

Osteopontin Induces Airway Remodeling and Lung Fibroblast Activation in a Murine Model of Asthma

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Airway remodeling is a central feature of asthma; however, the mechanisms underlying its development have not been fully elucidated. We have demonstrated that osteopontin, an inflammatory cytokine and an extracellular matrix glycoprotein with profibrotic properties, is up-regulated in a murine model of allergen-induced airway remodeling. In the present study, we determined whether osteopontin plays a functional role in airway remodeling. Osteopontin (OPN)-deficient (OPN^{-/-}) and wild-type mice were sensitized and exposed to inhaled ovalbumin (OVA) or saline for 5 weeks. Collagen production, peribronchial smooth muscle area, mucus-producing cell number, and bronchoalveolar cell counts were assessed. The functional behavior and phenotype of lung fibroblasts from OVA-treated OPN^{-/-} and from wild-type mice were studied using *ex vivo* cultures. OVA-treated OPN^{-/-} mice exhibited reduced lung collagen content, smooth muscle area, mucus-producing cells, and inflammatory cell accumulation as compared with wild-type mice. Reduced matrix metalloproteinase-2 activity and expression of transforming growth factor- β 1 and vascular endothelial growth factor were observed in OVA-treated OPN^{-/-} mice. Lung fibroblasts from OVA-treated OPN^{-/-} mice showed reduced proliferation, migration, collagen deposition, and α -smooth muscle actin expression in comparison with OVA-treated wild-type lung fibroblasts. Thus, OPN is key for the development of allergen-induced airway remodeling in mice. In response to allergen, OPN induces the switching of lung fibroblasts to a pro-fibrogenic myofibroblast phenotype.

Keywords: airway remodeling; fibroblast; mouse model; myofibroblast; osteopontin

Airway remodeling is a prominent pathophysiologic feature of chronic asthma. It is characterized by changes in tissue structural components, including damage and shedding of airway epithelium, mucus gland hypertrophy, increased myofibroblast number, subepithelial fibrosis, increase of airway smooth muscle (ASM) mass, and neovascularity (1–4). The poor response to treatment seen in patients with refractory asthma may be a consequence of the ongoing airway remodeling that leads to fixed airway obstruction (1, 2). Despite advances in understanding the inflammatory and immunologic features of asthma, there is limited understanding of the cellular and molecular mechanisms underlying the remodeling changes seen in chronic asthma.

An increased number of myofibroblasts has been reported in airways of patients with chronic asthma (5) and in lungs of mice chronically exposed to allergen (6). Fibroblasts/myofibroblasts

are a major source of collagens and glycoproteins, and after activation they account for the enhanced extracellular matrix (ECM) deposition that leads to the subepithelial fibrosis (7, 8). Among these up-regulated ECM proteins, collagen I, fibronectin, tenascin, laminins, and vitronectin have been shown to play a direct role in the adhesion, migration, and proliferation of lung structural cells and in the development of airway remodeling (7–10).

Osteopontin (OPN), a 44-kD ECM glycoprotein that functions both as an ECM molecule and as a cytokine, has been associated with inflammation and fibrosis/tissue repair in lung diseases (11, 12). OPN contributes to tissue fibrosis and wound healing by regulation of cell-ECM interactions, by modulation of transforming growth factor- β 1 (TGF- β 1) and matrix metalloproteinase (MMP) expression, and by enhancing angiogenesis (12–14).

OPN has recently been implicated in allergic inflammation. It regulates dendritic cell activation in a murine model of allergen-induced airway inflammation (15) and modulates IgE-dependent mast cell degranulation and migration (16). These studies suggest a role for OPN in the pathogenesis of acute airway inflammation in early stages of asthma; however, whether OPN plays a role in airway remodeling in chronic asthma has not been evaluated.

We have recently demonstrated that OPN is up-regulated in a murine model of allergen-induced airway remodeling and is expressed by eosinophils, T cells, and macrophages (17). In addition, we observed a significant correlation between lung OPN levels and airway collagen deposition and smooth muscle area (17). In the present study, we show that OPN contributes to the pathogenesis of airway remodeling and induces lung fibroblast activation and differentiation in a murine model of chronic allergen-induced experimental asthma.

MATERIALS AND METHODS

Animals

OPN^{-/-} (B6.Cg-Spp1tm1Blh/J) and C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and from Harlan Laboratories Ltd (Jerusalem, Israel). Mice were housed under specific pathogen-free conditions. The Hebrew University-Hadassah Medical School Animal Ethics Committee approved all experimental animal protocols. Ten mice per group were used.

