Airborne Particulate Matter Induces Nonallergic Eosinophilic Sinonasal Inflammation in Mice

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Abstract

Exposure to airborne particulate matter (PM) has been linked to aggravation of respiratory symptoms, increased risk of cardiovascular disease, and all-cause mortality. Although the health effects of PM on the lower pulmonary airway have been extensively studied, little is known regarding the impact of chronic PM exposure on the upper sinonasal airway. We sought to test the impact of chronic airborne PM exposure on the upper respiratory system *in vivo*. Mice were subjected, by inhalation, to concentrated fine (2.5 μm) PM 6 h/d, 5 d/wk, for 16 weeks. Mean airborne fine PM concentration was 60.92 $\mu\text{m}/\text{m}^3$, a concentration of fine PM lower than that reported in some major global cities. Mice were then killed and analyzed for evidence of inflammation and barrier breakdown compared with control mice. Evidence of the destructive effects of

chronic airborne PM on sinonasal health *in vivo*, including proinflammatory cytokine release, and macrophage and neutrophil inflammatory cell accumulation was observed. A significant increase in epithelial barrier dysfunction was observed, as assessed by serum albumin accumulation in nasal airway lavage fluid, as well as decreased expression of adhesion molecules, including claudin-1 and epithelial cadherin. A significant increase in eosinophilic inflammation, including increased IL-13, eotaxin-1, and eosinophil accumulation, was also observed. Collectively, although largely observational, these studies demonstrate the destructive effects of chronic airborne PM exposure on the sinonasal airway barrier disruption and nonallergic eosinophilic inflammation in mice.

Keywords: air pollution; inflammation; particulate matter; permeability; rhinitis

Air pollution continues to be a major worldwide environmental and public health concern. Exposure to airborne particulate matter (PM) has been linked to aggravation of respiratory symptoms, increased risk of diabetes and cardiovascular disease, and all-cause mortality (1). Although the health effects of PM on the lower pulmonary airway have been extensively studied, little is known regarding the impact of environmental exposures on the upper sinonasal airway (2). Cross-sectional and database studies have

linked chronic rhinosinusitis with poor air quality, yet there is no direct evidence that airborne PM contributes to poor sinonasal health *in vivo* (3–5). Furthermore, nasal mucosa explants exposed to PM have also been reported to demonstrate increased IL-8 secretion, and PM exposure has also been reported to destabilize sinonasal epithelial barrier function *in vitro* (6, 7).

Eosinophilic inflammation plays a significant role in allergy-mediated disease, and multiple animal models of allergen-

induced sinonasal eosinophilic inflammation have been described, including ovalbumin, house dust mite, and *Aspergillus* extract (8, 9). Interestingly, a subpopulation of patients with sinonasal eosinophilic inflammation and subjective symptoms of rhinitis has shown no evidence of allergy (10). The most severe form of this condition is known as nonallergic rhinitis with eosinophilia syndrome (NARES) for which animal models are lacking (10).

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Clinical Relevance

Exposure to airborne particulate matter (PM) has been linked to aggravation of respiratory symptoms, but little is known regarding the impact of environmental exposures on the upper sinonasal airway. The present study demonstrates the destructive effects of chronic airborne PM on sinonasal health *in vivo*, including epithelial barrier breakdown, proinflammatory cytokine release, inflammatory cell accumulation, and, surprisingly, eosinophilic inflammation.

The negative effects of PM on human health are dependent on particle size, composition, and induction of oxidative stress (11). Fine (2.5- μm) PM (PM_{2.5}) is particularly damaging. Exposure of human sinonasal epithelial cells to PM_{2.5} *in vitro* has been shown to disrupt epithelial barrier function and cell surface localization of cell junction proteins, including claudin-1 and junction adhesion molecule A (7). Indeed, epithelial barrier dysfunction has been proposed to contribute to the pathogenesis of multiple airway diseases, including asthma, chronic obstructive pulmonary disease, and sinonasal inflammatory disease (12, 13). As little is known about the direct effects of chronic PM_{2.5} exposure on the sinonasal airway *in vivo*, we sought to elucidate whether chronic airborne PM_{2.5} exposure at environmentally relevant levels results in destructive barrier breakdown and inflammation.

This study was presented at the European Rhinologic Society/International Society of Inflammation and Allergy of the Nose Meeting in Stockholm, Sweden in 2016 (14).

Materials and Methods

Animals

National Institutes of Health (Bethesda, MD) guidelines for the care and use of laboratory animals were strictly followed, and all experiments were approved by the Animal Care and Use Committee at the University of Maryland (Baltimore, MD).

