Full Paper

Effect of Diesel Exhaust Particles on House Dust Mite–Induced Airway Eosinophilic Inflammation and Remodeling in Mice

Go Takahashi^{1,2}, Hiroyuki Tanaka^{1,2,*}, Keiko Wakahara^{1,3}, Reishi Nasu¹, Mikiko Hashimoto¹, Kosuke Miyoshi¹, Hirohisa Takano⁴, Hirotaka Yamashita^{1,2}, Naoki Inagaki^{1,2}, and Hiroichi Nagai¹¹Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan²United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1193, Japan³Division of Respiratory Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai, Syowa-ku, Nagoya, Aichi 466-8550, Japan⁴National Institute for Environmental Studies, 16-2 Onokawa, Tsukuba, Ibaraki 305-8506, Japan

Received October 1, 2009; Accepted December 2, 2009

Abstract. Recent research has focused on the effects of ambient particulate pollution and much evidence has indicated that particulate pollution is associated with the onset of asthma and allergy; however, the effect of diesel exhaust particles (DEP) on the development of allergen-induced airway remodeling has not been fully investigated in vivo. In the present study, we examined the effects of DEP on *Dermatophagoides farinae* allergens (Der f)–induced asthma-like phenotypes in mice. Mice were administered i.t. 8 times with Der f. DEP were injected i.t. with Der f 4 times throughout the experiment or twice at the sensitization period. In both cases, DEP aggravated Der f–induced increases in airway responsiveness to acetylcholine, the number of eosinophils and neutrophils in the bronchoalveolar lavage fluid (BALF), serum Der f–specific IgG1 levels, Th2 cytokines and transforming growth factor- β 1 levels in BALF, and amount of hydroxyproline in the right lungs. Furthermore, goblet cell hyperplasia and subepithelial fibrosis were also markedly aggravated. These findings indicate that DEP can potentiate airway remodeling induced by repeated allergen challenge as well as Th2-driven airway hyperresponsiveness, eosinophilic inflammation, and IgG1 production and that DEP can exhibit adjuvant activity for airway remodeling, probably due to the enhancement of allergen sensitization and/or of Th2 polarizing pathways.

Keywords: bronchial asthma, *Dermatophagoides farinae*, goblet cell, subepithelial fibrosis, transforming growth factor- β 1

Introduction

Recently, bronchial asthma has become one of the most common health problems in the world, especially within industrialized societies; and the prevalence rates have been increasing considerably over the last few decades (1 – 3) for reasons that are not yet completely understood. Changes in lifestyle and an increase in indoor allergen exposure caused by higher indoor temperature and humidity have been suggested as potential determinants. Therefore, it is reasonable to consider that

environmental exposures to allergens are of primary importance for the prevalence and development of asthma, in genetically predisposed individuals, because genes controlling allergic phenotypes have not been changed in the last few decades. Among a variety of allergens, house dust mite including *Dermatophagoides farinae* (Der f) and *Dermatophagoides pteronyssinus* are known to be principle allergens for the induction of asthma (4 – 6); however, the precise molecular mechanisms underlying the allergenicity of the allergens are not fully understood.

Contrary to an increase in indoor allergen exposure, recent epidemiological studies show that ambient particulate matter is associated with an increase in hospital admission for asthma (7) and the development of

*Corresponding author. hirotnk@gifu-pu.ac.jp

Published online in J-STAGE on January 22, 2010 (in advance)

doi: 10.1254/jphs.09276FP

respiratory symptoms in childhood asthma (8). In fact, several studies have shown adverse effects of ambient air pollution on respiratory health (9, 10) and increased risk of atopic diseases and allergic sensitization when children are exposed to ambient particulate matter (11). Among these pollutants, diesel exhaust particles (DEP) are important components of the pollution respirable particles in the range of $2.5\text{ }\mu\text{m}$ or less. Muranaka et al. demonstrated that intranasal inoculation of DEP along with allergens enhanced the allergen-specific IgE response in mice (12). Subsequently, various experimental studies in mice have suggested that the injection of DEP with antigen can enhance airway hyper-responsiveness (AHR) and airway inflammation (13–16). However, the effect of DEP on the development of allergen-induced airway remodeling, especially subepithelial fibrosis associated with increases in the level of fibrogenic factors like transforming growth factor- $\beta 1$ (TGF- $\beta 1$) in the airways, has not been fully investigated in vivo.

Recently, we (17, 18) have established a mouse model for allergic asthma using a major allergen, Der f. This mouse model is characterized by eosinophil infiltrates into the airways, increases in the level of interleukin (IL)-13, decrease in the level of interferon- γ (IFN- γ) in the bronchoalveolar lavage fluid (BALF), AHR to acetylcholine (ACh), and increases in the serum allergen-specific immunoglobulin (Ig) G1 level by repetitive intratracheal administration of the allergen without a T helper type 2 (Th2)-skewing adjuvant. Furthermore, airway remodeling, characterized by severe goblet cell hyperplasia/hypertrophy, subepithelial fibrosis, and increases in the level of TGF- $\beta 1$ in the BALF were also demonstrated. These data demonstrate that repeated Der f inoculations into the airway directly skewed Th2-predominant responses in the airway where the indoor allergens invade. Therefore, our mouse model, which was locally immunized with Der f but devoid of additional adjuvants, may reflect the characteristics of atopic asthma, especially induced by Der f. It can be a useful model for investigating environmental factors such as DEP that are involved in the onset and development of the disease.

