Adrenomedullin insufficiency increases allergen-induced airway hyperresponsiveness in mice

Hiroshi Yamamoto,1 Takahide Nagase,2 Takayuki Shindo,3 Shinji Teramoto,1 Tomoko Aoki-Nagase,1 Yasuhiro Yamaguchi,1 Yoko Hanaoka,1 Hiroki Kurihara,4 and Yasuyoshi Ouchi1

Departments of 1Geriatric Medicine, 2Respiratory Medicine, and 4Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, Tokyo; and 3Department of Organ Regeneration, Shinshu University Graduate School of Medicine, Nagano, Japan

Submitted 2 June 2006; accepted in final form 25 February 2007

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 CT gene-related peptide (CGRP). ADM mRNA is demonstrated in a number of tissues, abundant in adrenal medulla, atrium, and lung (7), whereas ADM also circulates in the plasma (13). McLatchie et al. demonstrated that the CT-receptor-like receptor functions as an ADM receptor in the presence of receptor-activity-modifying protein 2 (17). They also demonstrated that the expression of receptor-activity-modifying protein 2 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 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 NH2-terminal 20 peptide, 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 1 s; 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, proadrenomedullin NH2-terminal 20 peptide, 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.

In the murine lung model of allergen-induced hyperresponsive-
ness, 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), T helper (Th) 1 and Th2 cytokines, and leukotrienes (LTs).

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 1–3, 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 two 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 0700 to 2000 at 23°C. Mice were fed with a standard laboratory diet and water ad libitum. Mutant mice and their littermate controls (AM+/+), between 4 and 12 wk 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 (ip) 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 min with aerosolized 1% OVA dissolved in saline, generated with an ultrasonic nebulizer (NE-U17, Omron). Others received ip 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 (25 mg/kg ip) and ketamine hydrochloride (25 mg/kg ip) in combination and then paralyzed with pancuronium bromide (0.3 mg/kg ip). 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 2 cmH₂O 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 piezoelectric micro transducer (Endevco 8510B-2, San Juan Capistrano) 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 100 Hz, and converted from analog to digital with a converter (DT2801-A, Data Translation, Marlborough, MA). The signals were sampled at a rate of 200 Hz and stored on an IBM-AT compatible computer. Lung resistance (Rl) and elastance were measured as previously described (19).

Airway responsiveness to methacholine 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-s duration were sampled during tidal ventilation 1 min after administration of saline aerosol. This represented the baseline measurement. Then, each dose of methacholine (MCh) aerosol was administered for 2 min, and measurements were performed 1 min 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 Rt to 200% of baseline values (EC₂₀₀Rl) (18).

**BAL fluid.** BAL was performed using 1 ml of PBS 5 times in each group. In each animal, ~90% (4.5 ml) of the total injected volume was consistently recovered. After BAL fluid (BALF) was centrifuged at 450 g 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://wwwbdbiosciences.com/pharmingen/protocols/Mouse_IgE_ELISA.shtml). The purified anti-mouse IgE capture monoclonal antibody (BD Pharmingen, catalog no. 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). 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, 96-well flat-bottomed ELISA plates were coated with the purified anti-mouse IgE capture monoclonal antibody (BD Pharmingen, catalog no. 553413, clone R35-72) and conjugated with biotinylated OVA and SAV-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 20 U/ml.

**Assay of OVA-specific IgG1 in serum**. An enhanced protein-binding ELISA plate was coated with the purified OVA. The plate was then blocked with 200 μl of blocking buffer per well. Samples or standards were put in each well at various dilutions in blocking buffer. Horse-radish peroxidase-conjugated anti-mouse IgG1 (BD Pharmingen, catalog no. 559626, clone X56) was diluted to 2 μg/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). The detection limit of the ELISA assays for IgG1 was 142 U/ml.

**Assays of LTs, IL-4, -5, and -13, and IFN-γ in BALF**. LTC4/D4/E4 was measured using enzyme immunoassay kit (GE Healthcare Bio-Sciences, catalog no. RPN 224). Detection limit of LTC4/D4/E4 was 10 pg/ml. IL-4 and -5 and IFN-γ were measured using ELISA kits (Pierce Biotechnology, catalog no. EMIL42 (IL-4), EMIL52 (IL-5), EM1001 (IFN-γ)). IL-13 in BALF was measured using mouse IL-13 immunoassay kit (Genzyme TECHNE, catalog no. 10003). Detection limits of the ELISA assays for IL-4, IL-5, IL-13, and IFN-γ were 15, 20, 7.8, and 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 ADM[i-50] (Mouse) RIA kit (Phoenix Pharmaceuticals, catalog no. RK-01031). Before the assay, tissue sections were soaked in the 1.0 ml of 0.1 M acetic acid solution and were bathed in the 100°C water bath for 10 min. Then they were cooled down with ice, homogenized, and centrifuged at 13,000 rpm for 5 min 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-μm sections. The sections were stained with hematoxylin and eosin, 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). In terms of the reproducibility of this assessment, inter- or intraobserver variances were <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), 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) Amono 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 (Ac) is calculated by Abm − Ai; the area of smooth muscles (Asm) is also calculated by Abm − Pbm; 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. Seventy-eight 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 extracellular 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 Pbm²; i.e., NCC/Pbm². The standardized number of nuclear cells in the airway smooth muscle layer was compared among four 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). After tissue sections were deparaffinized and rehydrated, antigen retrieval was accomplished by 15-min incubation at 121°C in 0.01 M citrate buffered solution (pH 6.0). Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H2O2. Anti-α-SM actin antibody of 1:50 dilution was prepared together with ENVISION+™horseradish peroxidase kit for Mouse (Dako Cytomation, catalog no. K40000), then incubated with normal mouse serum for 60 min. This conjugate was added on each section and visualized by DAB. Labeling controls were performed under the

