

Platelet-derived growth factor is involved in the augmentation of airway responsiveness through remodeling of airways in diesel exhaust particulate-treated mice

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Background: Thickening of the region adjacent to the basement membrane is a key component of the remodeling of the asthmatic airway and is caused by collagen deposition in the region.

Objective: We sought to clarify the role of platelet-derived growth factor (PDGF), a competence factor of fibroblast, in the enhanced airway responsiveness and remodeling in a murine model.

Methods: Diesel exhaust particulates (DEPs) were administered intranasally every other day for 2 weeks with or without anti-PDGF- β neutralizing antibody or goat IgG. Pulmonary function was then analyzed by using whole-body plethysmography before and after acetylcholine inhalation.

Results: Anti-PDGF- β neutralizing antibody significantly inhibited both the elevation of airway resistance elicited by 1.25 and 2.5 mg/mL acetylcholine and the increase in the airway wall thickening induced by DEPs. In addition, bronchoalveolar lavage fluid cell analysis revealed that anti-PDGF- β neutralizing antibody did not affect cellular infiltration at the airways.

Conclusion: PDGF plays an important role in the process of remodeling brought about by DEP exposure in mice. (*J Allergy Clin Immunol* 2001;107:135-42.)

Key words: Asthma, airway hyperresponsiveness, platelet-derived growth factor, airway remodeling, diesel exhaust particulate, acetylcholine

Thickening of the airway walls is a conspicuous pathologic feature among patients with asthma who have died.^{1,2} Information from bronchoalveolar lavage fluid (BALF) has shown that to a substantial degree, airway wall thickening (AWT) is associated with various stages of asthma, accompanying allergic inflammation and bronchial epithelial cell damage.³⁻⁵ Although airway remodeling begins as a healing process in response to epithelial injury in asthmatic patients, it often leads to

Abbreviations used

AWT:	Airway wall thickening
BALF:	Bronchoalveolar lavage fluid
DEP:	Diesel exhaust particulate
ECM:	Extracellular matrix
PDGF:	Platelet-derived growth factor
Raw:	Airway resistance

unwanted structural changes, including AWT, subepithelial fibrosis, and mucus cell hyperplasia, which act as irreversible factors in severe asthma.⁶⁻⁹

Much evidence suggests that AWT in asthma involves production of extracellular matrix (ECM) components, including collagen, by myofibroblasts.^{10,11} Platelet-derived growth factor (PDGF), which is a competence factor of fibroblast proliferation,¹² is reported to play a key role in such fibrotic changes in the lung as pulmonary fibrosis.¹³⁻¹⁵ In one study transgenic mice expressing the *PDGFB* gene driven by the lung-specific surfactant protein promoter were shown to develop significant lung fibrosis.¹⁶ In addition, eosinophils from asthmatic patients are known to produce the PDGF- β chain,¹⁷ and PDGF synthesis is augmented in asthmatic patients in BALF and bronchial epithelial cells.¹⁸ However, effects of PDGF on an asthmatic model in vivo remain unknown.

In the present study we used a murine model to clarify the role of PDGF and the relationship between AWT and airway hyperresponsiveness in vivo. To augment airway hyperresponsiveness, we used diesel exhaust particulates (DEPs), which are derived from diesel automobiles and trucks and are suggested to be responsible for airway inflammation.¹⁹⁻²¹ We previously showed that DEPs increased airway responsiveness to acetylcholine²² at the dose achievable in high ambient exposure or occupational exposure.²¹ The airway hyperresponsiveness persisted for as long as 13 days after termination of DEP exposure and was associated with morphologic changes in the bronchial epithelial cells.²² We have now evaluated whether airway remodeling occurs in this model and whether the remodeling is involved in acetylcholine-induced airway hyperrespon-