In the present study, to investigate the effect of DEP in the onset and development of allergen-induced asthma-like phenotypes including airway remodeling, we injected DEP 4 times into mouse trachea during allergen administration, in which a threshold dose of Der f can slightly induce airway eosinophilia, increases in serum specific IgG1, the level of IL-13 in BALF, the number of goblet cells in the epithelium, and the area of fibrosis lesion, but not AHR. Then, to address the question whether coexistence of DEP and Der f at the sensitization can affect the development of airway

remodeling after repeated Der f challenge, we injected DEP twice into mouse airway only during the first and second administration of the allergen.

Materials and Methods

Animals

Seven-week-old BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu) and housed in plastic cages in an air-conditioned room at $22 \pm 1^\circ\text{C}$ with relative humidity of $60 \pm 5\%$. The mice were fed a standard laboratory diet and given water ad libitum. All experiments were carried out following the rules and regulations for the care and use of experimental animals as stipulated by Gifu Pharmaceutical University in 2008.

Agents

The following biological agents, chemicals, and drugs were purchased commercially: halothane (Takeda Chemical Industries, Osaka), *Dermatophagoides farinae* (Der f; LSL Co., Tokyo), dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc., Kyoto), phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo), Tween 80 (Nacalai Tesque), acetylcholine chloride (ACh, Nacalai Tesque), bovine serum albumin (BSA; Seikagaku Kogyo, Tokyo), Türk solution (Wako Pure Chemical Industries, Ltd., Osaka), pancuronium bromide (Sigma, St. Louis, MO, USA), sodium pentobarbitone (Abbott Lab., Chicago, IL, USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na, Nacalai Tesque), Diff-Quick solution (Sysmex Corp., Kobe), monoclonal rat anti-mouse IgG1 antibody (MCA336P; Serotec Co., Ltd., Oxford, UK), peroxidase-conjugated streptavidin (Dakopatts a/s, Glostrup, Denmark), and hydroxy-L-proline (Nacalai Tesque).

DEP

A 2982-cc common rail direct injection diesel engine with oxidation catalyst and exhaust gas recirculation (EGR) system was used for the generation of DEP. This engine met Japan's long-term diesel emissions regulations in 1998. The engine was operated at 2040 rpm and 35 N·m by a 220 kW EDYC dynamometer (Meiden-Sha, Tokyo) and used fuel (LS light oil; ENEOS, Tokyo) containing 27 ppm sulfur and lubricating oil (CF-4 10W-30). DEP were collected from the accumulation in the dilution tunnel, and 39.4% of the organic fraction of DEP was soluble in CH_2Cl_2 . DEP were stored at -80°C until experimental use. The DEP were kindly provided by Japan Automobile Research Institute (Tsukuba).

Experimental protocol

To investigate the effect of DEP on allergen-induced

Fig. 1. Experimental protocols for intratracheal (i.t.) instillation. Protocol A: DEP (closed circle) were administered i.t. 4 times on day 0, 14, 21, and 28. Protocol B: DEP (closed circle) were administered i.t. twice on day 0 and 1. In each protocol, allergen (*Dermatophagoides farinae*, open square) was given i.t. 8 times. PBS, phosphate-buffered saline–injected; Der f, *Dermatophagoides farinae*–injected; DEP, diesel exhaust particles–injected.

airway inflammation and airway remodeling, we used two protocols. Based on the previous study that DEP at 100 $\mu\text{g}/\text{body}$ corresponds to a 5-fold–accumulation dose in lungs under the Japanese environmental standard [100 $\mu\text{g}/\text{m}^3$ as daily mean value of suspended particulate matters (19)], we chose dosages of 10, 30, and 100 $\mu\text{g}/\text{body}$ of DEP.

Protocol A: mice were divided into four groups of 8–9 mice: 1) PBS containing 0.05% Tween 80 alone, 2) Der f alone, 3) DEP alone, and 4) 4 μg Der f + DEP. Each agent was dissolved in PBS solution at pH 7.4 containing 0.05% Tween 80. Group 2 received intratracheal (i.t.) doses of 4 or 20 μg Der f. Group 3 received i.t. doses of 10, 30, or 100 μg DEP. Group 4 received i.t. doses of 4 μg Der f and 10, 30, or 100 μg DEP. In each group, DEP and/or Der f were injected i.t. through a polyethylene tube with 4% halothane as shown in Fig. 1 (Protocol A).

Protocol B: mice were divided into four groups: 1) PBS containing 0.05% Tween 80 alone, 2) Der f alone, 3) DEP alone, and 4) 4 μg Der f + DEP. Each agent was dissolved in a pH 7.4 PBS solution containing 0.05% Tween 80. Group 2 received an i.t. dose of 4 or 20 μg Der f. Group 3 received i.t. doses of 30 or 100 μg DEP. Group 4 received i.t. doses of 4 μg Der f and 30 or 100 μg DEP. In each group, DEP and/or Der f were injected i.t. as shown in Fig. 1 (Protocol B). At 48 h after the final challenge, airway responsiveness to ACh was measured; and BAL, histological evaluation, and the measurement of hydroxyproline content in right lungs were carried out.