Allergen Challenge Protocol and Lung Pathology

Mice were sensitized with intraperitoneal ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) (10 μ g OVA/1 mg Al (OH)₃ [Sigma] in 0.5 ml 0.9% saline), or saline on Days 1 and 10, and then challenged with inhaled saline or OVA (2% weight/volume diluted in 0.9% saline: 4 ml/inhalation) three times a week, starting on Day 15, for 5 weeks as described (17). Twenty-four hours after the last challenge, mice were anesthetized with 150 μ l pentobarbitone sodium (CTS Chemical Ind., Tel Aviv, Israel) and bronchoalveolar lavage (BAL) was performed as described (17). Mice were then killed by abdominal aortic transection and exsanguination. The right lung was removed and frozen in liquid nitrogen for collagen content (upper lobe) and enzyme-linked immunosorbent assay (ELISA) determination (lower lobe). The left lung was fixed by intrabronchial infusion with 4% formaldehyde (Biolab, Jerusalem, Israel). Sections of the left lung were stained with hematoxylin and eosin (H&E), Masson Trichrome, and Periodic Acid-Schiff (PAS).

(Received in original form June 8, 2008 and in final form December 17, 2008)

This study was supported by a grant from the Chief Scientist Office of the Ministry of Health, Israel (Grant N° 5878–1) and the Israel Lung Association, Tel Aviv.

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Am J Respir Cell Mol Biol Vol 41, pp 290–296, 2009

Originally Published in Press as DOI: 10.1165/rcmb.2008-0307OC on January 16, 2009

Internet address: www.atsjournals.org

Immunohistochemistry for α -Smooth Muscle Actin

Lung sections were prepared as described (17). Blocking of the sections was performed using Beat blocking solutions A and B from a Histo-mouse-SP bulk kit (Zymed Laboratories, San Francisco, CA). Slides were then exposed to monoclonal anti-mouse α -smooth muscle actin (SMA) antibody (Sigma). The primary antibody was revealed using a Histo-mouse-SP broad-spectrum (AEC) detection kit (Zymed) following the manufacturer's recommendations. All slides were counterstained with hematoxylin (Zymed).

Lung Fibroblast Isolation and Culture

Lungs were minced and incubated (37°C) for 45 minutes in collagenase solution (1 mg/ml; Sigma) and then passed through BD Falcon cell strainers (BD Biosciences, Bedford, MA), as performed in our laboratory (18). Cells were resuspended in RPMI 1640 culture medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Biological Industries Ltd, Kibbutz Beit Haemek, Israel). Cells between passages 2 and 4 were used for all experiments ($n = 3-4$).

BAL Cell Counts

BAL fluid was processed as previously described (17). A total of 200 cells were counted and classified for each slide using Neubauer Chambers (Marienfeld, Lauda-Königshofen, Germany). Results were expressed as number of cells per ml of BAL.

Determination of Lung Collagen Content

Total lung collagen content was determined using the Sircoll Collagen Assay kit (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer's recommendations. Briefly, upper lobes of right lungs were homogenized in 0.5 M acetic acid containing 1 mg of pepsin (Sigma) per 10 mg of tissue. Samples were mixed by gentle inversion for 24 hours. After centrifugation, 1 ml of Dye Sircoll reagent was added to 100 μ l of sample and mixed for 30 minutes. The collagen-dye complex was recovered by centrifugation and mixed with 1 ml of Sircoll alkali reagent. Optical density was then evaluated at 540 nm using a microtiter plate reader. Results were expressed as μ g of collagen per half lung.

Quantification of Peribronchial Smooth Muscle Area

The area of peribronchial α -SMA immunostaining was outlined and quantified using an Axiolab light microscope (Zeiss, Oberkochen, Germany), a Coolpix 990 camera (Nikon, Tokyo, Japan), and the image analysis software Image ProPlus 4.5 (Media Cybernetics, Inc., Silver Spring, MD). Results are expressed as the area of α -SMA stained per μ m of basement membrane of bronchi. Five bronchi per mouse were analyzed.

Determination of Metalloproteinase Activity

Metalloproteinase-2 (MMP-2) activity was assessed in BAL fluid by gelatin zymography as previously described (19). Briefly, equal amounts of total protein (7.5 μ g) were mixed with Laemmli buffer (Bio-Rad, Hercules, CA) and then loaded and run on an 8% SDS-polyacrylamide gel containing 1 mg/ml of gelatin for 90 minutes. The gel was then incubated in renaturing buffer for 30 minutes and transferred to developing buffer and incubated for 24 hours at 37°C. Nondigested gelatin was stained with 0.5% Coomassie brilliant blue (Sigma). Bands obtained as a result of gelatinolytic activity were analyzed by densitometry using the Quantity One 1-D Analysis software program, 2003 version (Bio-Rad).

Quantification of Mucus-Producing Cells

The number of periodic acid-Schiff (PAS)-positive and PAS-negative bronchial epithelial cells was determined in individual airways (6). Results were expressed as the percentage of PAS-positive cells per bronchus.