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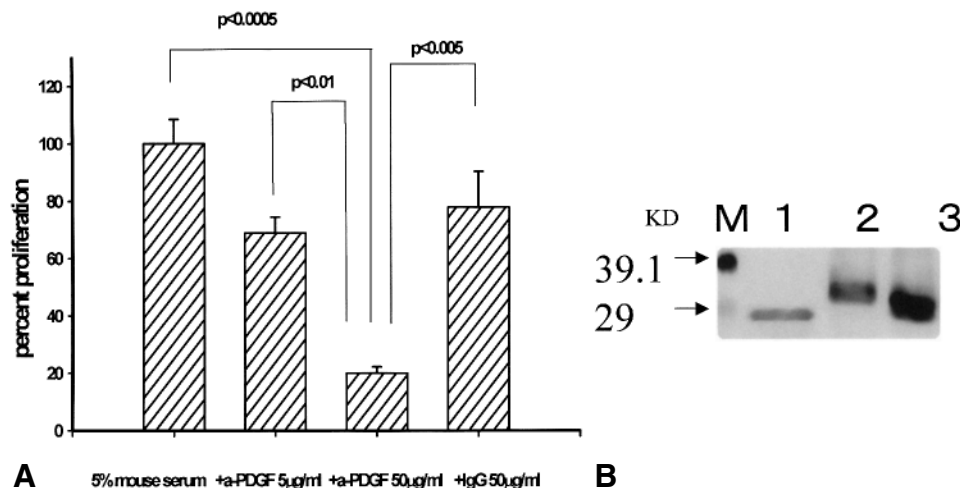


FIG 1. Effects of anti-human PDGF-neutralizing antibody on murine PDGF. **A**, Effects of anti-human PDGF on murine fibroblast proliferation. BALB/C/3T3 fibroblasts were cultured with or without anti-human PDGF-neutralizing antibody or goat IgG control in the presence of 5% mouse serum. The data were expressed as the proliferation only with mouse serum as 100%. The data were expressed as means $\pm$ SEM of 4 samples of triplicate determination in each group. **B**, Western blot analysis. Human (lane 2) and mouse (lane 3) serum was incubated with goat anti-PDGF-B neutralizing antibody (Becton Dickinson), followed by immunoprecipitation with protein G agarose. Then the sample was applied for Western blot analysis by using rabbit anti-PDGF-B antibody, which reacts with rat, human, and mouse PDGF, as described in the "Methods" section. M, Molecular weight marker; line 1, recombinant rat PDGF-BB; line 2, human serum immunoprecipitates; line 3, mouse serum immunoprecipitates. Data shown are representative of 3 similar experiments.

siveness. We found that anti-PDGF- β neutralizing antibody inhibited DEP-induced AWT and hyperresponsiveness to acetylcholine, suggesting that AWT is directly related to airway hyperresponsiveness and that by stimulating deposition of ECM beneath the basement membrane, PDGF plays a crucial role.

METHODS

Reagents

DEPs were kindly provided by Dr Fujimaki (National Institute for Environmental Studies) after being collected on glass fiber filters placed at the end of a dilution tunnel connected to a 4-cylinder diesel engine (model 4JB1; Isuzu Automobile Company, Tokyo, Japan).²³ It was 3 years old and still potent, as shown in the study by Ushio et al.²⁴ As measured with an Anderson Air Sampler, the mean diameter of the particles was 0.4 μ m, and their carbon content was 26% of the total concentration.²³ Goat anti-human PDGF-neutralizing antibody, which reacts with the PDGF-B subunit, was purchased from Becton Dickinson, Mountain View, Calif; rabbit anti-PDGF antibody used for immunostaining was from Santa Cruz Biotech Inc (Santa Cruz, Calif) and reacts with human, rat, and murine PDGF-B chains. Goat IgG was from Chemicon International, Inc (Temecula, Calif), pentobarbital sodium was from Pitman More Inc (Mundelein, Ill), pancuronium bromide was from Organ (Teknika, the Netherlands), and acetylcholine chloride was from Wako Chemicals (Tokyo, Japan).

Cell proliferation assay

BALB/3T3 cells (ATCC CCL163) were plated in 96-well plates and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, Md) supplemented with 10% FCS (Gibco).

Once the monolayers had grown to confluence, the Dulbecco's modified Eagle's medium was replaced with HD-F12 supplemented with 5% mice serum plus either goat anti-PDGF-B neutralizing antibody (Becton Dickinson) or control IgG. After incubation for 72 hours, cell proliferation was assessed by using alamar blue (BioSource International, Camarillo, Calif), a fluorometric-colorimetric redox indicator of metabolic activity. After adding 1:10 volume of alamar blue to each well, the plates were incubated for 3 hours, and absorbance was measured at 570 and 600 nm. The assay was carried out in triplicate.

