

Coexposure to Environmental Tobacco Smoke Increases Levels of Allergen-Induced Airway Remodeling in Mice¹

Myung Goo Min,^{2*} Dae Jin Song,^{2*†} Marina Miller,* Jae Youn Cho,* Shauna McElwain,* Paul Ferguson,* and David H. Broide^{3*}

Environmental tobacco smoke (ETS) can increase asthma symptoms and the frequency of asthma attacks. However, the contribution of ETS to airway remodeling in asthma is at present unknown. In this study, we have used a mouse model of allergen-induced airway remodeling to determine whether the combination of chronic exposure to ETS and chronic exposure to OVA allergen induces greater levels of airway remodeling than exposure to either chronic ETS or chronic OVA allergen alone. Mice exposed to chronic ETS alone did not develop significant eosinophilic airway inflammation, airway remodeling, or increased airway hyperreactivity to methacholine. In contrast, mice exposed to chronic OVA allergen had significantly increased levels of peribronchial fibrosis, increased thickening of the smooth muscle layer, increased mucus, and increased airway hyperreactivity which was significantly enhanced by coexposure to the combination of chronic ETS and chronic OVA allergen. Mice coexposed to chronic ETS and chronic OVA allergen had significantly increased levels of eotaxin-1 expression in airway epithelium which was associated with increased numbers of peribronchial eosinophils, as well as increased numbers of peribronchial cells expressing TGF- β 1. These studies suggest that chronic coexposure to ETS significantly increases levels of allergen-induced airway remodeling (in particular smooth muscle thickness) and airway responsiveness by up-regulating expression of chemokines such as eotaxin-1 in airway epithelium with resultant recruitment of cells expressing TGF- β 1 to the airway and enhanced airway remodeling. *The Journal of Immunology*, 2007, 178: 5321–5328.

Several epidemiologic studies in asthmatics have reported an association between environmental tobacco smoke (ETS)⁴ exposure and asthma symptoms (1–8). In asthmatic children, parental smoking increases levels of asthma symptoms and the frequency of asthma exacerbations (1–8). The Environmental Protection Administration estimates that exposure to ETS exacerbates asthma in up to a million children (1). Exposure to ETS has been linked to several adverse asthma outcomes including increased prevalence of asthma, increased severity of asthma symptoms, increased frequency of asthma medication use, and increased emergency room visits by asthmatic children (1, 3, 5). Although the results of epidemiological studies of ETS exposure and asthma have predominantly been based on self-reported passive smoke exposure (with potential recall bias of exposure to ETS), some epidemiologic studies have also used personal nicotine monitoring, hair nicotine, and cotinine assays to provide objective measures of passive smoke exposure (2, 3, 8). In addition to these epidemiologic studies of ETS exposure and asthma, experimental

ETS challenge studies in humans indicate that passive smoke exposure has adverse effects on airflow and/or airway responsiveness in asthma (9, 10).

Although these studies provide evidence for a role for ETS in increasing asthma symptoms and the frequency of asthma exacerbations, much less is known about the ability of ETS to exacerbate airway remodeling in asthma. Epidemiologic studies in asthma demonstrate that asthmatics who smoke have a greater decline in forced expiration volume in 1 s when followed over a 15-year period compared with asthmatics who do not smoke (11), suggesting that tobacco smoke may contribute to the decline in lung function and airway remodeling. However, no studies have directly assessed levels of remodeling in the airway in asthmatics who are exposed to ETS to determine whether exposure to ETS is associated with increased levels of airway remodeling. In this study, we have used a mouse model of allergen-induced airway remodeling to determine whether controlled chronic exposure of mice to the combination of ETS and allergen induces greater levels of airway remodeling than exposure to either stimulus alone. Insights from this study are relevant to allergic children who are coexposed to both indoor allergens and ETS from parents who smoke.

Materials and Methods

Mouse model of chronic OVA-induced airway remodeling

Eight- to 10-wk-old BALB/c mice (12 mice/group; The Jackson Laboratory) were immunized s.c. on days 0, 7, 14, and 21 with 25 μ g of OVA (grade V; Sigma-Aldrich) adsorbed to 1 mg of alum (Sigma-Aldrich) in 200 μ l of normal saline as previously described (12, 13). Intranasal OVA challenges were administered on days 27, 29, and 31 under isoflurane (Vedco) anesthesia and then repeated twice a week for 1 mo (see Fig. 1). Age- and sex-matched control mice were sensitized but not challenged with OVA during the 1-mo study. Mice were sacrificed 24 h after the final chronic OVA challenge and bronchoalveolar lavage (BAL) fluid and lungs were analyzed. Lungs from the different experimental groups were processed as a batch for either histologic staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified

*Department of Medicine, University of California-San Diego, La Jolla, CA 92093; and [†]Department of Pediatrics, College of Medicine, Korea University, Seoul, Korea
Received for publication August 29, 2006. Accepted for publication February 3, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Tobacco-Related Disease Research Program Grant 12RT-0071 (to D.H.B.).

² M.G.M. and D.J.S. contributed equally as first authors to this manuscript.

³ Address correspondence and reprint requests to Dr. David H. Broide, University of California-San Diego, Basic Science Building, Room 5090, 9500 Gilman Drive, La Jolla, CA 92093-0635. E-mail address: dbroide@ucsd.edu

⁴ Abbreviations used in this paper: ETS, environmental tobacco smoke; AHR, airway hyperreactivity; Mch, methacholine; BAL, bronchoalveolar lavage; MBP, major basic protein; CTGF, connective tissue growth factor; PAS, periodic acid-Schiff.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/\$2.00

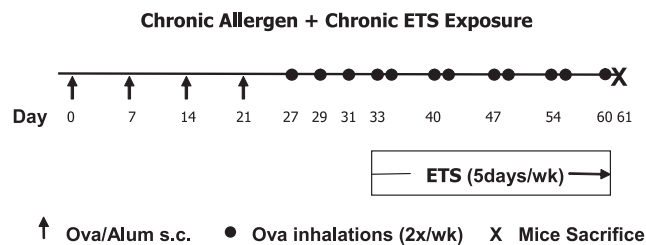


FIGURE 1. The experimental protocol. Mice were immunized s.c. on days 0, 7, 14, and 21 with OVA (arrows). Intranasal OVA challenges were administered on days 27, 29, and 31 and then repeated twice a week for 1 mo (●). Different groups of mice were either administered ETS alone, or ETS in combination with OVA challenges. ETS was started on day 33 (after mice had been sensitized and challenged on three occasions with intranasal OVA). The ETS was continued 5 days/wk for 1 mo. Mice were sacrificed 24 h after the final OVA challenge and BAL fluid and lungs were analyzed.

under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination. The quantitative histologic and image analysis of all coded slides was performed by research associates blinded to the coding of all the slides.