BAL study

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in BALF. Experiments were performed according to previously described methods (20). Mice were killed with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) and tracheas cannulated. The left bronchi were tied for histological examination. Then, the right air lumen was washed 4 times with 0.5 ml calcium- and magnesium-free PBS containing 0.1% BSA and 0.05 mM EDTA-2Na. This procedure was repeated three 3 times (total volume of 1.3 ml, recovery >85%). BALF from each animal was pooled in a plastic tube, cooled on ice, and centrifuged (150 $\times g$) at 4°C for 10 min. Cell pellets were resuspended in the same buffer (0.5 ml). BALF was stained with Türk solution and the number of nucleated cells was counted in a Burkner chamber. A differential count was made on a smear, prepared with a cytocentrifuge (Cytospin II; Shandon, Cheshire, UK) and stained with Diff-Quik solution (based on standard morphologic criteria), of at least 300 cells (magnification $\times 500$). The supernatant of BALF was stored at -30°C for determination of cytokine production.

Measurement of immunoglobulins

At day 30, blood was collected by cardiac puncture and sera were obtained by centrifugation and stored at -80°C . Antigen-specific IgG1 in the mouse serum was measured using the enzyme-linking immunosorbent assay (ELISA) according to previously described methods (17). Briefly, serum Der f–specific IgG1 was measured by coating Der f at a concentration of 20 $\mu\text{g}/\text{ml}$. After blocking with 1% BSA, serum dilutions were incubated for 1 day followed by peroxidase-conjugated monoclonal mouse IgG1 antibody (MCA336P). The 450-nm absorbance (reference 690 nm) of the enzymatic reactions were read using an automatic ELISA plate reader (Multiscan MS, ver 8; Labsystems Oy, Helsinki, Finland) and analyzed using Deltasoft 3. Because Der f–specific specific IgG1 antibody as a standard was not commercially available, all samples were tested simultaneously, and results were represented as absorbance at 450 nm after background correction.

Cytokine levels in BALF

The cytokine levels in the supernatant of BALF were measured using ELISA: for IFN- γ (Endogen, Inc., Woburn, MA, USA) and for IL-13 (R&D Systems, Inc., Minneapolis, MN, USA). The TGF- $\beta 1$ content in BALF was also measured by ELISA (R&D Systems, Inc.), which can detect mouse TGF- β protein, due to the high homology of TGF- β across species. The assay detects only the active form of TGF- $\beta 1$. Each sample was acti-

vated according to the manufacturer's recommendation prior to measurement. The detection limit of each kit was 10 pg/ml for IFN- γ , 1.5 pg/ml for IL-13, or 7 pg/ml for TGF- β 1.

Measurement of hydroxyproline content in right lungs

Whole collagen content of the right lung was evaluated by determining hydroxyproline content (20). Briefly, after recovery of BALF, the right lung lobes were removed and sliced into 1-mm-thick sections. The sliced lung samples were dried with acetone and then hydrolyzed with 2 ml of 6 N HCl at 120°C for 24 h in sealed glass tubes. The hydroxyproline content in the hydrolysate was measured according to Kivirikko et al. (21). Authentic hydroxyproline (hydroxy-L-proline) was used to establish a standard curve.

Histological study

The left lungs were distended by the injection of 10% buffered formalin via the trachea, excised, and immersed in the same fixative with the trachea clamped for 24 h. Tissues were sliced and embedded in paraffin, and 6- μ m sections were stained with periodic acid-Schiff (PAS) and Masson-Trichrome for light microscopy examination. Examination of goblet cell hyperplasia was carried out with PAS-stained histological preparations of the left lobe using a Leica image analysis system (Leica, Cambridge, UK). Using a 10-fold magnification objective, 4 representative areas were chosen from the largest visible airway which, in the horizontal sections through the hilus, was the left main bronchus. Subsequently, with a 40-fold magnification corresponding to one microscopic field, the hyperplasia of the goblet cells in the epithelial lining was recorded by a score based on the percentage of the goblet cells in the epithelial cells described below. The length of the epithelial basement membrane of the bronchus of one area was $\geq 500 \mu\text{m}$. To minimize sampling errors, a 5-point scoring system (grades 0–4) was adopted: grade 0, no goblet cells; grade 1, <25% goblet cells; grade 2, 25%–50% goblet cells; grade 3, 50%–75% goblet cells; and grade 4, $\geq 75\%$ goblet cells (20). The mean score of the total epithelial cells in the 4 areas of one mouse were counted. The mean scores of hyperplasia of the goblet cells were calculated for 8–9 mice.

Masson-trichrome-stained sections were used for the assessment of subepithelial fibrosis using a Leica image analysis system (Leica). As described above, using a 10-fold magnification objective, 3 representative areas were chosen, avoiding the selection of the furcation of the bronchus and the surrounding blood vessels in the largest airway. Subsequently, with a 40-fold magnification, epithelial basement membrane areas $\geq 250 \mu\text{m}$

were selected, and the thickness of the epithelial layer and the fibrotic area (stained in blue) 30 μm beneath the basement membrane of the standardized sampling points were measured (18). The means of the thickness of the epithelial layer and fibrotic area divided by basement membrane length were calculated for 8–9 mice.

Measurement of airway function

Measurement of bronchial responsiveness to intravenous ACh was performed as previously described (20, 22). Briefly, to measure airway responsiveness to ACh, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the jugular vein was cannulated for intravenous injection of ACh. Mice were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration and then ventilated with a rodent ventilator (New England Medical Instruments, Inc., Medway, MA, USA). Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. To measure airway responsiveness to ACh, changes in respiratory overflow volume were measured using an increasing dose of ACh. The increase in respiratory overflow volume induced by ACh was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The area under the curve (AUC) calculated from dose-response curves for ACh was used to express the magnitude of AHR. Briefly, each dose was converted logarithmically, and then AUC was calculated and represented as arbitrary units (20).