ELISA

ELISA for interleukin (IL)-4, IL-13, TGF- β 1, and vascular endothelial growth factor (VEGF) was performed on BAL fluid and supernatant from lung tissue homogenates using kits (R&D Systems, Minneapolis,

MN or Cytolab, Rehovot, Israel) following manufacturer's recommendations as previously described (17).

Fibroblast Migration Assay

Migration was determined using the "scratch-wound" assay (20). Briefly, 10^5 fibroblasts/well were seeding in 12-well plate and incubated (37°C, 5% CO₂) to confluence. A cell-free wound area was then created by scratching the cells with a pipette tip. The time of the scratching wound was designated as time 0. Cells were then allowed to migrate into the cell-free wound for 16 hours. Results were expressed as the percentage of the recovered wound area.

Fibroblast Proliferation Assay

Fibroblasts (10^5 /well) were seeded in 12-well plate in RPMI 1640 (Sigma) with 10% FCS (Biological Industries Ltd) on Day 0. On Days 1, 2, 3, and 4, cells were trypsinized and the number of viable cells was determined after trypan blue staining (21).

Immunofluorescence for α -SMA

Confluent fibroblasts were cultured on round coverslips and prepared as previously described (19). Cells were incubated with monoclonal anti-mouse α -SMA (Sigma) or irrelevant isotype-matched IgG antibodies (60 min at 4°C) in the presence or absence of recombinant mouse OPN or TGF- β 1 (R&D Systems). Binding was detected using Cy5-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Nuclei were counterstained with propidium iodide (Sigma). Tissue slides were examined using a 410 laser scanning confocal microscope (Zeiss) attached to a Zeiss Axiovert 135M inverted microscope. Quantification was assessed using Image-Pro Plus (Media Cybernetics) on photographs taken at a magnification of $\times 600$ and expressed as integrated optical density (IOD).

Collagen Deposition by Cultured Fibroblast Monolayers

Fibroblasts were seeded in 24-well plates and collagen deposition was determined using the Sircoll colorimetric assay as previously described (17) in the presence or absence of recombinant mouse OPN or TGF- β 1 (R&D Systems). Collagen deposition was expressed as fold increase relative to deposition by lung fibroblasts isolated from saline-treated OPN^{-/-} mice (internal control).

Data Analysis

The mean and standard error of mean (SEM) values are given for each group. Either one-way (Figure 4) or two-way Analysis of Variance (ANOVA) followed by Bonferroni post test was performed to compare groups. $P < 0.05$ was considered statistically significant.

RESULTS

Reduced Airway Remodeling in OPN^{-/-} Mice

OPN^{-/-} and wild-type (WT) mice were sensitized and then challenged with OVA or saline three times/week for 5 weeks. To determine whether OPN plays a role in the development of airway remodeling, we evaluated peribronchial fibrosis, peribronchial smooth muscle mass, and goblet cell number in OVA-treated OPN^{-/-} and WT mice.

Peribronchial fibrosis was evaluated by peribronchial trichrome staining and lung collagen content. Exposure to OVA increased the peribronchial trichrome-stained area and lung collagen levels in WT and OPN^{-/-} mice when compared with saline controls (Figures 1A and 1B). OVA-treated OPN^{-/-} mice showed reduced peribronchial collagen-stained area versus OVA-treated WT mice (Figure 1B). Lung collagen levels in OVA-treated OPN^{-/-} mice were also reduced (52.6 ± 3.7 μ g collagen/lung) versus OVA-treated WT mice (79.7 ± 9.9 μ g collagen/lung, $P < 0.01$) (Figure 1A).

Peribronchial smooth muscle was evaluated by α -SMA immunostaining. OVA challenge increased the α -SMA-stained peribronchial smooth muscle area in WT and OPN^{-/-} mice