PM Exposure

Male C57BL/6 mice (8 wk old) were exposed, by inhalation, to either filtered air

(FA) or concentrated PM_{2.5} (PM) for 6 h/d, 5 d/wk, for 16 weeks, as previously described (15). Mean ($\pm$ SD) levels of PM_{2.5} were 8.09 ($\pm$ 2.61) $\mu\text{g}/\text{m}^3$ in ambient air and 60.92 ($\pm$ 21.31) $\mu\text{g}/\text{m}^3$ in the PM exposure chamber. Mean ambient temperature was 13.26°C and mean humidity was 72%. Selection for FA or PM groups was randomized (total, $n = 38$; FA, $n = 19$; PM, $n = 19$). Inhalation exposure was conducted in a mobile exposure system. Calculation of PM_{2.5} concentrations in the exposure chambers was performed by collecting samples on Teflon filters (Teflo; 37 mm, 2- μm pore; PALL Life Sciences, Ann Arbor, MI) at least once each week. Gravimetric determinations were made using an MT-5 microbalance (Mettler Toledo, Columbus, OH) in a temperature- and humidity-controlled class 100 clean laboratory.

Nasal Histopathology

Sinonasal mucosa was extracted from mice as previously described (16). For immunostaining, primary antibodies used in this study were: rabbit anti-Krt5 (PRB-160P; Covance, Princeton, NJ); goat anti-eosinophilic major basic protein (EMBP; sc-33938; Santa Cruz, Dallas, TX); rabbit anti-myeloperoxidase (MPO; NBP1-42591; Novus Biologicals, Littleton, CO); goat anti-claudin-1 (sc-22932; Santa Cruz); and rat anti-E-cadherin (NB120-11512; Novus Biologicals). Confocal images were collected with an LSM700 laser scanning confocal microscope (Zeiss, Oberkochen, Germany). EMBP- and MPO-positive cells in sections of sinonasal tissue were counted by a blinded observer. Data are presented as mean ($\pm$ SEM) from 10 images per animal.

Subepithelial thickness was also measured by a blinded observer in hematoxylin and eosin-stained sections.

Nasal Airway Lavage Protein Analysis and Cytology

Transpharyngeal nasal airway lavage fluid (NALF) was collected as previously described (16). Nasal lavages were centrifuged at 6000 rpm for 5 minutes. Supernatants were collected and analyzed for IL-13, IL-1 β , and eotaxin-1 by sandwich ELISA (R&D Systems, Minneapolis, MN). Serum albumin was quantified by competitive ELISA (Abcam, Cambridge, UK). Cell pellets from lavage samples were resuspended in 100 μl PBS and total cells were counted. Cytospin slides were prepared using the StatSpin Cytofuge 12 (Beckman Coulter, Brea, CA) and stained with Diff-Quik stain (Dade Behring-Switzerland, Deerfield, IL), followed by a differential count of at least 200 cells per slide. Diff-Quik is a modified Wright-Giemsa involving brief methanol fixation and staining with a xanthene and thiazine dye.

Real-Time PCR

mRNA was first extracted from septal and turbinate mucosa and reverse transcribed using the Omniscript RT kit (Qiagen, Hilden, Germany) under conditions provided by the manufacturer. Real-time PCR was performed using StepOnePlus (Applied Biosystems, Foster City, CA) under standard cycling parameters. Amplicon expression of each target gene was normalized to its 18S RNA content, and the level of expression of target mRNA was

Figure 1. Chronic airborne fine (2.5- μm) particulate matter (PM_{2.5}) exposure induces sinonasal inflammatory cell accumulation in nasal airway lavage fluid. Differential cell analysis of nasal lavage fluid from filtered air (FA)- and PM_{2.5}-exposed mice ($n = 5$ per group). Data is presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

Figure 2. Chronic airborne PM_{2.5} exposure induces sinonasal inflammatory cell accumulation in sinonasal tissue. (A) Representative images of immunofluorescence analysis of sinonasal tissue for myeloperoxidase (MPO)-positive cells (green, upper row) and eosinophilic major basic protein (EMBP)-positive cells (red, lower row), indicated by white arrows; 4',6-diamidino-2-phenylindole (DAPI; blue); KRT5-positive cells as a reference for the location of horizontal basal cells (green, lower row). Scale bars: 20 μ m; dashed lines, basal lamina; L, airway lumen. (B) Quantification of MPO- and EMBP-positive cells ($n = 3$ mice per group). Data is presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

measured as the Δ Ct. Relative fold changes in gene expression compared with wild-type vehicle control were calculated as $2^{-\Delta\Delta C_t}$.