Effect of chronic ETS exposure on allergen-induced airway remodeling

To determine whether chronic ETS exposure would influence the development of allergen-induced airway remodeling, different groups of mice (12 mice/group) were exposed to either chronic ETS alone, chronic OVA allergen alone, or the combination of chronic ETS and chronic OVA allergen. ETS was first administered on day 33 after the mice had been sensitized with OVA s.c. (see Fig. 1) and received intranasal OVA challenges on days 27, 29, and 31 (see Fig. 1). Chronic ETS was continued daily for the subsequent duration of the 1-mo period of twice weekly intranasal OVA challenges. Mice were subjected to chronic ETS (side stream smoke from six cigarettes per day each administered over ~ 5 min with a 15-min break between cigarettes, 5 days/wk) generated by burning 2R4F reference cigarettes (2.45 mg of nicotine/cigarette; purchased from Tobacco Research Institute, University of Kentucky, Lexington, KY) using a smoking machine (McChesney-Jaeger CSM-SSM Single Cigarette Machine; CH Technologies) regulated by programmable controls provided with JASPER Windows 9x/2000 software over RS-232 communication ports (CH Technologies). Each smoldering cigarette is puffed for ~ 2 s, once every 25 s, for a total of 12 puffs/cigarette, at a flow rate of 5 L/min. The outflow from the smoking machine was adjusted to mimic an exposure to ETS by producing a mixture of room air (98%) and mainstream smoke (2%). The mice were exposed to the ETS in a 12-port nose-only directed flow inhalation exposure system (Jaeger-NYU 12 port). Nose ports were monitored for total suspended particulates using a gravimetric method (14). The concentration of total suspended particulates was $173 \pm 5.3 \mu\text{g}/\text{m}^3$. All animal experimental protocols were approved by the University of California-San Diego Animal Subjects Committee.

Measurement of smooth muscle layer thickness

The thickness of the airway smooth muscle layer was measured using an image analysis system as previously described (12, 13). In brief, the thickness of the smooth muscle layer (the transverse diameter) was measured from the innermost aspect to the outermost aspect of the smooth muscle layer. The smooth muscle layer thickness in at least 10 bronchioles of similar size (150–200 μm) were counted on each slide.

Lung sections were also immunostained with an anti- α -smooth muscle actin primary Ab (Sigma-Aldrich). The area of α -smooth muscle actin staining was outlined and quantified using a light microscope attached to an image analysis system as previously described (12, 13). Results are expressed as the area of α -smooth muscle actin staining per micrometer length of basement membrane of bronchioles 150–200 μm of internal diameter.

Effect of chronic ETS and chronic OVA allergen challenge on airway hyperreactivity (AHR)

Airway hyperresponsiveness to methacholine (Mch) was assessed 24 h after the final chronic OVA and/or chronic ETS challenge (after 1 mo

of repetitive OVA $\pm$ ETS challenges) in intubated and ventilated mice (flexiVent ventilator; Scireq) as previously described in this laboratory (15). The frequency independent airway resistance was determined in mice exposed to nebulized PBS and Mch (3, 24, 48 mg/ml) (15).

Effect of chronic ETS and chronic OVA allergen challenge on the development of peribronchial fibrosis

Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma-Aldrich) to preserve the pulmonary architecture. The area of peribronchial trichrome staining in the paraffin-embedded lung was outlined and quantified using a light microscope (Leica DMLS; Leica Microsystems) attached to an image analysis system (Image-Pro Plus; Media Cybernetics) as previously described (12, 13). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150–200 μm of internal diameter.

Assessment of BAL eosinophils and peribronchial eosinophilic inflammation

BAL total eosinophil counts and immunohistochemical detection of peribronchial major basic protein (MBP)⁺ cells were assessed as previously described (12, 13). In brief, lung sections were processed for MBP immunohistochemistry using an anti-mouse MBP Ab (provided by Dr. J. Lee, Mayo Clinic, Scottsdale, AZ). The number of individual cells staining positive for MBP in the peribronchial space were counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive for MBP per bronchiole with 150–200 μm of internal diameter. At least 10 bronchioles were counted in each slide.

Quantitation of peribronchial TGF- β 1 and connective tissue growth factor (CTGF) expression

The number of peribronchial cells expressing TGF- β 1 or CTGF were assessed in lung sections processed for immunohistochemistry using either an anti-TGF- β 1 primary Ab (vendor code SC146; Santa Cruz Biotechnology) or an anti-CTGF primary Ab (vendor code SC14939; Santa Cruz Biotechnology), the immunoperoxidase method, and image analysis quantitation as previously described (12, 13). Results are expressed as the number of TGF- β 1- or CTGF-positive cells/bronchus (12, 13).

BAL cytokine and chemokine levels

Levels of selected eosinophil active cytokines and chemokines (IL-5, eotaxin-1) were assayed in BAL fluid using an IL-5 (vendor code DY405) and eotaxin-1 (vendor code DY420) ELISA (R&D Systems).

Immunohistochemical localization of eotaxin-1 in lung sections

Lung sections were immunostained using the immunoperoxidase method as previously described (12, 13) with an anti-eotaxin-1 primary Ab (Santa Cruz Biotechnology), or a species- and isotype-matched control Ab, to investigate the cellular source of eotaxin-1 expression. The area of airway epithelial eotaxin-1 immunostaining was outlined and quantitated in bronchioles 150–200 μm of internal diameter by image analysis. Results are expressed as the area of airway epithelial eotaxin-1 staining in micrometer squared-per-micrometer length of airway epithelial basement membrane.

Quantitation of airway mucus expression

The number of periodic acid-Schiff (PAS)-positive and PAS-negative, as well as the number of Alcian blue-positive (pH 2.5 to detect acid mucosubstances) and Alcian blue-negative, airway epithelial cells in individual bronchioles was counted as previously described in this laboratory (12, 13). At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole (or Alcian blue-positive cells per bronchiole), which is calculated from the number of PAS-positive epithelial cells per bronchus (or Alcian blue-positive cells per bronchus) divided by the total number of epithelial cells of each bronchiole.

Statistical analysis

Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test followed by posttesting using Dunn's multiple comparison of means. All results are presented as mean $\pm$ SEM. A statistical software package (Graph Pad Prism) was used for the analysis. Values of $p < 0.05$ were considered statistically significant.