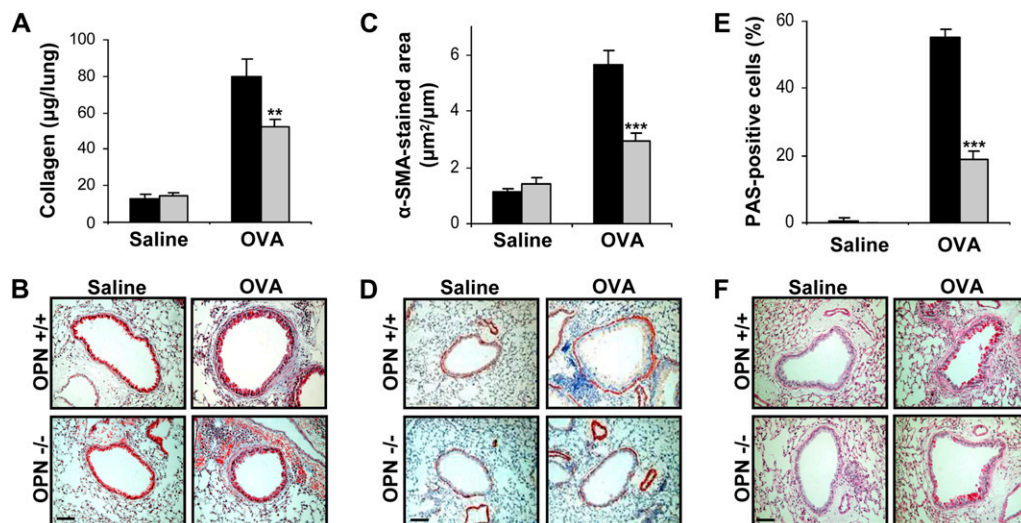


Figure 1. Reduced airway remodeling in osteopontin (OPN)^{-/-} mice. Wild-type (WT) (solid bars) and OPN^{-/-} mice (shaded bars) were exposed to ovalbumin (OVA) and saline for 5 weeks. OVA-treated OPN^{-/-} mice showed (A) reduced lung collagen content, (B) reduced peribronchial collagen deposition, (C and D) reduced peribronchial α -SMA-immunostained area, and (E and F) reduced airway mucus production versus WT mice. $^{**}P < 0.01$, $^{***}P < 0.001$; mean $\pm$ SEM; $n = 10$ /group. Bar = 50 μ m.

versus saline controls (Figure 1D). There was a reduction of the α -SMA-stained peribronchial smooth muscle area in OVA-treated OPN^{-/-} mice versus OVA-treated WT mice (2.9 ± 0.3 versus 5.6 ± 0.5 $\mu\text{m}^2/\mu\text{m}$ perimeter of bronchus, $P < 0.001$) (Figure 1C).

Lung sections of WT and OPN^{-/-} mice exposed to OVA showed increased number of PAS-positive cells in comparison with saline controls (Figures 1E and 1F). The percentage of PAS-positive cells of OVA-treated OPN^{-/-} mice was reduced (18.8 ± 2.4) versus OVA-treated WT mice (55.2 ± 2.2 , $P < 0.001$) (Figure 1E).

Reduced TGF- β 1 and VEGF Expression and MMP-2 Activity in OPN^{-/-} Mice

TGF- β 1 is an important profibrogenic mediator in asthma (4). We found a reduction of active TGF- β 1 in lungs homogenates from OVA-treated OPN^{-/-} mice versus WT mice (0.20 ± 0.02 versus 0.36 ± 0.04 ng/ml of TGF- β 1, $P < 0.01$) (Figure 2A).

VEGF levels in BAL fluid correlate with airway neovascularity (4). We found reduced VEGF in BAL fluid of OVA-treated OPN^{-/-} mice versus OVA-treated WT mice (0.09 ± 0.01 versus 0.15 ± 0.01 ng/ml, $P < 0.01$) (Figure 2B).

Increased MMP activity in BAL fluid has been reported in asthma (22). We observed reduced MMP-2 activity in BAL fluid of OVA-treated OPN^{-/-} mice versus WT mice (19.6 ± 5.5 versus 49.4 ± 7.6 units, $P < 0.05$) (Figure 2C).

OPN Deficiency Attenuates Allergen-Induced Fibroblast Activation

To examine whether OPN expression contributes to fibroblast activation in our model of allergen-induced airway remodeling, we evaluated migration, proliferation, differentiation, and collagen deposition of primary fibroblasts isolated from lungs of WT and OPN^{-/-} mice exposed to OVA.

Lung fibroblasts proliferation was reduced in OPN^{-/-} mice in comparison with WT fibroblasts (Figure 3A). Difference between cell number was maximal at Day 2 ($2.8 \pm 0.3 \times 10^5$ cells for OPN^{-/-} versus $5.5 \pm 0.4 \times 10^5$ cells for WT, $P < 0.05$).

Fibroblast migration was evaluated by the scratch wound assay (20). OPN^{-/-} lung fibroblasts exhibited reduced migration capacity versus WT lung fibroblasts as determined by percentage of recovered wound area (49.2 ± 3.2 for OPN^{-/-} versus 71.3 ± 2.1 for WT, $P < 0.001$) (Figure 3B).

Fibroblast differentiation to a myofibroblast phenotype was evaluated by immunostaining for α -SMA followed by immuno-

fluorescence quantification. α -SMA expression was increased in fibroblasts from lungs of OVA-treated WT mice versus saline controls. Fibroblasts from OVA-treated OPN^{-/-} mice exhibited reduced α -SMA levels in comparison with OVA-treated WT mice (13.3 ± 5.0 units for OPN^{-/-} versus 73.9 ± 4.6 units for WT, $P < 0.001$, $n = 6$ –8 pictures/group) (Figure 4A). To investigate whether OPN affects fibroblast synthetic capacity, we evaluated collagen deposition in fibroblasts from WT and OPN^{-/-} mice. Saline-treated WT cells and OVA-treated OPN^{-/-} cells did not show increased collagen levels, whereas OVA-treated WT cell monolayers exhibited an increase of $32.1 \pm 1.5\%$ over control ($P < 0.001$ versus OVA-treated OPN^{-/-} cells, $n = 4$ experiments) (Figure 4B). Addition of recombinant mouse OPN (50 ng/ml, 48 h) or TGF- β 1 (10 ng/ml, 48 h) to OVA-treated OPN^{-/-} lung fibroblasts restores an activated phenotype similar