Statistical analyses

Results are represented as the mean $\pm$ S.E.M. Statistical significance between two groups was estimated by using the two-tailed Student's *t*-test or the Mann-Whitney's *U*-test after the variances of the data were evaluated with the *F*-test. To define statistically significant differences among control mice and DEP-treated mice, the data were subjected to Bartlett's analysis followed by a parametric or a non-parametric Dunnett's multiple range test. *P* values less than 0.05 were considered significant.

Results

Co-administration of DEP with Der f during the allergen instillations (Protocol A)

Effect of DEP on allergen-induced AHR, airway eosinophilia, specific IgG1 production in serum, and cytokine production in BALF: To investigate the effect of DEP on the onset and development of allergen-induced AHR, inflammatory infiltrates in the airway, Th1/Th2 cytokine balance, and specific IgG1 production, we

Fig. 2. Effect of DEP on allergen-induced increases in airway responsiveness to ACh, the numbers of eosinophils in BALF, serum antigen-specific IgG1 level, IL-13 production in BALF, and decrease in IFN- γ production in BALF in BALB/c mice (Protocol A). Values represent the mean or mean $\pm$ S.E.M. of 8–9 mice in each group. AUC, area under the curve for the dose-response to ACh (range: 31.25–2000 μ g/kg); PBS, phosphate-buffered saline-injected; Der f, *Dermatophagoides farinae*-injected; DEP, diesel exhaust particles-injected; N.D., not detected; * P < 0.05, ** P < 0.01, *** P < 0.001 (vs. PBS group); † P < 0.05, †† P < 0.01, ††† P < 0.001 (vs. 4 μ g Der f group); # P < 0.05, ### P < 0.01, #### P < 0.001 (vs. DEP group).

injected DEP i.t. into mice four times at designated points as shown in Fig. 1 (Protocol A). As shown in Fig. 2, repeated Der f instillations induced dose-dependent increases in airway responsiveness to ACh (A and B), the number of eosinophils and neutrophils in BALF (C and D), IL-13 and eotaxin production in BALF (E and F), decreases in IFN- γ production in BALF (G), and serum antigen-specific IgG1 production (H) compared with

those in PBS-treated mice. In contrast to the 4 μ g of Der f-injected mice, co-administration of DEP with the allergen clearly aggravated all responses in a dose-dependent manner. Intratracheal inoculations of DEP at a dose of 10 or 30 μ g showed no effect on these parameters, whereas administration of DEP at a dose of 100 μ g induced slight increases in the number of eosinophils and neutrophils in BALF (Fig. 2: C and D), IL-13 production in BALF,

and the decreases in IFN- γ production in BALF (Fig. 2: E and G).

Effect of DEP on allergen-induced airway remodeling: To investigate the effect of DEP in the onset and development of allergen-induced airway remodeling, we examined the histological changes in airways, TGF- β 1 (known as a fibrogenic cytokine) production in BALF, and the amount of hydroxyproline content (a collagen-specific amino acid) content in the right tissue 48 h after the final allergen challenge. Figure 3 shows the representative sections of each group stained with PAS for detection of goblet cells (A – D) and stained with Masson-Trichrome for detection of connective tissue (E – H). The quantitative findings of the histological examination, TGF- β 1 production, and the amount of hydroxyproline in each group are shown in Table 1. As indicated in Fig. 3 and Table 1, repeated Der f injections caused not only goblet cell hyperplasia and hypertrophy, but also subepithelial fibrosis, observed beneath the basement membrane of the bronchi and peripheral bronchiole, associated with increases in the amount of TGF- β 1 in BALF and hydroxyproline content in the right lung tissue, whereas mice inoculated with 4 μ g of Der f showed marked goblet cell hyperplasia, which was comparable to those of mice with 20 μ g of Der f, and a weak but significant TGF- β 1 production in BALF and fibrotic changes compared with PBS-injected mice (Fig. 3: A vs. B). In contrast to the 4 μ g of Der f-instilled group, co-administration of DEP with lower dose of the allergen 4 times, at designated points as shown in Fig. 1A, clearly aggravated the goblet cell hyperplasia (Fig. 3D and Table 1), increases in TGF- β 1 production, and the development of subepithelial fibrosis (Fig. 3: D and H, and Table 1). Administration of DEP at a dose of 10 or 30 μ g alone did not show any marked effect on the epithelial changes

Fig. 3. Histological analysis of lung sections stained with PAS (A – D) and with Masson-trichrome (E – H) 48 h after the final antigen challenge ($\times 100$) in BALB/c mice (Protocol A). A and E: PBS-injected mice; B and F: 4 μ g Der f-injected mice; C and G: 100 μ g DEP-injected mice; D and H: 4 μ g Der f + 100 μ g DEP-injected mice.