Western blot analysis

Mouse and human sera were first precleared by using protein A columns (Pharmacia Biotech, Wilstrom, Sweden) followed by incubation with goat anti-PDGF-B antibody (Becton Dickinson) and protein G microbeads (Miltenyi Biotec, Gladbach, Germany) for 30 minutes. Subsequently, immunoprecipitates were separated in the magnetic field of a μ MACS separator. The immunoprecipitates were eluted with SDS sample buffer, after which they were separated by SDS-PAGE and electrotransferred onto Hybond-P membranes (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The membranes were then blocked in buffer containing 4% BSA and incubated first with rabbit anti-PDGF-B (Santa Cruz) and then with horseradish peroxidase-conjugated F(ab')₂ donkey anti-rabbit antibody (1:2000, Amersham Pharmacia Biotech Inc). The immunoblotted proteins were visualized by using an enhanced chemiluminescence kit from Amersham Pharmacia Biotech Inc.

Sensitization of mice

Pathogen-free, male A/J mice (10-12 weeks old), whose airways are inherently hyperresponsive to acetylcholine,^{25,26} were purchased from Charles River (Sulzfeld, Germany). DEPs were resuspended in saline solution with continuous circulation for 30 minutes

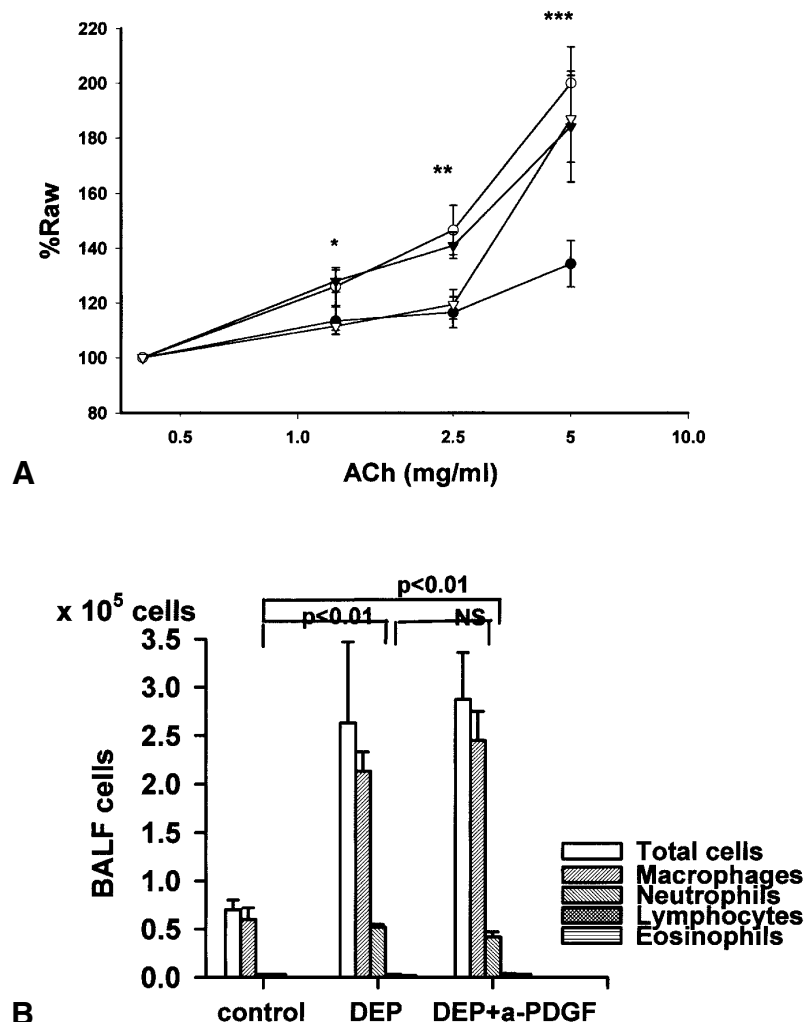


FIG 2. Effects of neutralization of PDGF on murine model. **A**, Effects of neutralization of PDGF on bronchoconstriction induced with acetylcholine (ACh) inhalation. Acetylcholine-evoked changes in Raw are expressed as a percentage of the Raw observed before acetylcholine inhalation (100%). Data shown are means $\pm$ SEM for 8 mice in each group. *Filled circles*, Control mice with intranasal administration of saline only; *open circles*, mice treated with DEPs intranasally for 2 weeks; *filled triangles*, mice treated with both DEPs and sham IgG (goat IgG); *open triangles*, mice treated with both DEPs and anti-PDGF. ***Control versus DEPs, DEPs plus IgG, and DEPs