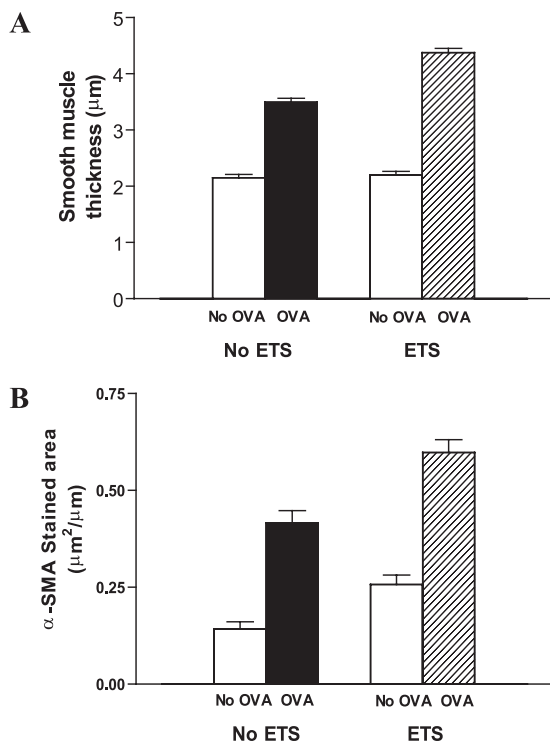


FIGURE 2. The thickness of the peribronchial smooth muscle layer in micrometers was assessed by image analysis (A). Mice chronically challenged with OVA alone had a significant increase in thickness of the peribronchial smooth muscle layer compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.0001$). The combination of chronic ETS and chronic OVA challenge increased the thickness of the peribronchial smooth muscle layer to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.001$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$) (A). Mouse lungs were also immunostained with an α -smooth muscle actin Ab and the area of peribronchial immunostaining quantitated in micrometers squared-per-micrometer length of the basement membrane of the bronchus by image analysis (B). Mice chronically challenged with OVA alone had a significant increase in levels of peribronchial α -smooth muscle actin immunostaining compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.001$). The combination of chronic ETS and chronic OVA challenge increased the levels of the peribronchial α -smooth muscle actin immunostaining to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.01$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$).

Results

Chronic ETS increases the thickness of the smooth muscle layer and the area of peribronchial α -smooth muscle actin immunostaining induced by chronic OVA

Exposure of mice to chronic ETS alone did not induce an increase in thickness of the peribronchial smooth muscle layer compared with non-ETS-exposed mice (2.2 ± 0.08 vs 2.1 ± 0.06 μm ; ETS plus no OVA vs no ETS plus no OVA; $p = \text{NS}$) (Fig. 2A). In contrast, exposure of mice to chronic OVA alone induced an increase in thickness of the peribronchial smooth muscle layer compared with non-ETS-exposed mice (3.5 ± 0.07 vs 2.1 ± 0.06 μm ; no ETS plus OVA vs no ETS plus no OVA; $p = 0.0001$) (Fig. 2A). The combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of thickness of the peribronchial smooth muscle layer compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.0001$) (Fig.

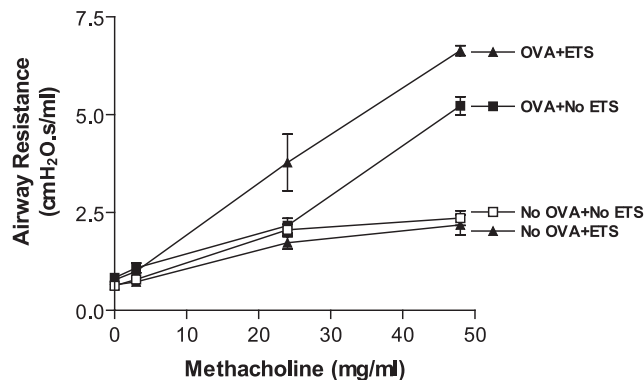


FIGURE 3. AHR to Mch was assessed 24 h after the final OVA and/or ETS chronic challenge in intubated and ventilated mice. Results are expressed as airway resistance in mice exposed to nebulized Mch (3, 24, 48 mg/ml). Mice chronically challenged with OVA alone had a significant increase in levels of AHR compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.001$, Mch 48 mg/ml). The combination of chronic ETS and chronic OVA challenge increased the levels of the AHR to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.05$, Mch 48 mg/ml) or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$, Mch 48 mg/ml).

2A), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.001$) (Fig. 2A).

Exposure of mice to chronic ETS alone induced a slight but statistically insignificant increase in the area of peribronchial α -smooth muscle actin immunostaining compared with non-ETS-exposed mice (ETS plus no OVA vs no ETS plus no OVA; $p = \text{ns}$) (Fig. 2B). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the area of peribronchial α -smooth muscle actin immunostaining compared with non-OVA-challenged mice (0.42 ± 0.03 vs 0.14 ± 0.02 $\mu\text{m}^2/\mu\text{m}$ circumference of bronchiole; no ETS plus OVA vs no ETS plus no OVA; $p = 0.001$) (Fig. 2B). The combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of peribronchial α -smooth muscle actin immunostaining compared with either chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig. 2B), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.01$) (Fig. 2B).

Chronic ETS exposure increases levels of AHR in chronic OVA-challenged mice

To determine whether the increased thickness of the smooth muscle layer associated with exposure of mice to chronic ETS and chronic OVA was associated with increased responsiveness of airway smooth muscle, we determined levels of airway responsiveness to Mch in mice exposed to chronic ETS and/or chronic OVA allergen. Exposure of mice to chronic ETS alone did not induce a change in AHR to Mch compared with non-ETS-exposed mice (ETS plus no OVA vs no ETS plus no OVA; $p = \text{ns}$) (Fig. 3). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in AHR compared with non-OVA-challenged mice (OVA plus no ETS vs no-OVA plus no ETS; Mch 48 mg/ml, $p = 0.001$) (Fig. 3). The combination of chronic ETS and chronic OVA allergen exposure induced a significantly greater increase in AHR compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; Mch 48 mg/ml; $p = 0.001$) (Fig. 3), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; Mch 48 mg/ml; $p = 0.05$) (Fig. 3).

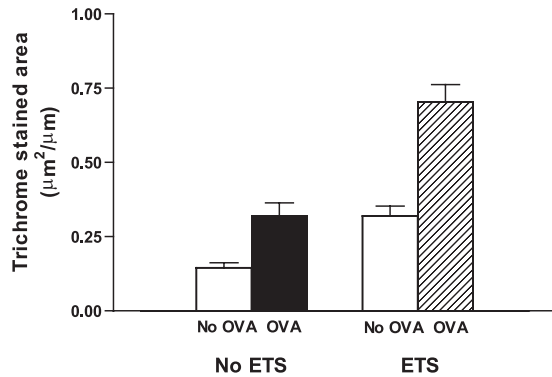


FIGURE 4. Mouse lungs were stained with trichrome and the area of peribronchial trichrome staining was quantitated in micrometers squared-per-micrometer length of bronchus by image analysis. Mice chronically challenged with OVA alone had an increase in levels of peribronchial trichrome staining compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.01$). The combination of chronic ETS and chronic OVA challenge increased the levels of the peribronchial trichrome staining to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.001$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$).

Chronic ETS increases levels of chronic OVA-induced peribronchial collagen deposition

An increase in peribronchial trichrome staining was noted in mice following exposure to chronic OVA alone compared with non-OVA-exposed mice (0.32 ± 0.04 vs 0.14 ± 0.02 $\mu\text{m}^2/\mu\text{m}$ circumference of bronchiole; no ETS plus no OVA vs no ETS plus OVA; $p = 0.01$) (Fig. 4). However, the combination of chronic ETS and chronic OVA allergen exposure induced a significantly greater increase in levels of peribronchial trichrome staining compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig. 4), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.001$) (Fig. 4).