---

**Table 1. Total cell counts and cell fractions in BALF in each experimental group**

<table>
<thead>
<tr>
<th>TCC (×10⁶)</th>
<th>Mφ, %</th>
<th>Lym, %</th>
<th>Eos, %</th>
<th>PMN, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM&lt;sup&gt;−/−&lt;/sup&gt; saline (n = 5)</td>
<td>4.94±1.91</td>
<td>96.02±2.82</td>
<td>4.32±2.67</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>AM&lt;sup&gt;−/−&lt;/sup&gt; saline (n = 5)</td>
<td>4.82±0.54</td>
<td>91.96±2.51</td>
<td>7.24±2.25</td>
<td>0.10±0.00</td>
</tr>
<tr>
<td>AM&lt;sup&gt;−/−&lt;/sup&gt; OVA (n = 5)</td>
<td>40.20±18.22†</td>
<td>70.58±15.00</td>
<td>2.84±1.87</td>
<td>25.88±14.88*</td>
</tr>
<tr>
<td>AM&lt;sup&gt;−/−&lt;/sup&gt; OVA (n = 6)</td>
<td>21.13±8.75</td>
<td>60.02±12.41†</td>
<td>4.00±1.60</td>
<td>35.30±12.84†</td>
</tr>
</tbody>
</table>

Values are means ± SE. TCC: total cell count; Mφ, macrophages; Lym, lymphocytes; Eos, eosinophils; PMN, polymorphonuclear cells; BALF, bronchoalveolar lavage fluid. †P < 0.05 compared with the saline group in the same strain of animals. *P < 0.01 compared with the saline group in the same strain of animals.
same conditions other than the usage of mouse IgG2a negative control (DAKO Cytomation, catalog no. X0943) instead of anti-α-SM actin antibody at the same immunoglobulin concentration.

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

Data analysis. Comparisons of data among the experimental groups were carried out with one-way ANOVA (Scheffé’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 SPSS II (SPSS Japan, version 14.0).

RESULTS

Airway responsiveness to MCh administration. There were no significant differences in baseline Rt and lung elastance among each group. MCh dose response curves for Rt are demonstrated in Fig. 1. *AM*+/− mice showed the elevation of Rt. to MCh dose of 2.5–5 mg/ml, whereas the other groups exhibited no significant responses.

Airway responsiveness was also assessed using EC20RL (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 ± 0.98* ng/ml; OVA-treated *AM*+/+, 2.41 ± 0.63 ng/ml, respectively (*P* < 0.05 vs. the other groups). Although bronchial hyperresponsiveness to MCh was observed in the OVA-challenged wild-type mice, responses in the OVA-challenged mutant mice were significantly enhanced compared with the OVA-treated wild-type mice.

Assessment of the BALF. 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 mice. 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 μg/ml (*n* = 6); saline-treated *AM*+/−, 43.35 ± 7.00 μg/ml (*n* = 5); OVA-treated *AM*+/+, 82.47 ± 23.60 μg/ml (*n* = 9); OVA-treated *AM*+/−, 69.64 ± 14.70 μg/ml (*n* = 7), respectively], but no differences were found among each group. IL-4, 5, IL-13, and IFN-γ levels of the mutant groups before MCh challenge did not significantly differ from those of OVA-untreated groups. 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 levels 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, whereas no difference was found between OVA-treated mutant mice and OVA-treated wild-type mice. Meanwhile, the OVA-specific IgE in serum was not detectable (Table 3).

**ir-ADM.** 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 levels of the OVA-treated mutant mice after MCh challenge was lower than that of the OVA-treated wild-type mice (*P* < 0.01).
AM<sup>1/2</sup> 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 significantly greater 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 RL in AM<sup>1/2</sup> mice, was very lower than that of the 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

![Fig. 4. Photomicrographs of small airways and lung parenchyma from saline-treated wild-type (A), saline-treated ADM mutant (B), OVA-treated wild-type (C), or OVA-treated ADM mutant mice (D). Specimens were stained with hematoxylin and eosin. Scale bar, 100 μm.](image)

![Fig. 5. Semi-quantitative assessment of eosinophil infiltration around the airways. #P < 0.05 compared with the saline group in the same strain.](image)

![Fig. 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.](image)
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.

First, 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 effect on antigen-induced airway eosinophilia in mice.

Second, we examined the potential proinflammatory mediators including immunoglobulins (OVA-specific IgE and IgG1), Th1 and Th2 cytokines, and LTs. 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, TNF-β, and 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 not affect 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-type 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-type 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.

Third, 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 was not related to the ADM gene disruption, suggesting that goblet cell hyperplasia itself does not contribute the AHR in this animal model. However, our morphometric analysis data on each histological section revealed that the internal airway lumen area (Ai/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 (Ae/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 (Asm/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 ADM 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 an in vitro experiment concerning the effects of extracellular ADM on the airway smooth muscle proliferation. However, there are plenty of data about the inhibitory effects of ADM on smooth muscle cell proliferation in vitro and in vivo. Rossi and coworkers have demonstrated that ADM 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 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 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. Furthermore, 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.

ACKNOWLEDGMENTS

We are grateful to S. Ishii (Department of Biochemistry and Molecular Biology, Graduate School of Medicine, University of Tokyo) and T. Yokomizo (Department of Medical Biochemistry, Graduate School of Medicine, University of Kyusyu) for excellent technical assistance and valuable suggestions. We are also grateful to R. Nagai (Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo) for great assistance.

GRANTS

This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (no. 16590737), for the Respiratory Failure Research Group from the Ministry of Health, Labour and Welfare, Japan, and for Comprehensive Research on Aging and Health from the Ministry of Health, Labour and Welfare, Japan.

REFERENCES