plus anti-PDGF: $P < .01$. **Control and DEPs plus anti-PDGF versus DEPs and DEPs plus IgG: $P < .01$. *Control and DEPs plus anti-PDGF versus DEPs and DEPs plus IgG: $P < .05$. **B**, BALF cell analysis. *Control*, mice with intranasal administration of saline only; *DEP*, mice treated with DEPs; *DEP + anti-PDGF*, mice treated with both DEPs and anti-PDGF. The data shown are means $\pm$ SEM of 8 mice in each group.

before administration. The mice were intranasally administered 0.25 mg/mL DEP in 40 μ L of saline every other day for 2 weeks, as previously reported.²² This dose of DEPs reflects high ambient exposure and is relevant to the dose used in human studies.^{20,21} The control mice were administered saline solution alone. To assess the role of PDGF, anti-PDGF-B neutralizing antibody (50 μ L of 1 mg/mL stock) was administered intranasally immediately before each administration of DEPs, or an identical volume and concentration of goat IgG was administered to sham control animals.

Assessment of airway responsiveness

Under pentobarbital-induced anesthesia, mice were administered pancuronium bromide, after which they were thoracotomized and placed on a Harvard ventilator operating with a 0.3-mL tidal volume

and a respiratory frequency of 120 breaths/min. The mice were then placed into whole-body plethysmographs (Buxo Electronics, Inc, Troy, NY) to measure airway resistance (Raw). Increasing doses of acetylcholine (1.25–5.0 mg/mL) was administered intranasally by ultranebulization for 3 minutes. Data are expressed as percentage of Raw, which is defined by the following formula:

$$(\text{Raw after inhalation of acetylcholine} / \text{Raw before inhalation}) \times 100 (\%)$$

BALF cell analysis and histologic examination

After measuring pulmonary function, BALF was obtained by washing the lungs with 1 mL of saline 5 times. The BALF cells were pelleted and resuspended in 1 mL of saline and then counted.