Chronic ETS exposure increases levels of peribronchial eosinophilic inflammation in chronic OVA-challenged mice

Exposure of mice to chronic ETS alone did not induce an increase in BAL eosinophils (Fig. 5A) or MBP⁺ peribronchial eosinophils (Fig. 5B) compared with non-ETS-exposed mice (ETS plus no OVA vs no ETS plus no OVA; $p = \text{NS}$). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the number of BAL eosinophils compared with non-OVA-challenged mice ($12.8 \pm 3.2 \times 10^4$ vs $0.02 \pm 0.01 \times 10^4$ BAL eosinophils; OVA plus no ETS vs no OVA plus no ETS; $p = 0.01$) (Fig. 5A). A similar significant increase in the number of MBP⁺ peribronchial eosinophils was noted in mice exposed to chronic OVA challenge alone compared with non-OVA-challenged mice (31.1 ± 2.0 vs 0.2 ± 0.1 peribronchial MBP⁺ eosinophils; OVA plus no ETS vs no OVA plus no ETS; $p = 0.005$) (Fig. 5B).

The combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of BAL eosinophils compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig. 5A), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.05$) (Fig. 5A). Similarly, the combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of peribronchial MBP⁺ eosinophils compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig.

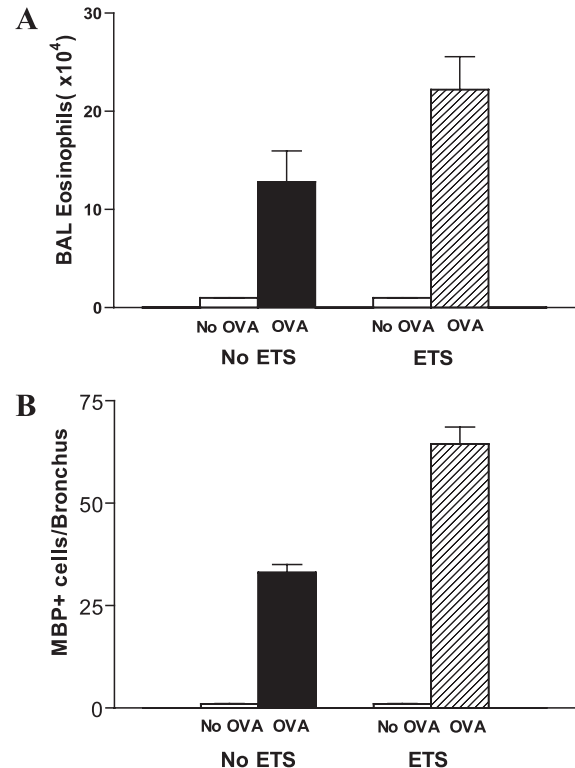


FIGURE 5. The number of eosinophils in BAL fluid was quantitated by Wright-Giemsa staining (A), and the number of peribronchial eosinophils by immunostaining lung sections with an anti-MBP Ab (B). Mice chronically challenged with OVA alone had a significant increase in levels of BAL eosinophils ($p = 0.01$) and peribronchial eosinophils ($p = 0.005$) compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS). The combination of chronic ETS and chronic OVA challenge increased the levels of BAL eosinophils ($p = 0.001$, vs ETS; $p = 0.05$, vs OVA) (A) and peribronchial eosinophils ($p = 0.001$, vs ETS; $p = 0.005$, vs OVA) (B) to a greater degree than either stimulus alone.

5B), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.005$) (Fig. 5B).

Chronic ETS exposure increases levels of peribronchial cells expressing TGF- β 1 in chronic OVA-challenged mice

Exposure of mice to chronic ETS alone did not induce an increase in the number of peribronchial TGF- β 1-positive cells (Fig. 6) compared with non-ETS-exposed mice (ETS plus no OVA vs no ETS plus no OVA; $p = \text{NS}$). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the number of peribronchial TGF- β 1-positive cells compared with non-OVA-challenged mice (12.1 ± 2.0 vs 0.3 ± 0.02 TGF- β 1-positive cells/bronchus; OVA plus no ETS vs no OVA plus no ETS; $p = 0.01$) (Fig. 6). The combination of chronic ETS and chronic OVA allergen exposure induced a significant increase in the number of peribronchial TGF- β 1-positive cells compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig. 6), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.005$) (Fig. 6).

Chronic ETS exposure increases levels of peribronchial cells expressing CTGF in chronic OVA-challenged mice

Exposure of mice to chronic ETS alone did not induce an increase in the number of peribronchial CTGF-positive cells (Fig. 7) compared with non-ETS-exposed mice (ETS plus no OVA vs no ETS plus no OVA; $p = \text{NS}$). In contrast, exposure of mice to chronic

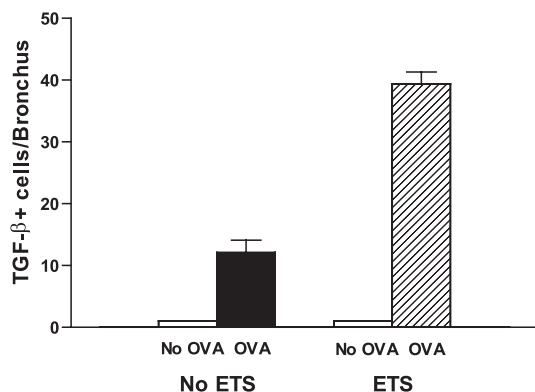


FIGURE 6. Mouse lungs were immunostained with an anti-TGF- β 1 Ab and the number of peribronchial cells immunostaining positive for TGF- β 1 quantitated by image analysis. Mice chronically challenged with OVA alone had a significant increase in the numbers of peribronchial TGF- β 1-positive cells compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.01$). The combination of chronic ETS and chronic OVA challenge increased the numbers of peribronchial TGF- β 1-positive cells to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.005$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$).

OVA challenge alone induced a significant increase in the number of peribronchial CTGF-positive cells compared with non-OVA-challenged mice (4.9 ± 0.4 vs 0.6 ± 0.1 CTGF-positive cells/bronchus; OVA plus no ETS vs no OVA plus no ETS; $p = 0.001$) (Fig. 7). The combination of chronic ETS and chronic OVA allergen exposure induced a significant increase in the number of peribronchial CTGF-positive cells compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig. 7), or compared with chronic OVA allergen alone (ETS plus OVA vs no ETS plus OVA; $p = 0.01$) (Fig. 7).