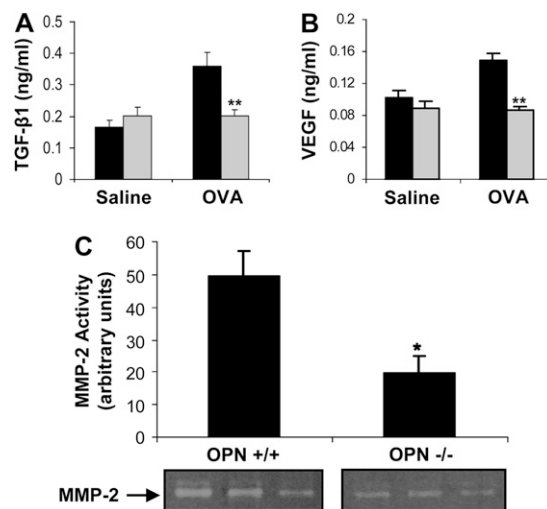


Figure 2. Reduced active transforming growth factor (TGF)- β 1, vascular endothelial growth factor (VEGF), and matrix metalloproteinase (MMP)-2 activity in OPN^{-/-} mice. OVA-treated OPN^{-/-} mice (shaded bars) showed decreased levels of lung active (A) TGF- β 1 and (B) VEGF versus WT mice (solid bars), as determined by ELISA ($^{**}P < 0.01$; mean $\pm$ SEM; $n = 10$ /group). (C) Reduction of MMP-2 activity was observed in BAL fluid from OVA-treated OPN^{-/-} mice versus WT mice, as assessed by zymography (representative images from three different mice/group) and by quantitation using densitometry ($^{*}P < 0.05$; mean $\pm$ SEM; $n = 6$ /group).

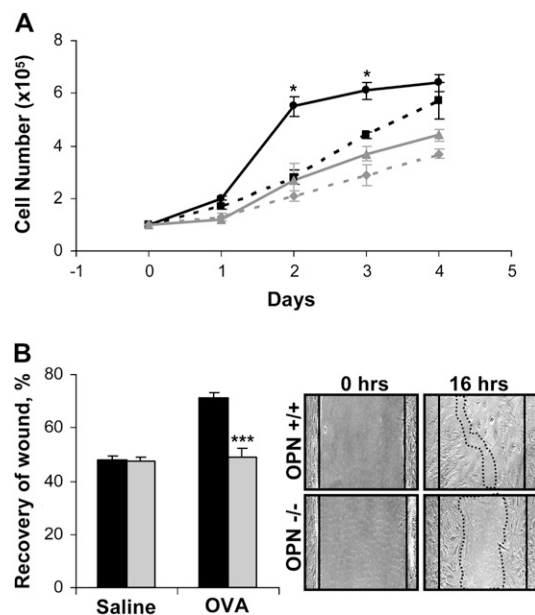


Figure 3. Impaired proliferation and migration in lung fibroblasts from OVA-treated OPN^{-/-} mice. Lung fibroblasts from OPN^{-/-} and WT mice exposed to OVA (black lines) and saline (gray lines) were isolated and cultured as described in MATERIALS AND METHODS. (A) Reduced number of OVA-treated OPN^{-/-} fibroblasts (squares, dotted lines) versus WT fibroblasts (circles, solid lines) was observed (**P* < 0.05 at Days 2 and 3) (mean ± SEM, *n* = 4). (B) Reduced fibroblast migration was observed in OVA-treated OPN^{-/-} fibroblasts (shaded bars) versus WT fibroblasts (solid bars). Results are expressed as percentage of recovered wound area. Representative images (×100) of OVA-treated lung fibroblasts at time 0 and at 16 hours of incubation are shown (****P* < 0.001; mean ± SEM, *n* = 8 pictures/group).

to that observed for OVA-treated WT lung fibroblasts (α-SMA expression: 57.7 ± 5.1 units for OPN and 69.4 ± 4.7 units for TGF-β1; and increase in collagen deposition of 31.0 ± 2.8% for OPN and 37.1 ± 1.7% for TGF-β1; Figures 4A and 4B).