Statistical Analysis

All values are shown as mean ($\pm$ SEM). mRNA and cell counts were evaluated by using a two-tailed Student's t test. Cytokine

concentrations in nasal lavages were compared by employing the nonparametric Mann–Whitney U test. Statistical significance was considered to be P less than 0.05. Results were analyzed with the use of the statistical software, Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Figure 3. Chronic airborne PM_{2.5} exposure induces increased expression of IL-1 β , IL-13, oncostatin M (OSM), and eotaxin-1 and decreased expression of T helper type 1 (Th1) cytokines in sinonasal tissue. RT-PCR analysis of sinonasal tissue represented as fold change in mRNA ($n = 6$ –7 mice per group). ND, nondetectable levels. Data is presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

Results

To test the impact of chronic airborne PM exposure on the upper respiratory system, we subjected mice to PM_{2.5} for 16 weeks. This was performed 6 h/d, 5 d/wk, at an average airborne PM_{2.5} concentration lower than the reported annual average PM_{2.5} concentration of some large cities, including New Delhi, Cairo, and Beijing (9). To assess the sinonasal inflammatory response to chronic PM_{2.5} exposure, we first performed differential cell counts of NALF. Differential cell counts demonstrated that PM-exposed mice had

Figure 4. Chronic airborne PM_{2.5} exposure induces increased secretion of IL-1β, IL-13, and eotaxin-1 in sinonasal tissue. (A–C) ELISA analysis of IL-1β, IL-13, and eotaxin-1 content in nasal airway lavage fluid (NALF) from FA- and PM_{2.5}-exposed mice ($n = 6$ per group). Data is presented as mean $\pm$ SEM. * $P < 0.05$.

significantly elevated numbers of macrophages ($P < 0.05$), neutrophils ($P < 0.05$), and eosinophils ($P < 0.01$) in NALF compared with control mice exposed to FA (Figure 1). Next, the maxilloturbinate sinonasal mucosa was examined by immunofluorescence staining for MPO-

positive cells, and inflammatory cell infiltrates were found to be significantly increased in PM-exposed mice ($P < 0.01$; Figures 2A and 2B). The maxilloturbinate sinonasal mucosa was also examined by immunofluorescence staining for EMBP-positive cells, and eosinophil

accumulation was found to be significantly increased in PM-exposed mice ($P < 0.05$; Figures 2A and 2B).

As an inflammatory response is often orchestrated by secreted inflammatory cytokines and chemokines, we performed quantitative PCR for a panel of cytokines

Figure 5. Chronic airborne PM_{2.5} exposure induces sinonasal epithelial cell barrier dysfunction assessed by immunofluorescence. (A) Representative images of immunofluorescence analysis for nasal septal epithelial junctional protein disruption for claudin-1 (green, left column) and E-cadherin (red, middle column), as well as DAPI (blue merged image, right column). Areas of junctional protein disruption are indicated by white arrows ($n = 3$ mice). (B) Mean fluorescence intensity (MFI) quantification of immunofluorescence images ($n = 3$ mice). * $P < 0.05$. (C) Quantification of barrier dysfunction by ELISA for serum albumin in NALF ($n = 10$ per condition). Data is presented as mean $\pm$ SEM. ** $P < 0.01$.

Figure 6. Chronic airborne PM_{2.5} exposure increases sinonasal subepithelial cell thickness assessed by histology. (A) Representative images of hematoxylin and eosin staining analysis of nasal subepithelial cell thickness with high-magnification *inset*. Dashed line, basal lamina; scale bars: 10 μm. (B) Quantification of nasal subepithelial cell thickness ($n = 3$ mice per group). Data is presented as mean $\pm$ SEM. * $P < 0.05$.

and chemokines attributed to various T helper groups to characterize the scope of the inflammatory response. This analysis included IL-1 β , TNF- α , IFN- γ , IL-12p40, IL-4, IL-5, IL-13, oncostatin M, eotaxin-1, IL-17, and IL-22. We found significantly increased expression of IL-1 β , IL-13, oncostatin M, and eotaxin-1, and significantly decreased expression in IFN- γ and IL-12p40 (Figure 3). To validate our quantitative PCR findings, we analyzed nasal lavage fluids for key inflammatory protein content that was up-regulated at the transcript level. Compared with FA-exposed mice, PM exposure elicited a significant increase in the secretion of the proinflammatory cytokine, IL-1 β ($P < 0.05$; Figure 4A). Furthermore, a significant increase in the secretion of the eosinophil-

promoting cytokine, IL-13 ($P < 0.05$), and eosinophil chemokine eotaxin-1 ($P < 0.05$) was observed in PM-exposed mice (Figures 4B and 4C).