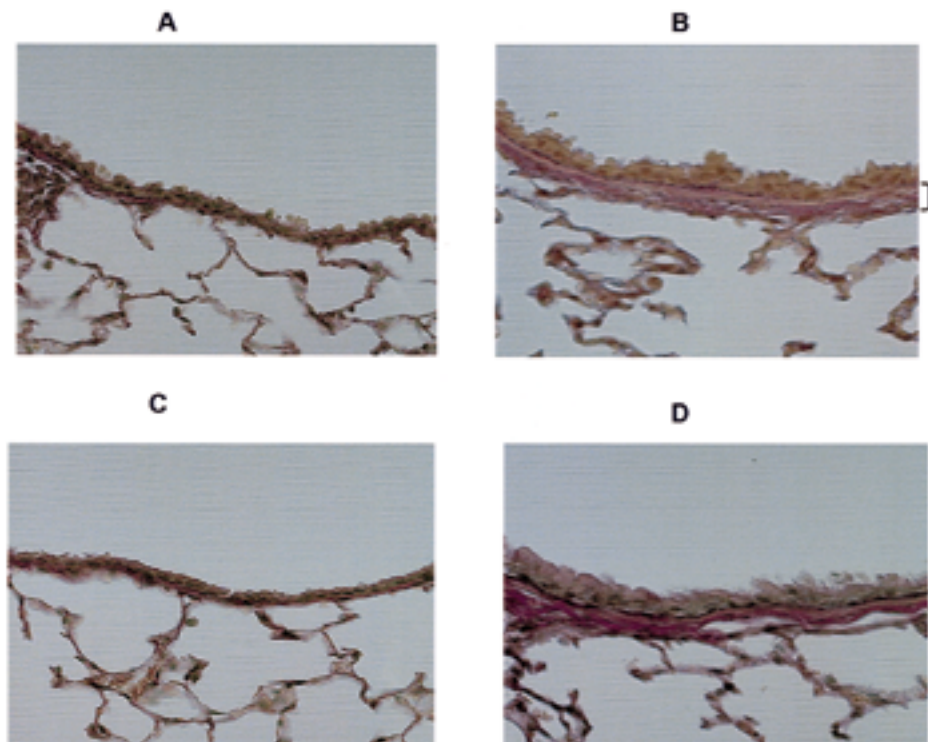


FIG 3. Histologic analysis of lung stained with Elastica van Gieson stain. **A**, Tissue specimen from an untreated control mouse; **B**, tissue specimen from a DEP-treated mouse; **C**, tissue specimen from a mouse treated with both DEPs and anti-PDGF; **D**, tissue specimen from a mouse treated with both DEPs and sham IgG (goat IgG). Increasing elastic tissues are indicated by brackets.

BALF cells were pelleted onto glass slides by using a cytospin centrifuge. Differential leukocyte counts were made by counting 200 cells on cytospin preparation stained with Diff-Quik (Kokusai Shiyaku, Tokyo, Japan) by light microscopy using standard morphologic criteria.

The lungs were then fixed in 20% formaldehyde, inflated under 10 cm of H₂O pressure, and stained with Elastica van Gieson stain. Thickening beneath the basement membrane was analyzed by using an image analyzer and standardized. The AWT index was calculated as follows: (External perimeter – Inner perimeter)/Inner perimeter.

Collagen deposition was assessed indirectly from the production of hydroxyproline. To measure hydroxyproline, the excised lung was washed in saline solution and freeze-dried at –70°C. The hydroxyproline content was then determined in triplicate by using the method of Woessner,²⁷ as previously described.²⁸

Immunohistochemistry

BALF cells were pelleted onto glass slides by using a cytospin centrifuge and then fixed for 15 minutes in ice-cold acetone. Paraffin tissue section was deparaffinized and rehydrated. Samples were treated with Target Retrieval (Dako Corp, Glodstrup, Denmark) at 95°C, blocked at room temperature by using Protein Block Serum-Free (Dako), and incubated with either rabbit anti-human PDGF-B (Santa Cruz) or rabbit IgG (control). Antigen-antibody complexes were detected by using a streptavidin-biotin-alkaline phosphatase technique (InnoGenex, San Ramon, Calif), followed by visualization with Fast Red alkaline phosphatase substrate. Endogenous alkaline phosphatase was removed by adding levamisole (Dako) at the time of color development.

Statistics

Data are expressed as the mean ± SEM. Comparisons of multiple parameters were made by using ANOVA. A *P* value of less than .05 was considered statistically significant.

RESULTS

Inhibitory effects of anti-PDGF-B neutralizing antibody on proliferation of mouse 3T3 fibroblast

Our anti-PDGF-B neutralizing antibody was originally raised against the human PDGF-B subunit. We therefore initially determined whether the antibody would cross-react with mouse PDGF-B. 3T3 fibroblasts, which were used for this assay because they are known to require PDGF for proliferation,^{29,30} were incubated for 72 hours in medium containing 5 or 50 µg/mL anti-PDGF-B neutralizing antibody or with 50 µg/mL IgG, after which proliferation was assessed. That our anti-human PDGF-B neutralizing antibody did indeed neutralize mouse PDGF-B was confirmed by the observation that proliferation of 3T3 fibroblast was almost inhibited in the presence of 50 µg/mL anti-PDGF-B, whereas the same concentration of IgG had little or no effect (Fig 1, A).