OPN Deficiency Affects Airway Inflammation and Th2 Cytokine Expression Levels

We analyzed the number and type of inflammatory cells in BAL fluid. The total number of cells and number of eosinophils, macrophages, lymphocytes, and neutrophils were increased in BAL fluid of WT and OPN^{-/-} mice exposed to OVA as compared with saline-treated mice (controls) (Table 1). OVA-treated OPN^{-/-} mice showed reduced total cell number (781.3 ± 62.2 × 10³/ml) versus OVA-treated WT mice (1,457.3 ± 70.8 × 10³, *P* < 0.001) and reduced number of eosinophils versus OVA-treated WT mice (82.8 ± 17.4 × 10³ versus 289.3 ± 27.6 × 10³, *P* < 0.001) (Table 1). H&E-stained lung sections from OVA-treated OPN^{-/-} mice also showed a reduction in perivascular and peribronchial inflammatory infiltrates compared with WT mice (data not shown).

We also evaluated the concentrations of Th2-associated cytokines (IL-4 and IL-13) in lung tissue homogenates and BAL fluid from WT and OPN^{-/-} mice exposed to OVA and saline (Figure 5). Levels of IL-4 and IL-13 were significantly reduced in lung tissue and BAL fluid from OVA-treated OPN^{-/-} mice compared with OVA-treated WT mice.

DISCUSSION

In the present study we demonstrate that OPN is an important contributor to the development of different aspects of airway

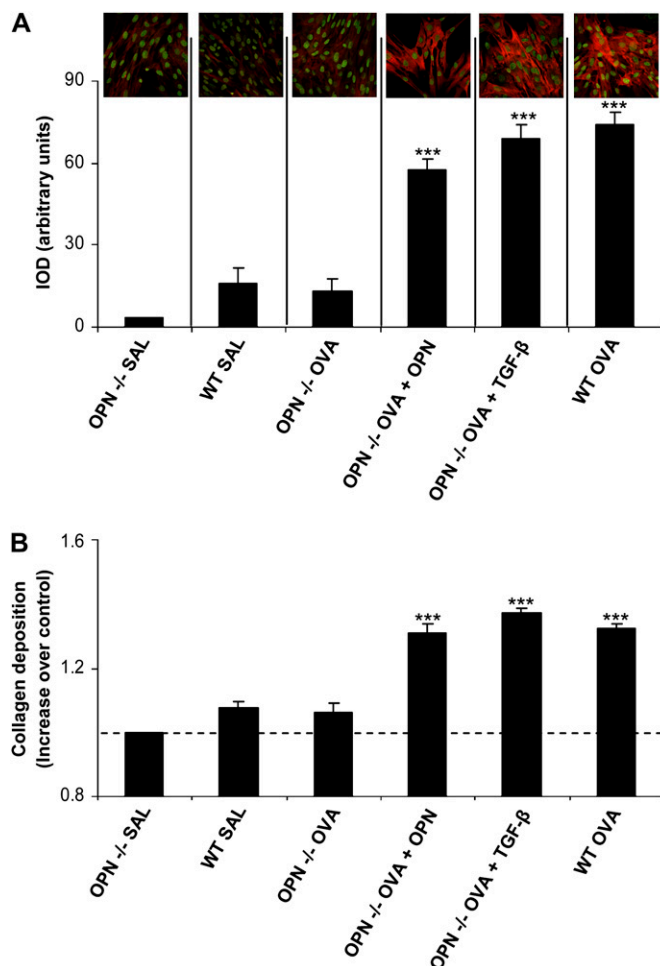


Figure 4. Lung fibroblasts isolated from OVA-treated OPN^{-/-} mice exhibit an impaired myofibroblast phenotype that is restored by the addition of "extrinsic" OPN. (A) Reduced immunofluorescence for α-SMA, expressed as integrated optical density (IOD), and (B) reduced collagen deposition, assessed by the Sircoll colorimetric assay, were observed in OVA-treated OPN^{-/-} fibroblasts versus WT fibroblasts. Addition of recombinant OPN (50 ng/ml, 48 h) or TGF-β1 (10 ng/ml, 48 h) to OVA-treated OPN^{-/-} lung fibroblasts restored α-SMA expression and collagen deposition to levels comparable to OVA-treated WT fibroblasts (****P* < 0.001, mean ± SEM, *n* = 6–8 pictures/group for α-SMA, *n* = 4 for collagen deposition).

remodeling in a murine model of chronic allergen-induced experimental asthma. In comparison with OVA-treated WT mice, OPN^{-/-} mice chronically exposed to OVA show reduced subepithelial fibrosis, peribronchial smooth muscle area, and airway mucus-producing cell number. We also show that OPN is required for allergen-induced lung fibroblast activation, as demonstrated by the impaired proliferation, migration, α-SMA expression, and collagen synthesis observed in fibroblasts from lungs of OVA-treated OPN^{-/-} mice.