To further understand how PM_{2.5} may be contributing to sinonasal damage, we next investigated the effect of airborne PM_{2.5} on the sinonasal epithelial barrier. Indeed, the sinonasal epithelial cell layer forms a critical interface between the external environment and underlying sinonasal tissue, and protects against inappropriate exposure of the underlying tissue to external inflammatory stimuli. At the center of epithelial barrier integrity are intercellular junction proteins, including tight junctions, such as claudin-1, and adherens junction proteins, such as epithelial cadherin (E-cadherin) (10).

Immunofluorescence analysis of the epithelial barrier in PM-exposed mice revealed a significant decrease in expression of both claudin-1 and E-cadherin (Figures 5A and 5B). To assess the degree of epithelial barrier breakdown in mice exposed to airborne PM, we analyzed NALF for serum albumin content. Serum albumin in lavage fluids was significantly elevated in PM-exposed mice compared with control FA-exposed mice ($P < 0.01$; Figure 5C). Finally, hematoxylin and eosin-stained tissue sections demonstrated a significant increase in subepithelial thickness ($P < 0.001$; Figures 6A and 6B). Collectively, these data demonstrate that chronic PM_{2.5} exposure leads to significant sinonasal epithelial cell barrier dysfunction *in vivo*.

Discussion

The data in this study are the first *in vivo* evidence to demonstrate that chronic airborne PM_{2.5} exposure results in significant sinonasal inflammation and sinonasal epithelial barrier breakdown. The model of PM exposure we used to answer this question offers multiple advantages. First, the model closely mimics physiologic conditions. Mice were subjected to airborne PM_{2.5} via inhalation rather than intranasal instillation or an alternative dosing mechanism. This exposure was chronic, and occurred over a 16-week time course. Although the PM was concentrated in our study, the concentration used (60.92 µg/m³) was less than the average annual level in some major cities, including New Delhi (143.0 µg/m³), Cairo (109.6 µg/m³), and Beijing (~85 µg/m³), and comparable to a number of other major cities in Asia (17). Second, we were able to remove particles larger than PM_{2.5} and allergens, before subjecting mice to inhalation.

The sinonasal mucosa is one of the first systems of the body to be exposed to inhaled external and environmental stimuli. The sinonasal epithelial layer provides an initial physical barrier to these stimuli, and barrier dysfunction has been previously proposed to contribute to sinusitis (18). The initial inciting event *in vivo* is unclear, in terms of whether airborne PM directly disrupts epithelial barrier function, leading to increased exposure to the underlying tissue

and resultant inflammatory response, or conversely, if the inflammatory cytokines and inflammatory cell infiltration itself cause the sinonasal epithelial barrier breakdown observed in these studies. Inflammatory cytokines have also been previously reported to disrupt sinonasal epithelial barrier function (19, 20). Additional human studies have also demonstrated that *in vivo* stimulation with pollutants, such as diesel exhaust particles, can enhance nasal cytokine expression, and potentiate allergen-specific IgE production (21, 22). Further studies are necessary to clarify these potential mechanisms and to continue to identify additional health concerns from air pollutants and PM exposure.

Finally, a significant increase in eosinophilic inflammation and eosinophil-promoting cytokine and chemokine release was observed upon chronic airborne PM exposure. It is unclear as to the mechanism whereby eosinophilic inflammation is induced in this setting. However, as sinonasal epithelial cells represent a major source of eotaxin (18), a potential explanation is the direct or indirect induction of eosinophil-promoting cytokines and chemokines in response to chronic PM exposure, ultimately leading to eosinophil accumulation. Interestingly, using RPMI 2,650 cells as a model of nasal epithelial cells, one group recently reported a significant increase in IL-13 and eotaxin-1 expression after exposure to PM_{2.5} *in vitro* (23). Another potential contributing

mechanism could include potentiation of innate lymphoid cell (ILC) group 2 activation and cytokine expression. ILCs have been demonstrated to contribute to respiratory airway hyperreactivity via production of IL-13 (24). Furthermore, ILC2s interact with multiple resident cell types, and have been linked to eosinophilic inflammation (25). It would be interesting to investigate whether air pollutant-mediated potentiation of ILC activity could contribute to the findings reported in our study. As animal models of nonallergic eosinophilic inflammation are lacking, chronic airborne PM exposure may represent a novel model of NARES. The conclusions of this study are limited by one time point (16 wk) and by the lack of mechanistic studies, such as the use of knockout or tissue-specific deletion of inflammatory mediators in mice. For example, the contribution of eotaxin-1 secretion in response to PM exposure could be directly investigated through repeating these experiments in eotaxin-1-deficient mice. This study, although observational in nature, is necessary to lay the foundation for further mechanistic exploration of NARES. Future experiments, employing additional time points and use of knockout mice, will help us further understand the sinonasal inflammatory response to PM exposure *in vivo*. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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