Effect of chronic ETS on chronic OVA-induced BAL IL-5 and eotaxin-1 levels

As coexposure of mice to chronic OVA and chronic ETS induced an increase in the number of peribronchial eosinophils, we evalu-

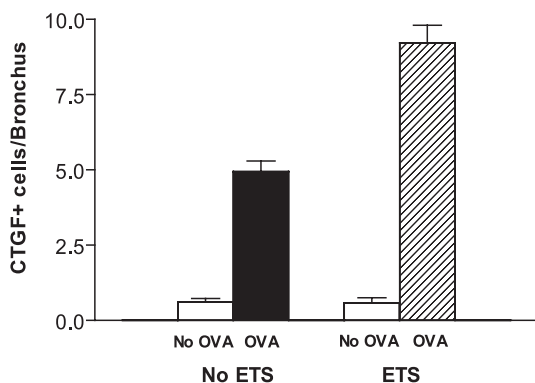


FIGURE 7. Mouse lungs were immunostained with an anti-CTGF Ab and the number of peribronchial cells immunostaining positive for CTGF quantitated by image analysis. Mice chronically challenged with OVA alone had a significant increase in the numbers of peribronchial CTGF-positive cells compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.001$). The combination of chronic ETS and chronic OVA challenge increased the numbers of peribronchial TGF- β 1-positive cells to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.01$) or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$).

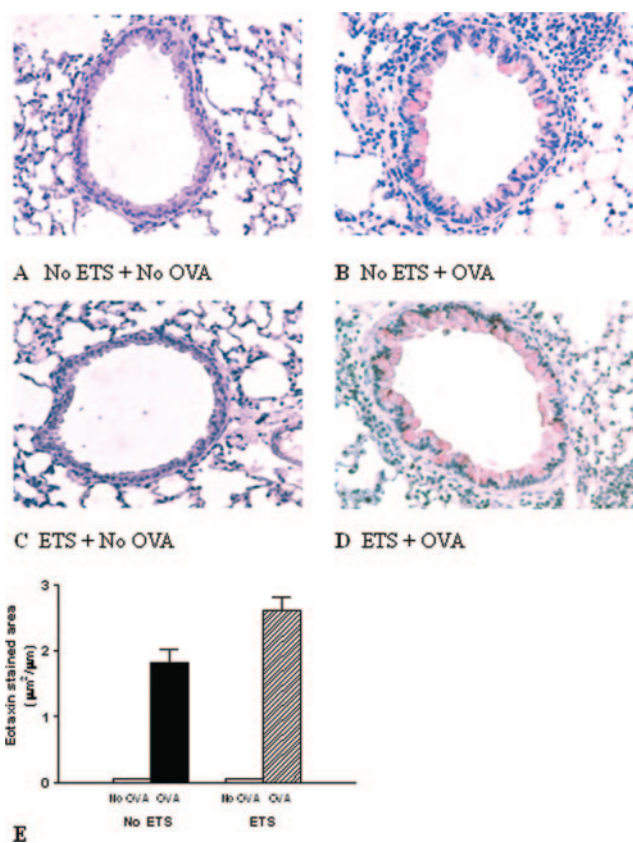


FIGURE 8. Mouse lungs were immunostained with an anti-eotaxin-1 Ab (A, no ETS plus no OVA; B, no ETS plus OVA; C, ETS plus no OVA; D, ETS plus OVA) and the area of epithelial eotaxin-1 immunostaining was quantitated by image analysis (E). Mice chronically exposed to OVA alone had a significant increase in the area of epithelial eotaxin-1 immunostaining compared with control non-OVA-exposed mice (OVA plus no ETS vs no OVA plus no ETS; $p = 0.001$). The combination of chronic ETS and chronic OVA coexposure increased the area of epithelial eotaxin-1 immunostaining to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.01$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.0001$).

ated whether cytokines which induce eosinophil proliferation or migration into tissues were modulated by coexposure of mice to chronic OVA and chronic ETS. Chronic ETS exposure alone did not induce either IL-5 or eotaxin-1, whereas chronic OVA challenge alone induced significantly increased levels of both BAL IL-5 (74.4 ± 8.8 vs 15.3 ± 12.2 pg/ml IL-5; OVA plus no ETS vs no OVA plus no ETS; $p = 0.05$), and BAL eotaxin-1 (68.5 ± 15.2 vs 6.0 ± 2.5 pg/ml eotaxin-1; OVA plus no ETS vs no OVA plus no ETS; $p = 0.05$). The combination of chronic OVA and chronic ETS coexposure induced significantly higher levels of BAL IL-5 (113.6 ± 23.5 vs 2.2 ± 2.2 pg/ml IL-5; $p = 0.001$, vs ETS) and BAL eotaxin-1 (122.6 ± 44.4 vs 9.3 ± 2.0 pg/ml eotaxin-1; $p = 0.001$, vs ETS) compared with levels noted following chronic ETS challenge alone. Although mice exposed to the combination of chronic OVA and chronic ETS had higher levels of BAL IL-5 (113.6 ± 23.5 vs 74.4 ± 8.8 pg/ml IL-5) and BAL eotaxin-1 (122.6 ± 44.4 vs 68.5 ± 15.2 pg/ml eotaxin-1) compared with levels noted following chronic OVA challenge alone, this did not reach statistical significance.

Effect of chronic ETS exposure and chronic OVA coexposure on airway epithelial eotaxin-1 immunostaining

Mouse lungs were immunostained with an anti-eotaxin-1 Ab (Fig. 8, A–D) and the area of airway epithelial eotaxin-1 immunostaining

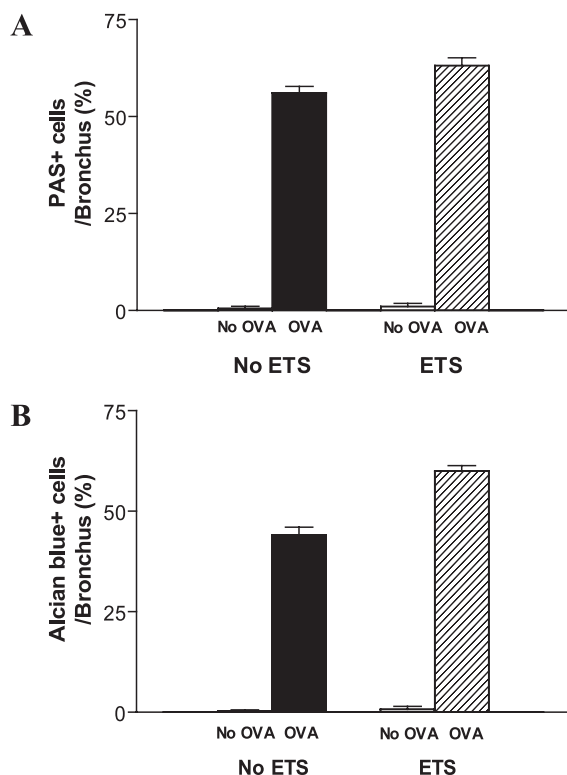


FIGURE 9. Mouse lungs were stained with either PAS (A) or Alcian blue (B) and the percentage of bronchial epithelial cells staining positive for either PAS or Alcian blue was quantitated by light microscopy. Mice chronically challenged with OVA alone had a significant increase in levels of bronchial epithelial staining with either PAS ($p = 0.0005$) (A) or Alcian blue ($p = 0.0001$) (B) compared with control non-OVA-challenged mice (OVA plus no ETS vs no OVA plus no ETS). The combination of chronic ETS and chronic OVA coexposure increased the levels of the bronchial epithelial PAS staining to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.05$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.0005$) (A). Alcian blue staining demonstrated that the combination of chronic ETS and chronic OVA coexposure increased the levels of the bronchial epithelial Alcian blue staining to a greater degree than either chronic OVA alone ($p = 0.01$) or chronic ETS alone ($p = 0.001$) (B).

quantitated by image analysis (Fig. 8E). Mice chronically challenged with OVA alone had a significant increase in the area of airway epithelial eotaxin-1 immunostaining compared with control non-OVA-challenged mice (1.82 ± 0.21 vs $0.01 \pm 0.01 \mu\text{m}^2/\mu\text{m}$ circumference of bronchiole; OVA plus no ETS vs no OVA plus no ETS; $p = 0.001$) (Fig. 8, A, B, and E). The combination of chronic ETS and chronic OVA challenge increased the area of epithelial eotaxin-1 immunostaining to a greater degree than either chronic OVA alone (OVA plus ETS vs OVA plus no ETS; $p = 0.01$), or chronic ETS alone (OVA plus ETS vs no OVA plus ETS; $p = 0.001$) (Fig. 8).