We also confirmed that the anti-human PDGF-B neutralizing antibody used cross-reacted with mouse PDGF-

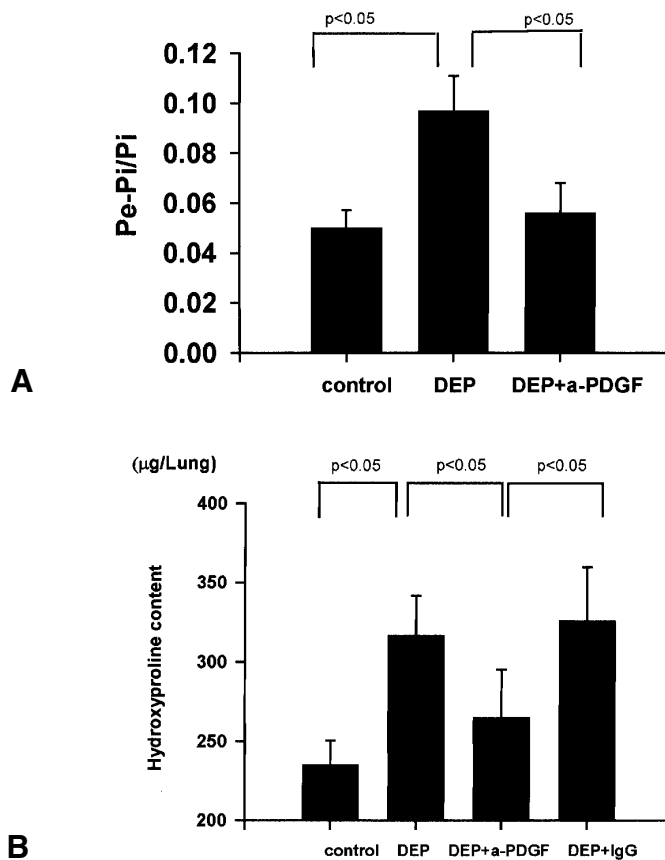


FIG 4. Effects of neutralization of PDGF on AWT. **A**, AWT index in mice. Samples stained with Elastica van Gieson stain were analyzed by using an image analyzer. AWT index was calculated as follows: external perimeter – internal perimeter/internal perimeter of bronchioles. *Control*, Control mice with intranasal administration of saline only; *DEP*, mice treated with DEPs; *DEP + a-PDGF*, mice treated with both DEPs and anti-PDGF. Data are means $\pm$ SEM of 6 bronchioles from at least 3 different mice in each group. **B**, Hydroxyproline content in the lung. *Control*, Mice with intranasal administration of saline only; *DEP*, mice treated with DEPs; *DEP + a-PDGF*, mice treated with both DEPs and anti-PDGF; *DEP + IgG*, mice treated with goat IgG with DEP exposure. Data are means $\pm$ SEM of 5 mice in each group.

B by means of immunoprecipitation with the antibody. Mouse serum treated with the anti-PDGF-B antibody showed a 30-kd band of PDGF-B (Fig 1, *B*, lane 3), as well as human serum treated with the anti-PDGF-B antibody (Fig 1, *B*, lane 2) and recombinant rat PDGF-BB (Fig 1, *B*, lane 1). These data indicated that the anti-human PDGF-B neutralizing antibody we used really cross-reacts with mouse PDGF-B.

Analysis of airway hyperresponsiveness

Baseline Raw was not different among untreated mice and mice exposed to DEPs for 2 weeks before analysis. On inhalation of 1.25 or 2.5 mg/mL acetylcholine, however, DEP-treated mice exhibited higher Raw than control mice ($P < .05$ and $P < .01$, respectively; Fig 2, *A*), which is consistent with earlier reports.²²

To determine whether PDGF activity played a role in the DEP-induced hyperreactivity to acetylcholine, anti-

PDGF-B neutralizing antibody was administered intranasally just before each administration of DEPs. The airway response to acetylcholine was then measured 24 hours after the final administration of antibody and DEPs. At an acetylcholine concentration of 5 mg/mL, mice treated with either DEPs, DEPs plus goat IgG, or DEPs plus anti-PDGF-B antibody exhibited larger evoked increases in Raw than control mice ($P < .01$; Fig 2, *A*). However, the airway responses elicited by 2.5 or 1.25 mg/mL acetylcholine were significantly attenuated by the anti-PDGF-B antibody, suggesting that PDGF-B does play a role in the increased airway responsiveness to acetylcholine ($P < .05$; Fig 2, *A*). Sham IgG did not affect the response induced by 2.5 or 1.25 mg/mL acetylcholine (Fig 2, *A*). These data suggested that the treatment with anti-PDGF-B neutralizing antibody inhibited the bronchoconstriction of small airways induced by acetylcholine.