Increased subepithelial fibrosis, a hallmark of airway remodeling in chronic asthma, is characterized by the increased deposition of collagens, primarily type I and III, and other extracellular matrix proteins (1–4). OPN is up-regulated in a number of fibrotic pulmonary diseases and is likely to play a role in their pathogenesis (12). We have previously demonstrated that OPN expression is increased in lungs of mice chronically exposed to OVA and significantly correlates with lung collagen production (17). In the present study, we show

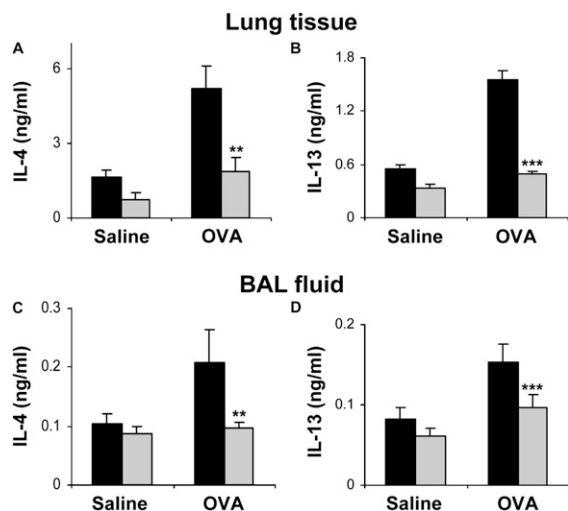


Figure 5. Reduced Th2 cytokine release in OPN^{-/-} mice. (A and C) IL-4, (B and D) IL-13 levels were reduced in lung tissue homogenates and BAL fluid from OVA-treated OPN^{-/-} mice (shaded bars) versus WT mice (solid bars) (***P* < 0.01, ****P* < 0.001; mean ± SEM; *n* = 10/group).

that the absence of OPN led to reduced peribronchial collagen deposition in response to chronic exposure to OVA. Our observations are consistent with previous studies performed with OPN^{-/-} mice that showed less collagen production in the kidney in a model of interstitial renal fibrosis (23), in the heart after myocardial infarction (24), and in the lung in a model of pulmonary fibrosis (14). After skin incisional injury, Liaw and coworkers showed that OPN^{-/-} mice had abnormal matrix organization due to less collagen fibrillogenesis, which may be a consequence of altered collagen synthesis and/or turnover (25). TGF-β1 and MMP-2 are important modulators of lung collagen homeostasis and are up-regulated in murine models of allergen-induced asthma (2, 4, 26). In our study, we observed reduced expression of active TGF-β1 and impaired MMP-2 activity in OPN^{-/-} mice after chronic exposure to OVA. OPN may modulate TGF-β1 expression (11, 14), enhances MMP-2 activation (27), and has been shown to be associated with TGF-β1 and MMP-2 in the fibroblastic foci isolated from lung tissue of patients with idiopathic pulmonary fibrosis (28). Using a bleomycin-induced lung fibrosis model, Berman and colleagues reported reduced active TGF-β1 and MMP-2 activity in OPN^{-/-} mice and proposed a role for OPN in fibrosis (14). Although our results provide evidence for a role for OPN in OVA-induced fibrosis by TGF-β1- and MMP-2-dependent signals *in vivo*, additional studies are necessary to clarify the pathophysiological role of OPN in this context.

Increased fibroblast activation and differentiation to myofibroblasts has been reported in patients with asthma (5) as well

as in lungs of mice chronically exposed to OVA *in vivo* (6) and *ex vivo* (21). Although *ex vivo* cellular responses may not necessarily predict their behavior *in vivo*, we found that lung fibroblasts isolated from OVA-treated OPN^{-/-} mice have a less pro-fibrotic phenotype, as shown by their reduced migration, proliferation rate, collagen deposition, and α-SMA expression compared with WT fibroblasts. OPN-dependent fibroblast activation and differentiation in our model may be via both a direct and an indirect effect of OPN on fibroblasts. Evidence in support of a *direct* role for OPN includes the ability of OPN to restore pro-fibrotic responses (collagen deposition and α-SMA expression) in OVA-treated OPN^{-/-} fibroblasts to levels comparable to WT fibroblasts, as well as previous studies that demonstrate a direct pro-fibrotic effect for OPN on lung fibroblasts *in vitro* including enhanced migration, adhesion, proliferation, and collagen synthesis (17, 29, 30). Other studies have also demonstrated a direct activatory role for OPN on cardiac fibroblasts (31). OPN-induced lung fibroblast activation is mediated via binding to CD44 and/or αv integrin receptors located on the cell surface (29, 30). OPN may also activate fibroblasts *indirectly* via inducing the expression of pro-fibrogenic factors from inflammatory and structural cells. This notion is supported by two findings: first, OVA-treated OPN^{-/-} mice show reduced expression of TGF-β1, IL-4, and IL-13; and second, the addition of TGF-β1 to fibroblasts isolated from OVA-treated OPN^{-/-} mice restores the activated phenotype observed in fibroblasts isolated from WT mice. TGF-β1 as well as IL-4 and IL-13 have direct effects on fibroblasts (1), promote proliferation of bronchial fibroblasts derived from patients with asthma (32), and are critical for the induction of OVA-induced airway remodeling in animal models (1, 4). Lenga and coworkers demonstrated that OPN is critical for TGF-β1-induced activation and differentiation of primary cardiac fibroblasts (33). Our results strongly suggest that OPN^{-/-} fibroblasts *in vivo* are less activated because they are not exposed to the same pro-fibrotic milieu present in OVA-treated WT mice, but these fibroblasts retain the potential to undergo activation when stimulated. This pro-fibrotic milieu includes multiple growth factors and cytokines, including OPN, TGF-β1, IL-4, IL-13, and many others.