Table 1. Effect of DEP on allergen-induced airway remodeling in BALB/c mice (Protocol A)

Treatment	Goblet cell hyperplasia (arbitrary units)	Fibrotic area (area/BM)	TGF- β 1 (pg/ml)	Hydroxyproline (μ g/lung)
PBS	0.4 $\pm$ 0.1	7.1 $\pm$ 1.0	12.2 $\pm$ 1.7	109.6 $\pm$ 2.9
Der f 4 μ g	3.4 $\pm$ 0.1***	11.2 $\pm$ 0.9*	34.7 $\pm$ 7.5	134.0 $\pm$ 5.7**
Der f 20 μ g	3.9 $\pm$ 0.1***	20.1 $\pm$ 1.5***	376.7 $\pm$ 52.0***	169.1 $\pm$ 4.0***
DEP 10 μ g	0.7 $\pm$ 0.1	7.5 $\pm$ 0.6	16.3 $\pm$ 2.6	116.9 $\pm$ 6.9
DEP 30 μ g	0.6 $\pm$ 0.1	8.8 $\pm$ 0.8	18.0 $\pm$ 1.6	131.8 $\pm$ 6.1*
DEP 100 μ g	1.8 $\pm$ 0.3**	9.5 $\pm$ 0.4	75.3 $\pm$ 18.2***	143.4 $\pm$ 4.7**
Der f 4 μ g + DEP 10 μ g	3.2 $\pm$ 0.2###	10.5 $\pm$ 0.6##	111.6 $\pm$ 30.3##	146.7 $\pm$ 6.3###
Der f 4 μ g + DEP 30 μ g	3.8 $\pm$ 0.1###	13.8 $\pm$ 0.8###	130.8 $\pm$ 15.2###,†	153.0 $\pm$ 5.0 †
Der f 4 μ g + DEP 100 μ g	3.8 $\pm$ 0.1###,†	18.0 $\pm$ 1.2###,††	423.8 $\pm$ 36.2###,†††	175.0 $\pm$ 2.7###,†††

Values represent the mean $\pm$ S.E.M. of 8–9 mice in each group. * P < 0.05, ** P < 0.01, *** P < 0.001 (vs. PBS); † P < 0.05, †† P < 0.01, ††† P < 0.001 (vs. Der f 4 μ g); # P < 0.05, ## P < 0.01, ### P < 0.001 (vs. DEP group).

Fig. 4. Effect of DEP on allergen-induced increases in airway responsiveness to ACh, the numbers of eosinophils in BALF, serum antigen-specific IgG1 level, IL-13 production in BALF, and decrease in IFN-γ production in BALF in BALB/c mice (Protocol B). Values represent the mean or mean ± S.E.M. of 8–9 mice in each group. AUC, area under the curve for the dose-response to ACh (range: 31.25–2000 μg/kg); PBS, phosphate-buffered saline-injected; Der f, *Der-matophagoides farinae*-injected; DEP, diesel exhaust particles-injected; N.D., not detected. ** $P < 0.01$, *** $P < 0.001$ (vs. PBS group); † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ (vs. 4 μg Der f group); # $P < 0.05$, ### $P < 0.01$, ### $P < 0.001$ (vs. DEP group).

and fibrotic changes, whereas administration of DEP at a dose of 100 μg by itself showed significant increases in the number of goblet cells in the epithelium, TGF-β1 production, and collagen deposition in the lung compared with PBS-treated mice (Fig. 3: C and G, and Table 1).

Co-administration of DEP with Der f at sensitization on day 0 and 1 (Protocol B)

Effect of DEP on allergen-induced AHR, airway

eosinophilia, IgG1 production in serum, and cytokine production in BALF: To investigate the effect of DEP on the onset of allergen-induced AHR, inflammatory infiltrates in airway, IgG1 production, and Th1/Th2 cytokine balance, we injected DEP i.t. twice during the sensitization as shown in Fig. 1 (Protocol B). As depicted in Fig. 4, repeated Der f injections induced AHR (A and B), airway eosinophilia and neutrophilia (C and D), Th2/Th1 imbalance (E and G), eotaxin production (F), and

serum specific IgG1 production (H) dose-dependently compared with those in PBS-treated mice. In contrast to the 4 μ g Der f-injected mice, co-administration of DEP with Der f at the allergen sensitization prominently aggravated all responses except for the number of neutrophils in BALF (Fig. 4D) in a dose-dependent manner. In contrast, DEP at a dose of 30 or 100 μ g by itself showed no effect on these parameters except for slight increases in the number of neutrophils in BALF (Fig. 4D).

Effect of DEP on allergen-induced airway remodeling: Figure 5 shows the representative sections of each group stained with either PAS (A – D) or Masson-Trichrome (E – H). The quantitative findings are summarized in Table 2. In contrast to 4 μ g Der f-injected mice, simultaneous injection of DEP with the lower dose of Der f clearly aggravated the goblet cell hyperplasia, increases in TGF- β 1 production, and the development of subepithelial fibrosis, which were almost as much as those in the high dose of Der f-injected mice (Fig. 5D and Table 2). The administration of DEP at a dose of 30 and 100 μ g showed no effect on the epithelial changes (Fig. 5C and Table 2) and only slightly affected subepithelial fibrosis compared with PBS-injected mice (Fig. 5G and Table 2). These results were confirmed by the findings in the fibrotic area around the airways and the amount of hydroxyproline in the right lung tissues (Table 2).