Effect of chronic ETS on chronic OVA-induced airway mucus expression

Exposure of mice to chronic ETS alone did not induce a change in the percentage of airway epithelium which stained positive with PAS compared with non-ETS-exposed mice (ETS plus no OVA vs no ETS plus no OVA; $p = \text{NS}$) (Fig. 9A). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the percentage of airway epithelium which stained positive with PAS compared with non-OVA-challenged mice (56.1 ± 1.7 vs $0.6 \pm 0.6\%$ PAS-positive cells/bronchus; OVA plus no ETS

vs no-OVA plus no ETS; $p = 0.0005$) (Fig. 9A). The combination of chronic ETS and chronic OVA allergen exposure induced a significant increase in the percentage of airway epithelium which stained positive with PAS compared with chronic ETS alone (ETS plus OVA vs ETS plus no OVA; $p = 0.001$) (Fig. 9A). Although the combination of chronic ETS and chronic OVA induced a statistically significant increase in the percentage of airway epithelium which stained positive with PAS compared with chronic OVA allergen alone, the absolute level of increased PAS-positive cells/bronchus was relatively small compared with chronic OVA alone ($63.2 \pm 2.0\%$ vs $56.1 \pm 1.7\%$ PAS-positive cells/bronchus; ETS plus OVA vs no ETS plus OVA; $p = 0.05$) (Fig. 9A).

Alcian blue staining was also used to detect acidic mucosubstances in airway epithelial cells. Results of Alcian blue staining (Fig. 9B) demonstrated that OVA challenge induced a significant increase in the percentage of airway epithelial cells which were Alcian blue positive ($p = 0.001$; OVA vs no OVA), and this increase in the percentage of airway epithelial cells which were Alcian blue positive was further increased by the combined exposure of mice to OVA and ETS ($p = 0.01$; OVA plus ETS vs OVA).

Discussion

In this study, we have used a mouse model of allergen-induced airway remodeling to demonstrate that chronic coexposure of mice to the combination of ETS and allergen induces significantly increased levels of airway remodeling compared with levels of airway remodeling noted in mice from chronic exposure to either stimulus alone. In particular, the combination of chronic ETS and chronic allergen coexposure significantly increased the thickness of the peribronchial smooth muscle layer and this was associated with a significant increase in AHR to Mch. As studies in mice deficient in eosinophils have demonstrated that airway eosinophils are important to the development of AHR (16), and also contribute to the thickening of the peribronchial smooth muscle layer (12) and airway remodeling (12), we determined whether mice exposed to the combination of chronic ETS and chronic allergen had enhanced airway eosinophilic inflammation. Mice exposed to the combination of chronic ETS and chronic OVA had significantly increased numbers of BAL and peribronchial eosinophils compared with mice exposed to chronic OVA alone. The increased numbers of peribronchial eosinophils in mice exposed to chronic ETS and chronic OVA was associated with a trend to increased levels of eosinophil active cytokines and chemokines such as IL-5 and eotaxin-1. Increased expression of Th2 cytokines such as IL-5 have previously been noted in studies of mice exposed to ETS and acute OVA allergen (17, 18). Increased expression of eotaxin-1 in mice coexposed to ETS and OVA has not been previously reported. Our immunostaining of lung sections demonstrated that airway epithelial cells are the predominate source of eotaxin-1 in mice exposed to chronic ETS and chronic OVA allergen. Eotaxin-1 is a NF- κ B-regulated chemokine (19) that can be stimulated to be expressed from airway epithelial cells by stimuli including cytokines and oxidant stress (20). The gas phase of ETS contains free radicals such as superoxide radicals, hydroxyl radicals, and H_2O_2 (21) that are known to activate redox-sensitive transcription factors, such as NF- κ B (22), a regulator of eotaxin-1 expression. Interestingly, mice exposed to chronic ETS alone express minimal eotaxin-1, suggesting that chronic ETS exposure alone does not activate NF- κ B sufficiently in airway epithelial cells to detect measurable changes in eotaxin-1 in BAL fluid. The importance of NF- κ B-regulated genes in airway epithelial cells to

airway remodeling is suggested from studies in which *cre-lox* molecular techniques have been used to selectively inactivate NF- κ B in airway epithelium (23). Mutant mice unable to activate NF- κ B in airway epithelial cells have reduced levels of expression of eotaxin-1, reduced peribronchial expression of TGF- β 1⁺ eosinophils, and reduced levels of airway remodeling following chronic OVA challenge (23).

Previous studies of allergen-induced airway remodeling in mice (12) and in humans with asthma and airway remodeling (24) have also demonstrated the importance of eosinophil expression of TGF- β 1 in mediating airway remodeling. Interestingly, in this study, the combination of chronic ETS and chronic allergen coexposure induced an influx into the airway of eosinophils and TGF- β 1⁺ cells that was significantly greater than that noted with exposure to chronic allergen alone. In contrast, chronic ETS exposure alone did not induce the recruitment of eosinophils or TGF- β 1⁺ cells into the airway, or induce airway remodeling or increased AHR. The degree to which either stimulus (ETS and/or OVA), alone or combined, increased levels of peribronchial eosinophilic inflammation and the number of peribronchial TGF- β 1⁺ cells correlated well with levels of airway remodeling. For example, mice exposed to chronic ETS alone had low levels of peribronchial eosinophils, few TGF- β 1⁺ cells, and low levels of airway remodeling, whereas mice exposed to chronic OVA alone had intermediate levels of peribronchial eosinophils, numbers of TGF- β 1⁺ cells, and levels of airway remodeling. The combination of chronic ETS and chronic OVA coexposure induced the highest levels of peribronchial eosinophils, TGF- β 1⁺ cells, and airway remodeling. Prior studies in murine models of allergen-induced airway remodeling have demonstrated that either depletion of eosinophils (12), or neutralization of TGF- β (25), significantly inhibits airway remodeling underscoring the importance of eosinophils and TGF- β to airway remodeling in this mouse model of exposure to chronic ETS and chronic allergen. In addition to the increased levels of expression of TGF- β in the remodeled airway, we also noted increased levels of expression of CTGF in the remodeled airway. Although eosinophils were a significant source of TGF- β , CTGF was not localized to eosinophils and appeared to be localized to fibroblasts, a known source of CTGF (26). As CTGF has been linked to fibrotic disorders due to its ability to induce fibroblast proliferation as well as induce expression of fibronectin, and collagen I (26), CTGF may be an additional cytokine mediating the profibrotic effects noted in mice exposed to ETS and OVA. In contrast to several studies demonstrating expression of TGF- β in the remodeled airway in human asthma (24, 27), at present there is limited information regarding the role of CTGF in airway remodeling in human asthma. Thus, further studies are needed to determine the significance to asthma of detection of CTGF in the remodeled airway in mice.