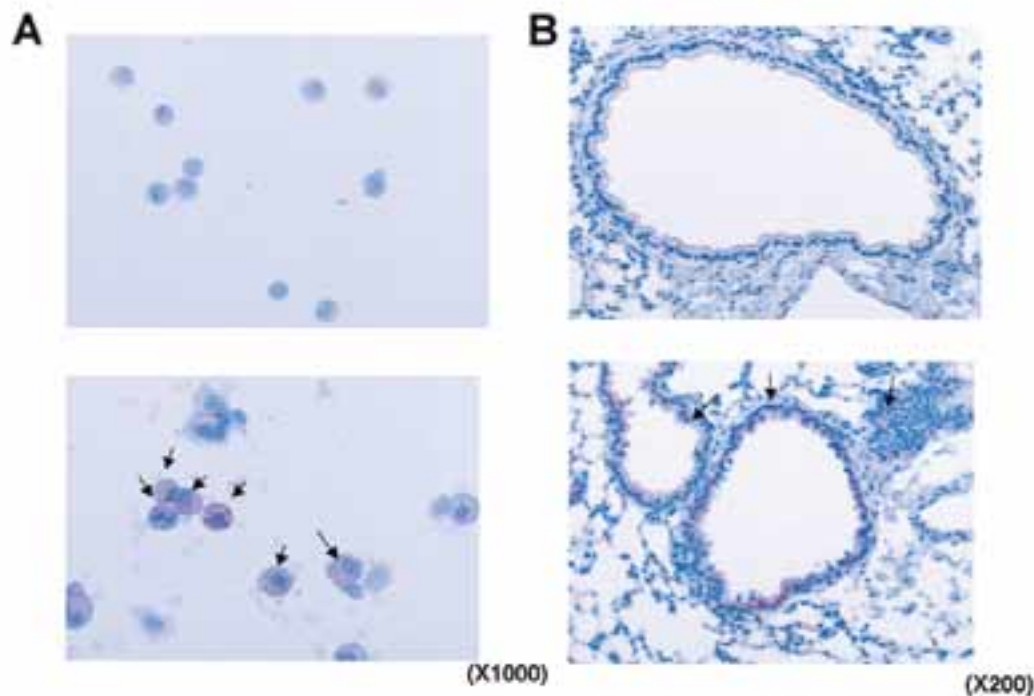


FIG 5. Immunostaining of PDGF. **A**, PDGF expression on BALF cells. PDGF protein expression was examined by immunostaining of BALF cells with rabbit anti-PDGF-B antibody. *Upper panel*, Specimen from control mice; *lower panel*, specimen from DEP-treated mice. **B**, PDGF expression on lung tissue. PDGF protein expression was examined by immunostaining of lung tissue with rabbit anti-PDGF-B antibody. *Upper panel*, Specimen from control mice; *lower panel*, specimen from DEP-treated mice. Arrows indicate positive cells for immunostaining.

BALF cell analysis

As reported previously, DEP treatment increased the total number of cells present in the BALF, which consisted mainly of macrophages (Fig 2, *B*). The increase in the number of BALF cells was unaffected by administration of anti-PDGF-B antibody, indicating that the inhibitory effects of the antibody on airway responsiveness to acetylcholine cannot be ascribed to a diminished airway inflammatory response.

Histopathologic analysis

Elastica van Gieson staining revealed that DEP treatment induced a thickening of the airway wall, with deposition of elastic fibers (Fig 3, *B* and *D*). Furthermore, as compared with mice exposed to DEPs alone and DEPs plus sham IgG (Fig 3, *B* and *D*), treating mice with anti-PDGF-B antibody diminished deposition of elastic fibers in the area beneath the epithelial cells (Fig 3, *C*).

To examine the AWT more quantitatively, we analyzed the AWT index, which enabled us to evaluate wall thickness independently of overall airway size.³¹ As shown in Fig 4, *A*, the AWT index in DEP-treated mice was significantly greater than that in control mice, but administration of anti-PDGF-B antibody completely blocked the DEP-induced increase in wall thickness (DEPs vs DEPs plus anti-PDGF in Fig 4, *A*).