Discussion

Bronchial asthma is a chronic airway inflammatory disease that is characterized by eosinophilic inflammation, bronchial hyperresponsiveness, and airway remodeling in pathophysiological studies (23). Characteristics of structured changes of airway remodeling include goblet cell hyperplasia/hypertrophy, subepithelial fibrosis, and smooth muscle hyperplasia/hypertrophy (24 – 26). Although these structural changes have been considered to be characteristics of chronic and severe asthma, the

Fig. 5. Histological analysis of lung sections stained with PAS (A – D) and with Masson-trichrome (E – H) 48 h after the final antigen challenge ($\times 100$) in BALB/c mice (Protocol B). A and E: PBS-injected mice; B and F: 4 μ g Der f-injected mice; C and G: 100 μ g DEP-injected mice; D and H: 4 μ g Der f + 100 μ g DEP-injected mice.

recent clinical studies, using bronchial biopsy sampling, have demonstrated that they may exist even in patients

Table 2. Effect of DEP on allergen-induced airway remodelling in BALB/c mice (Protocol B)

Treatment	Goblet cell hyperplasia (arbitrary units)	Fibrotic area (area/BM)	TGF- β 1 (pg/ml)	Hydroxyproline (μ g/lung)
PBS	0.3 $\pm$ 0.1	4.6 $\pm$ 0.8	32.3 $\pm$ 2.8	98.7 $\pm$ 3.8
Der f 4 μ g	2.3 $\pm$ 0.3*	11.6 $\pm$ 1.1***	57.9 $\pm$ 12.7	120.4 $\pm$ 6.2
Der f 20 μ g	3.8 $\pm$ 0.1***	18.2 $\pm$ 1.0***	367.0 $\pm$ 87.7**	179.4 $\pm$ 11.7**
DEP 30 μ g	0.4 $\pm$ 0.1	8.7 $\pm$ 0.5**	36.9 $\pm$ 4.5	97.0 $\pm$ 7.2
DEP 100 μ g	0.7 $\pm$ 0.2	11.0 $\pm$ 1.3***	34.6 $\pm$ 3.2	139.5 $\pm$ 11.4**
Der f 4 μ g + DEP 30 μ g	3.7 $\pm$ 0.1 ^{###,†}	14.2 $\pm$ 0.7 ^{###}	126.1 $\pm$ 22.4 ^{##}	175.9 $\pm$ 14.6 ^{###,††}
Der f 4 μ g + DEP 100 μ g	3.9 $\pm$ 0.0 ^{###,†††}	17.1 $\pm$ 1.5 ^{##,††}	286.4 $\pm$ 56.4 ^{###,††}	185.8 $\pm$ 11.0 ^{#,††}

Values represent the mean $\pm$ S.E.M. of 8 – 9 mice in each group. * P < 0.05, ** P < 0.01, *** P < 0.001 (vs. PBS); [†] P < 0.05, ^{††} P < 0.01, ^{†††} P < 0.001 (vs. Der f 4 μ g); [#] P < 0.05, ^{##} P < 0.01, ^{###} P < 0.001 (vs. DEP group).

with mild asthma and early in the disease process. Roche et al. reported that the deposition of collagen beneath the bronchial epithelium was also observed in young patients with mild atopic asthma (25), and Boulet et al. demonstrated that the degree of subepithelial collagen deposition in patients with recently diagnosed mild asthma was not significantly different from those of long-standing mild asthma (27). Therefore, comprehending the mechanisms underlying airway remodeling is becoming increasingly important, and concurrent development of new anti-remodeling agents is strongly desired.

In the present study, we investigated the effect of DEP on development of Der f-induced airway remodeling in a murine model of allergic asthma. First, we administered DEP to mice during Der f injection. As a result, DEP aggravated Der f-induced increases in airway responsiveness to acetylcholine, the number of eosinophils and neutrophils in BALF, serum Der f-specific IgG1 levels, Th2 cytokines and TGF- β 1 levels in BALF, and amount of hydroxyproline in the right lungs. Furthermore, goblet cell hyperplasia and subepithelial fibrosis were also aggravated. Surprisingly, similar results were observed by co-administration of DEP with Der f at the sensitization on day 0 and day 1. These findings demonstrate that DEP can aggravate not only airway eosinophilic inflammation but airway remodeling, probably through its adjuvant effect on allergen presentation and/or on the alteration of allergen-specific immune responses. To our knowledge, this is the first report that DEP dramatically augment Der f-induced subepithelial fibrosis associated with the increases in the level of TGF- β 1 in BALF and that the existence of DEP at the sensitization is required and enough to enhance asthma-like phenotypes subsequently.

In the present study, the treatment with DEP during Der f injection aggravated the increased number of eosinophils in BALF compared with the Der f-alone injection group. In addition, IL-5 and eotaxin production in BALF of the DEP + Der f group were higher than that in the Der f-alone group. Eotaxin is produced by airway epithelial cells, smooth muscle cells, and fibroblasts, and the production is known to be enhanced by Th2 cytokines like IL-13. Therefore, the increased level of Th2 cytokine depicted in Figs 2 and 5 could enhance the production of eotaxin. Furthermore, Sadakane et al. (28) demonstrated that Der f + DEP markedly increased the levels of RANTES, eotaxin, and GM-CSF compared to those of Der f alone. These findings suggest that the aggravation of the increased number of eosinophils in the BALF may explain the increase in Th2 cytokine levels and chemokine production.

Moreover, DEP aggravated Der f-induced AHR. The precise mechanisms underlying allergen-induced AHR still remain unclear; however, recent studies have dem-

onstrated that the AHR is dependent on Th2 cytokine using IL-4 gene-knockout mice (29, 30) and IL-13 gene-knockout mice (31). Taken together, these observations indicate that Th2 cytokines play important roles in the development of allergen-induced AHR. Therefore, in the present study, the increases in the production of Th2 cytokine in the BALF may cause the aggravated AHR by DEP injection.