Although several prior studies examining ETS exposure in combination with allergen exposure in mice have demonstrated enhanced Th2 responses (17, 18, 28–30), other studies have demonstrated that the combination of ETS exposure and OVA allergen can inhibit Th2 responses and AHR (31–33). The differences in the results in these studies may be due to differences in ETS and OVA exposure protocols, differences in exposure to mainstream smoke vs ETS, as well as differences in timing of ETS exposure during the sensitization compared with the allergen challenge period. This study differs from previous studies by examining the effect of coexposure to ETS and allergen on the development of airway remodeling end points which have not been previously studied. Thus, the novel findings in this study include the demonstration that chronic exposure to the combination of ETS and allergen induces increased expression of eotaxin-1 by airway epithelial cells,

recruitment of TGF- β 1⁺ cells and eosinophils into the airway, and that this is associated with increased airway remodeling, in particular, increased smooth muscle layer thickness and increased AHR, as well as increased subepithelial fibrosis and mucus.

Although no chronic ETS challenge studies have been reported in humans with asthma, acute ETS challenge studies in humans have demonstrated that a significant percentage of smoke-sensitive asthmatics who were challenged on one occasion with ETS for 4 h developed increased AHR to Mch (10). Acute ETS exposure increased AHR in 32% of smoke-sensitive asthmatics at 6 h, 29% at 24 h, and the increased AHR was sustained in up to 13% of asthmatics to day 14 after ETS challenge (10). Our study in mice suggests that more chronic exposure to ETS in combination with chronic allergen exposure may increase levels of AHR further than that induced by either stimulus alone. This may be particularly relevant to the development of AHR in either allergic children or adults exposed chronically indoors to both allergens and ETS.

Passive smoke consists of a complex mixture of >4000 different chemicals (34) and thus the specific chemical ingredient(s) in ETS responsible for interacting with allergen to induce increased airway remodeling are currently unknown. Interestingly, recent studies suggest that specific genes may exist that increase the susceptibility to develop asthma in the presence of exposure to tobacco smoke (35, 36). Linkage studies, which have stratified asthmatics based on ETS exposure, have demonstrated that certain chromosomal regions which show strong linkage with asthma and airway hyperresponsiveness (e.g., 1p, 3p, 5q, 9q) may harbor genes that exert their effects mainly in combination with ETS exposure (35, 36). Thus, a gene environment interaction between passive smoking and a genetic susceptibility may be causally involved in the development of asthma in some, but not all, children with asthma. Whether similar gene environment interactions between passive smoking and a genetic susceptibility contribute to airway remodeling in a subset of asthmatics is at present unknown.

In summary, in this study, we have demonstrated that chronic exposure to the combination of ETS and allergen induces greater levels of airway remodeling than that noted with either stimulus alone. If exposure to ETS and allergen in humans with asthma also induced increased thickness of the smooth muscle layer and increased AHR as noted in this study in a mouse model, this could provide one potential explanation for the increased frequency of asthma exacerbations (1–8), and emergency room visits (1, 3, 5) in asthmatic children whose parents smoke. Our study also suggests a potential mechanism by which mice chronically exposed to the combination of ETS and allergen develop increased levels of airway remodeling and AHR. Constituents of ETS in combination with allergen stimulate airway remodeling by inducing increased expression of eosinophil chemoattractants such as eotaxin-1 by airway epithelial cells, and increased Th2 cytokines (17, 18), which recruit larger numbers of TGF- β 1⁺ eosinophils to the airway than that induced by allergen alone. The increased levels of peribronchial eosinophils and cells expressing TGF- β 1 are likely to contribute to airway remodeling (12, 24, 25). Thus, reduced exposure to ETS in allergic individuals with asthma may contribute to reductions in levels of airway remodeling and airway responsiveness in such subjects.

Disclosures

The authors have no financial conflict of interest.

References

1. Assessment, Office of Health and Environment. 1992. Respiratory health effects of passive smoking: lung cancer and other disorders. U.S. Environmental Protection Agency, Washington, D.C.