Consistent with the increased wall thickness, DEPs increased the hydroxyproline content of the lung, suggesting the increased thickening was due at least in part to collagen deposition (Fig 4, *B*). Like wall thickness, hydroxyproline content was inhibited by administration of anti-PDGF-B antibody and was not affected by administration of goat IgG, the control immunoglobulin.

Immunostaining of PDGF-B

By immunolabeling BALF cells with anti-PDGF-B antibody, we identified macrophages to be the cells expressing the PDGF-B (Fig 5, *A*, *lower panel*). Immunohistologic analysis with anti-PDGF-B antibody revealed that airway epithelial cells treated with DEPs also produced PDGF-B (Fig 5, *B*, *lower panel*).

DISCUSSION

In this study DEP-treated A/J mice served as a model to analyze the role of PDGF in airway remodeling. Anti-PDGF-B neutralizing antibody inhibited DEP-induced increases in airway responsiveness elicited by 1.25 or 2.5 mg/mL acetylcholine and increased the airway wall thickness. Moreover, our findings indicate that there is a direct relationship between airway wall thickness and airway hyperresponsiveness.

Allergic inflammation is recognized as pathognomonic for asthma and is related to airway hyperresponsiveness.^{32,33} Nonetheless, some reports have shown that airway inflammation and hyperresponsiveness are not always well correlated.^{34,35} Several recent clinical studies have shown good correlations between airway remodeling and airway hyperresponsiveness.^{36,37}

Airway remodeling is characterized as AWT, subepithelial fibrosis, and mucus cell hyperplasia,³⁻¹¹ which act as irreversible factors in severe asthma. Focusing on subepithelial fibrosis, we examined the role of PDGF, a competence factor of fibroblasts.¹² The PDGF dimer, which occurs in 3 isoforms (AA, AB, and BB),³⁸ was first identified as a smooth muscle growth factor released from platelets.¹² It is now known that in addition to platelets, macrophages, monocytes, eosinophils, epithelial cells, and endothelial cells also produce PDGF.^{17,39} We found that PDGF was produced by alveolar macrophages and bronchial epithelial cells stimulated by DEPs (Fig 5). Although eosinophils have been reported to be a potent source for PDGF,¹⁷ we did not observe significant changes in the number of eosinophils in this model (Fig 2, B).

The usefulness of the internal perimeter as a marker of airway size and as a parameter with which to calculate smooth muscle shorting have been reported.^{31,40} To compare airway wall thickness, we used the inner perimeter to calculate an airway wall index that normalized airway size, enabling wall thickness to be analyzed independently of overall airway size. Our study revealed that PDGF reduced airway wall thickness in the small airways but did not affect airway inflammation. This suggests a direct relation between AWT and the increase in Raw induced by low concentrations of acetylcholine (1.25 and 2.5 mg/mL). At a higher concentration (5 mg/mL), effects of acetylcholine were unaffected by airway thickness and were thus more likely related to increased smooth muscle contractility. This scenario is consistent with observations made in human subjects by using bronchial tree models and in autopsy samples.^{31,40} Wiggs et al⁴⁰ have used a model of tracheobronchial tree to demonstrate that AWT of asthmatic subjects accentuate the airway narrowing induced by physiologic amounts of airway smooth muscle shorting and that the amplifying effects of encroachment and loss of recoil occur despite minimal changes in baseline resistance.

Type IV collagen and laminin deposition have been reported to be deposited in a layer beneath the basement membrane,^{3,41,42} and it is also possible that proteoglycan, which responds to PDGF, is also increased beneath the basement membrane.⁴³ We measured hydroxyproline content of the lung as an index of collagen deposition and confirmed that DEP exposure induced significant increases in hydroxyproline content, which were suppressed by the administration of anti-PDGF-B neutralizing antibody. Airway remodeling in asthma is also reported to involve thickening of the airway smooth muscle, which is also related to airway hyperresponsiveness.^{44,45} In our model increased smooth muscle mass

was not obvious because of the way of sensitization. We therefore focused only on ECM deposition beneath the basement membrane as a marker of airway remodeling.

Reversibility of airway remodeling has been intensively analyzed,^{46,47} and it appears that inhalation of corticosteroids can control airway remodeling.⁴⁶ Our study indicates that neutralization of stimulus-induced PDGF production may also help to control airway wall thickness.

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