In the Der f + DEP-treated group, the development of airway remodeling was aggravated compared with that in the Der f-alone group. In vitro, the exposure of human bronchial epithelial cells to DE induced the expression of TGF- β 1 mRNA. Moreover, the gas obtained by filtration of DE alone did not show the induction of TGF- β 1 mRNA (32). Thus, DEP are thought to play an important role in the induction of this mRNA in DE. Furthermore, the production of TGF- β 1 is partially dependent on Th2 responses (18, 30) and/or eosinophils (33, 34). These findings suggest that the synergistic effect of the action of DEP upon bronchial epithelial cells and antigen-induced Th2 responses cause the increase of TGF- β 1 levels in the BALF, leading to the aggravation of allergen-induced subepithelial fibrosis.

In the present study, DEP aggravated Der f-induced airway eosinophilic inflammation and remodeling regardless of the timing of administration (Fig. 1: Protocol A vs. Protocol B), and the magnitude of each parameter was comparable. So far, various experimental studies have suggested that DEP can exhibit adjuvant activity for allergen-induced airway inflammation (13–16) and that antioxidants can inhibit the adjuvant effect of DEP (35). In addition, Bommell et al. had reported that pyrene, a major compound of DEP, may promote allergic diseases by inducing the production of IL-4 (36). Furthermore, a decrease in IFN- γ expression induced by allergen plus DEP injection had also been reported (37). DEP could have the ability to bind allergen and act as carriers of several allergens (38). These findings suggest that DEP have potencies not only to induce airway inflammation by themselves but also to augment the efficacy of antigen presentation (39) by acting as carriers. However, in contrast to the results from Protocol A, DEP were administered with allergen only on day 0 and day 1 in Protocol B, and mice were continuously exposed to low dose of allergen. Therefore, DEP may epigenetically affect the Th2-polarizing pathway as reported in an in vivo study (40), although further investigation is needed to clarify the precise mechanisms underlying this phenotype.

In conclusion, we have demonstrated that DEP aggravated AHR as well as the number of inflammatory cells and enhanced Th2 cytokines and TGF- β 1 levels in BALF, serum antigen-specific IgG1 levels, and amount of hydroxyproline in the right lungs. Furthermore, goblet

cell hyperplasia and subepithelial fibrosis were also aggravated. These results suggest that that DEP can exhibit adjuvant activity not only for allergen-induced AHR or airway eosinophilic inflammation, but also for airway remodeling, probably due to the enhancement of allergen sensitization and/or of Th2 polarizing pathways.

Acknowledgements

We would like to thank Drs. Kazuhito Maejima and Hiroki Okumura (Japan Automobile Research Institute) for providing DEP and their helpful comments during this study and thank Dr. Sachithanantham Srikantha for skilful assistance in preparation of this manuscript. This work was supported in part by a Ministry of Health, Labour, and Welfare (Japan), Health and Labour Science Research Grant, Research on Allergic Disease and Immunology (to H.T.). This study was also supported in part by a donation from the late Professor Emeritus, Akihiko Koda, for which we are very grateful.