2. Chilmonczyk, B. A., L. M. Salmun, K. N. Megathlin, L. M. Neveux, G. E. Palomaki, G. J. Knight, A. J. Pulkkinen, and J. E. Haddow. 1993. Association between exposure to environmental tobacco smoke and exacerbations of asthma in children. *N. Engl. J. Med.* 328: 1665–1669.
3. Britton, J. 2005. Passive smoking and asthma exacerbation. *Thorax* 60: 794–795.
4. Evans, D., M. J. Levison, C. H. Feldman, N. M. Clark, Y. Wasilewski, B. Levin, and R. B. Mellins. 1987. The impact of passive smoking on emergency room visits of urban children with asthma. *Am. Rev. Resp. Dis.* 135: 567–572.
5. Cook, D. G., and D. P. Strachan. 1997. Health effects of passive smoking. 3. Parental smoking and prevalence of respiratory symptoms and asthma in school age children. *Thorax* 52: 1081–1094.
6. Strachan, D. P., and D. G. Cook. 1998. Health effects of passive smoking. 6. Parental smoking and childhood asthma: longitudinal and case-control studies. *Thorax* 53: 204–212.
7. Cook, D. G., D. P. Strachan, and I. M. Carey. 1998. Health effects of passive smoking. 9. Parental smoking and spirometric indices in children. *Thorax* 53: 884–893.
8. Eisner, M. D., J. Klein, S. K. Hammond, G. Koren, G. Lactao, and C. Iribarren. 2005. Directly measured second hand smoke exposure and asthma health outcomes. *Thorax* 60: 814–821.
9. Stankus, R. P., P. K. Menon, R. J. Rando, H. Glindmeyer, J. E. Salvaggio, and S. B. Lehrer. 1988. Cigarette smoke-sensitive asthma: challenge studies. *J. Allergy Clin. Immunol.* 82: 331–338.
10. Menon, P., R. J. Rando, R. P. Stankus, J. E. Salvaggio, and S. B. Lehrer. 1992. Passive cigarette smoke-challenge studies: increase in bronchial hyperreactivity. *J. Allergy Clin. Immunol.* 89: 560–566.
11. Lange, P., J. Parner, J. Vestbo, P. Schnohr, and G. Jensen. 1998. A 15-year follow-up study of ventilatory function in adults with asthma. *N. Engl. J. Med.* 339: 1194–1200.
12. Cho, J. Y., M. Miller, K. J. Baek, J. W. Han, J. Nayar, S. Y. Lee, K. McElwain, S. McElwain, S. Friedman, and D. H. Broide. 2004. Inhibition of airway remodeling in IL-5-deficient mice. *J. Clin. Invest.* 113: 551–560.
13. Cho, J. Y., M. Miller, K. J. Baek, J. W. Han, J. Nayar, S. Y. Lee, K. McElwain, S. McElwain, E. Raz, and D. H. Broide. 2004. Immunostimulatory DNA reverses established allergen-induced airway remodeling. *J. Immunol.* 173: 7556–7564.
14. Yang, Z., C. A. Knight, M. M. Mamerow, K. Vickers, A. Penn, E. M. Postlethwait, and S. W. Ballinger. 2004. Prenatal environmental tobacco smoke exposure promotes adult atherogenesis and mitochondrial damage in apolipoprotein E^{-/-} mice fed a chow diet. *Circulation* 110: 3715–3720.
15. Lim, D. H., J. Y. Cho, M. Miller, K. McElwain, S. McElwain, and D. H. Broide. 2006. Reduced peribronchial fibrosis in allergen-challenged MMP-9-deficient mice. *Am. J. Physiol.* 291: L265–L271.
16. Lee, J. J., D. Dimina, M. P. Macias, S. I. Ochkur, M. P. McGarry, K. R. O'Neill, C. Protheroe, R. Pero, T. Nguyen, S. A. Cormier, et al. 2004. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305: 1773–1776.
17. Rumold, R., M. Jyrala, and D. D. Diaz-Sanchez. 2001. Second hand smoke induces allergic sensitization in mice. *J. Immunol.* 167: 4765–4770.
18. Seymour, B. W., K. E. Pinkerton, K. E. Frieberthausen, R. L. Coffman, and L. J. Gershwin. 1997. Second hand smoke is an adjuvant for Th2 responses in a murine model of allergy. *J. Immunol.* 159: 6169–6175.
19. Rothenberg, M. E., A. D. Luster, and P. Leder. 1995. Mouse eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc. Natl. Acad. Sci. USA* 92: 8960–8964.
20. Henderson, W. R. Jr., E. Y. Chi, J. L. Teo, C. Nguyen, and M. Kahn. 2002. A small molecule inhibitor of redox-regulated NF- κ B and activator protein-1 transcription blocks allergic airway inflammation in a mouse asthma model. *J. Immunol.* 169: 5294–5299.
21. Vayssier, M., N. Banzet, D. Francois, K. Bellmann, and B. S. Polla. 1998. Tobacco smoke induces both apoptosis and necrosis in mammalian cells: differential effects of HSP70. *Am. J. Physiol.* 275: L771–L779.
22. Manna, S. K., T. Rangasamy, K. Wise, S. Sarkar, S. Shishodia, S. Biswal, and G. T. Ramesh. 2006. Long term environmental tobacco smoke activates nuclear transcription factor- κ B, activator protein-1, and stress responsive kinases in mouse brain. *Biochem. Pharmacol.* 71: 1602–1609.
23. Broide, D. H., T. Lawrence, T. Doherty, J. Y. Cho, M. Miller, K. McElwain, S. McElwain, and M. Karin. 2005. Allergen-induced peribronchial fibrosis and mucus production mediated by I κ B kinase β -dependent genes in airway epithelium. *Proc. Natl. Acad. Sci. USA* 102: 17723–17728.
24. Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M. S. Ludwig, N. Barnes, D. Robinson, and A. B. Kay. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J. Clin. Invest.* 112: 1029–1036.
25. McMillan, S. J., G. Xanthou, and C. M. Lloyd. 2005. Manipulation of allergen-induced airway remodeling by treatment with anti-TGF- β antibody: effect on the Smad signaling pathway. *J. Immunol.* 174: 5774–5780.
26. Burgess, J. K. 2005. Connective tissue growth factor: a role in airway remodeling in asthma? *Clin. Exp. Pharmacol. Physiol.* 32: 988–994.
27. Sagara, H., T. Okada, K. Okumura, H. Ogawa, C. Ra, T. Fukuda, and A. Nakao. 2002. Activation of TGF- β /Smad2 signaling is associated with airway remodeling in asthma. *J. Allergy Clin. Immunol.* 110: 249–254.
28. Seymour, B. W., E. S. Schelegle, K. E. Pinkerton, K. E. Frieberthausen, J. L. Peake, V. P. Kurup, R. L. Coffman, and L. J. Gershwin. 2003. Second-hand smoke increases bronchial hyperreactivity and eosinophilia in a murine model of allergic aspergillosis. *Clin. Dev. Immunol.* 10: 35–42.
29. Moerloose, K. B., R. A. Pauwels, and G. F. Joos. 2005. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 172: 168–172.
30. Moerloose, K. B., L. J. Robays, T. Maes, G. G. Brusselle, K. G. Tournoy, and G. F. Joos. 2006. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir. Res.* 7: 49.
31. Melgert, B. N., D. S. Postma, M. Geerlings, M. A. Luinge, P. A. Klok, B. W. Van der Strate, H. A. Kerstjens, W. Timens, and M. N. Hylkema. 2004. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am. J. Respir. Cell Mol. Biol.* 30: 880–885.
32. Bowles, K., D. Horohov, D. Paulsen, C. Leblanc, M. Littlefield-Chabaud, T. Ahlert, K. Ahlert, S. Pourciau, and A. Penn. 2005. Exposure of adult mice to environmental tobacco smoke fails to enhance the immune response to inhaled antigen. *Inhal. Toxicol.* 17: 43–51.
33. Robbins, C. S., M. A. Pouladi, R. Fattouh, D. E. Dawe, N. Vujicic, C. D. Richards, M. Jordana, M. D. Inman, and M. R. Stampfli. 2005. Mainstream cigarette smoke exposure attenuates airway immune inflammatory responses to surrogate and common environmental allergens in mice, despite evidence of increased systemic sensitization. *J. Immunol.* 175: 2834–2842.
34. Lofroth, G. 1989. Environmental tobacco smoke: overview of chemical composition and genotoxic components. *Mutat. Res.* 222: 73–80.
35. Colilla, S., D. Nicolae, A. Pluzhnikov, M. N. Blumenthal, T. H. Beaty, E. R. Bleecker, E. M. Lange, S. S. Rich, D. A. Meyers, C. Ober, and N. J. Cox. 2003. Evidence for gene-environment interactions in a linkage study of asthma and smoking exposure. *J. Allergy Clin. Immunol.* 111: 840–846.
36. Meyers, D. A., D. S. Postma, O. C. Stine, G. H. Koppelman, E. J. Ampleford, H. Jongepier, T. D. Howard, and E. R. Bleecker. 2005. Genome screen for asthma and bronchial hyperresponsiveness: interactions with passive smoke exposure. *J. Allergy Clin. Immunol.* 115: 1169–1175.