, IgE synthesis, Th1 or Th2 cytokines, and cysteinyl LTs were not affected by the ADM gene modulation either. On the other hand, ADM insufficiency caused the considerable airway smooth muscle hyperplasia probably due to promotion of peribronchial smooth muscle cell proliferation in the lungs. Thus, ADM gene may be related to the antigen-induced airway responsiveness in association with airway smooth muscle proliferation. Further, ADM might be a candidate for the novel therapeutic agent to prevent airway remodeling and asthmatic death in severe asthma. The ADM mutant mice used in this study may contribute to the study regarding the genetic roles of ADM on airway wall thickening in severe bronchial asthma and may further provide novel insights to study the physiological role of ADM in vivo.

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Figure legends

FIGURE 1. Methacholine (MCh) dose response curves for lung resistance (R_L) in the wild type and the heterozygous adrenomedullin (ADM) mutant mice.

[#] $P < 0.05$ compared with the R_L in the same MCh dosage of $AM^{+/-}$ SAL group. SAL, saline.

* $P < 0.05$ compared with the R_L in the same MCh dosage of $AM^{+/+}$ OVA group. OVA, ovalbumin.

FIGURE 2. EC_{200R_L} , Concentration of MCh required to increase R_L to 200% of baseline values.

[#] $P < 0.05$ compared with the value of $AM^{+/-}$ SAL group.

* $P < 0.05$ compared with the value of $AM^{+/+}$ OVA group.

FIGURE 3. Immunoreactive (Ir-) ADM in the lung before and after MCh challenge (RIA). $P < 0.01$ compared with the post-inhalation value of $AM^{+/+}$ OVA group.

[#] $P < 0.01$ compared with the pre-inhalation value.

FIGURE 4. Photomicrographs of small airways and lung parenchyma from (A) saline-treated wild type, (B) saline-treated ADM mutant, (C) OVA-treated wild type, (D) OVA-treated ADM mutant mice. Specimens were stained with hematoxylin and eosin. Scale bar, 100 μ m.

FIGURE 5. Semi-quantitative assessment of eosinophil infiltration around the airways.

[#] $P < 0.05$ compared with the saline group in the same strain.

FIGURE 6. Semi-quantitative assessment of goblet cell hyperplasia and airway hypersecretion of wild type and mutant mice with OVA inhalation challenge.

[#] $P < 0.05$ compared with the saline group.

FIGURE 7. Area of internal airway lumen (Ai/Abm) following OVA- or SAL-inhalational challenge. Ai/Abm indicates the standardized internal airway lumen area by Abm. P values are shown where significant ($P < 0.05$).

FIGURE 8. Area of airway epithelial cell layer (Ae/Abm) following OVA- or SAL- inhalational challenge. Ae/Abm indicates the standardized area of airway epithelial cell layer by Abm. P values are shown where significant ($P<0.05$).

FIGURE 9. Area of airway smooth muscle cell layer (Asm/Abm) following OVA- or SAL- inhalational challenge. Asm/Abm indicates the standardized area of airway smooth muscle cell layer by Abm. P values are shown where significant ($P<0.05$).

FIGURE 10. NCC/Pbm² around the respiratory bronchioles following OVA- or SAL- inhalational challenge. NCC/Pbm² indicates the standardized nuclear cell count by Abm. P values are shown where significant ($P<0.05$).

FIGURE 11. Photomicrographs of small airways and lung parenchyma from (A) saline-treated wild type, (B) saline-treated ADM mutant, (C) OVA-treated wild type, (D) OVA-treated ADM mutant mice after OVA- or SAL- inhalational challenge. Specimens were stained with anti- α -SM actin antibody immunohistochemically. Scale bar, 100 μ m.

FIGURE 12. Area of airway smooth muscle cell layer (Asm/Abm) following OVA- or SAL- inhalational challenge in the immunohistochemically stained sections with anti- α -SM actin antibody. P values are shown where significant ($P<0.05$).