References

- Mannino DM, Homa DM, Akinbami LJ, Moorman JE, Gwynn C, Redd SC. Surveillance for asthma—United states, 1980–1999. *MMWR Surveill Summ*. 2002;51:1–13.
- Robertson CF, Roberts MF, Kappers JH. Asthma prevalence in Melbourne schoolchildren: Have we reached the peak? *Med J Aust*. 2004;180:273–276.
- Verlato G, Corsico A, Villani S, Cerveri I, Migliore E, Accordini S, et al. Is the prevalence of adult asthma and allergic rhinitis still increasing? Results of an Italian study. *J Allergy Clin Immunol*. 2003;111:1232–1238.
- Huss K, Adkinson NF Jr, Eggleston PA, Dawson C, Van Natta ML, Hamilton RG. House dust mite and cockroach exposure are strong risk factors for positive allergy skin test responses in the childhood asthma management program. *J Allergy Clin Immunol*. 2001;107:48–54.
- Platts-Mills TA, Vervloet D, Thomas WR, Aalberse RC, Chapman MD. Indoor allergens and asthma: Report of the third international workshop. *J Allergy Clin Immunol*. 1997;100:S2–S24.
- Sporik R, Holgate ST, Platts-Mills TA, Cogswell JJ. Exposure to house-dust mite allergen (der p i) and the development of asthma in childhood. A prospective study. *N Engl J Med*. 1990;323:502–507.
- Schwartz J, Slater D, Larson TV, Pierson WE, Koenig JQ. Particulate air pollution and hospital emergency room visits for asthma in Seattle. *Am Rev Respir Dis*. 1993;147:826–831.
- Romieu I, Meneses F, Ruiz S, Sienna JJ, Huerta J, White MC, et al. Effects of air pollution on the respiratory health of asthmatic children living in Mexico city. *Am J Respir Crit Care Med*. 1996;154:300–307.
- Goldsmith CA, Kobzik L. Particulate air pollution and asthma: a review of epidemiological and biological studies. *Rev Environ Health*. 1999;14:121–134.
- McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, et al. Respiratory effects of exposure to diesel traffic in persons with asthma. *N Engl J Med*. 2007;357:2348–2358.
- Morgenstern V, Zutavern A, Cyrys J, Brockow I, Koletzko S, Kramer U, et al. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am J Respir Crit Care Med*. 2008;177:1331–1337.
- Muranaka M, Suzuki S, Koizumi K, Takafuji S, Miyamoto T, Ikemori R, et al. Adjuvant activity of diesel-exhaust particulates for the production of IgE antibody in mice. *J Allergy Clin Immunol*. 1986;77:616–623.
- Miyabara Y, Takano H, Ichinose T, Lim HB, Sagai M. Diesel exhaust enhances allergic airway inflammation and hyperresponsiveness in mice. *Am J Respir Crit Care Med*. 1998;157:1138–1144.
- Ohta K, Yamashita N, Tajima M, Miyasaka T, Nakano J, Nakajima M, et al. Diesel exhaust particulate induces airway hyperresponsiveness in a murine model: essential role of gm-csf. *J Allergy Clin Immunol*. 1999;104:1024–1030.
- Takafuji S, Suzuki S, Koizumi K, Tadokoro K, Miyamoto T, Ikemori R, et al. Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for ige production in mice. *J Allergy Clin Immunol*. 1987;79:639–645.
- Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, Sagai M. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. *Am J Respir Crit Care Med*. 1997;156:36–42.
- Hirose I, Tanaka H, Takahashi G, Wakahara K, Tamari M, Sakamoto T, et al. Immunomodulatory effects of CpG oligodeoxynucleotides on house dust mite-induced airway inflammation in mice. *Int Arch Allergy Immunol*. 2008;147:6–16.
- Wakahara K, Tanaka H, Takahashi G, Tamari M, Nasu R, Toyohara T, et al. Repeated instillations of *Dermatophagoides farinae* into the airways can induce th2-dependent airway hyperresponsiveness, eosinophilia and remodeling in mice: Effect of intratracheal treatment of fluticasone propionate. *Eur J Pharmacol*. 2008;578:87–96.
- Sagai M, Saito H, Ichinose T, Kodama M, Mori Y. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radic Biol Med*. 1993;14:37–47.
- Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y, et al. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res*. 2001;50:616–624.
- Kivirikko KI, Laitinen O, Prockop DJ. Modifications of a specific assay for hydroxyproline in urine. *Anal Biochem*. 1967;19:249–255.
- Nagai H, Maeda Y, Tanaka H. The effect of anti-il-4 monoclonal antibody, rapamycin and interferon-gamma on airway hyperactivity to acetylcholine in mice. *Clin Exp Allergy*. 1997;27:218–224.
- Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol*. 2004;22:789–815.
- Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest*. 1992;101:916–921.
- Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet*. 1989;1:520–524.
- Heard BE, Hossain S. Hyperplasia of bronchial muscle in asthma. *J Pathol*. 1973;110:319–331.
- Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, et al. Airway hyperresponsiveness, inflammation, and

- subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med*. 2000;162:1308–1313.
- 28 Sadakane K, Ichinose T, Takano H, Yanagisawa R, Sagai M, Yoshikawa T, et al. Murine strain differences in airway inflammation induced by diesel exhaust particles and house dust mite allergen. *Int Arch Allergy Immunol*. 2002;128:220–228.
- 29 Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol*. 1995;12:254–259.
- 30 Komai M, Tanaka H, Masuda T, Nagao K, Ishizaki M, Sawada M, et al. Role of th2 responses in the development of allergen-induced airway remodelling in a murine model of allergic asthma. *Br J Pharmacol*. 2003;138:912–920.
- 31 Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, et al. Critical role for il-13 in the development of allergen-induced airway hyperreactivity. *J Immunol*. 2001;167:4668–4675.
- 32 Abe S, Takizawa H, Sugawara I, Kudoh S. Diesel exhaust (de)-induced cytokine expression in human bronchial epithelial cells: a study with a new cell exposure system to freshly generated de in vitro. *Am J Respir Cell Mol Biol*. 2000;22:296–303.
- 33 Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, et al. Inhibition of airway remodeling in il-5-deficient mice. *J Clin Invest*. 2004;113:551–560.
- 34 Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwaru D, Takatsu K, et al. Role of interleukin-5 and eosinophils in allergen-induced airway remodeling in mice. *Am J Respir Cell Mol Biol*. 2004;31:62–68.
- 35 Whitekus MJ, Li N, Zhang M, Wang M, Horwitz MA, Nelson SK, et al. Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J Immunol*. 2002;168:2560–2567.
- 36 Bommel H, Li-Weber M, Serfling E, Duschl A. The environmental pollutant pyrene induces the production of il-4. *J Allergy Clin Immunol*. 2000;105:796–802.
- 37 Diaz-Sanchez D, Tsien A, Fleming J, Saxon A. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a t helper cell 2-type pattern. *J Immunol*. 1997;158:2406–2413.
- 38 Ormstad H, Johansen BV, Gaarder PI. Airborne house dust particles and diesel exhaust particles as allergen carriers. *Clin Exp Allergy*. 1998;28:702–708.
- 39 Inoue K, Koike E, Takano H, Yanagisawa R, Ichinose T, Yoshikawa T. Effects of diesel exhaust particles on antigen-presenting cells and antigen-specific th immunity in mice. *Exp Biol Med (Maywood)*. 2009;234:200–209.
- 40 Liu J, Ballaney M, Al-alem U, Quan C, Jin X, Perera F, et al. Combined inhaled diesel exhaust particles and allergen exposure alter methylation of t helper genes and ige production in vivo. *Toxicol Sci*. 2008;102:76–81.