The mechanism of OPN-induced fibroblast activation (and remodeling as a whole) may also be secondary to its effect on allergen-induced airway inflammation. In the present study, we observed that OPN deficiency leads to a reduction in the number and type of inflammatory cells after chronic allergen exposure. It has recently been shown that OPN modulates different subpopulations of dendritic cells after acute OVA sensitization and challenge (15), thereby altering cytokine profiles in the airways. Eosinophil numbers are reduced in OVA-treated OPN^{-/-} mice, and these cells produce TGF-β1 and Th2 cytokines (34), thereby contributing to the remodeling process. Inflammatory cells are also an important source of OPN. OPN is expressed by circulating CD4⁺ T cells from

TABLE 1. DIFFERENTIAL CELL COUNTS IN BRONCHOALVEOLAR LAVAGE FLUID

		Total Cells	Eosinophils	Macrophages	Lymphocytes	Neutrophils
Wild-type	Saline	140.5 ± 44.1	1.3 ± 0.9	135.8 ± 42.0	3.1 ± 1.5	0.3 ± 0.2
	OVA	1457.3 ± 70.8	289.3 ± 27.6	519.0 ± 46.2	191.3 ± 14.9	457.7 ± 45.2
OPN ^{-/-}	Saline	122.9 ± 36.9	0.1 ± 0.1	115.2 ± 35.1	7.6 ± 3.3	0 ± 0
	OVA	781.3 ± 62.2*	82.8 ± 17.4*	355.6 ± 42.3	132.2 ± 15.8†	210.7 ± 34.7*

Definition of abbreviations: OPN, osteopontin; OVA, ovalbumin.

Total and differential cell counts were performed as described in MATERIALS AND METHODS; counts shown are number of cells (×10³) per ml. Data are mean ± SEM of 10 mice per group.

* *P* < 0.001 (OVA WT vs. OVA OPN^{-/-} mice).

† *P* < 0.01 (OVA WT vs. OVA OPN^{-/-} mice).

individuals with asthma (35) and by mast cells (16). OPN is expressed by T cells, eosinophils, and macrophages in lungs of mice chronically exposed to OVA (17). IL-4 and IL-13 regulate OPN expression by human monocytes and monocyte-derived dendritic cells (36).

The association between OPN, airway inflammation, and airway remodeling *in vivo* is therefore complex, as is the link between inflammation and remodeling in asthma in general. Whether airway inflammation and airway remodeling are sequential or parallel processes is as yet unresolved and has not been investigated adequately in animals due to the lack of suitable models (37). Our study does not allow us to definitively determine whether the predominant effect of OPN on airway remodeling is via a direct effect on fibroblasts or a consequence of altered inflammation; however, our results suggest that both mechanisms are likely to be important.

Although the present study focuses primarily on OPN-induced fibrosis and fibroblast activation, we also demonstrate that OVA-treated OPN^{-/-} mice show attenuation of additional parameters of remodeling. Airway smooth muscle (ASM) cell hyperplasia and hypertrophy is observed in airways of patients with chronic asthma (2–4) and in animal models of chronic allergen challenge (4, 6). We have previously demonstrated that increased OPN expression correlates with increased peribronchial smooth muscle area (17), and now demonstrate that the absence of OPN leads to a reduction of airway smooth muscle. These findings implicate OPN as a key mediator of allergen-induced ASM cell hyperplasia and hypertrophy. OPN has previously been shown to induce vascular smooth muscle cell migration via β_3 -integrin (38) and arterial smooth muscle cell proliferation *in vitro* (39). IL-4 and IL-13, as well as TGF- β 1, are associated with increased human bronchial smooth muscle cell proliferation (40). Further studies will be helpful to clarify whether OPN stimulates ASM cell hyperplasia and hypertrophy in airway remodeling by acting directly through ASM cells or by modulating the effect of other mediators. Goblet cell hyperplasia and enhanced mucus production have been reported in murine models of allergen-induced airway inflammation and remodeling and were associated with IL-13 expression (41, 42). We observed that OPN^{-/-} mice had reduced numbers of mucus-containing airway goblet cells. Thus, OPN may contribute to goblet cell hyperplasia and mucus production via its effect on IL-13 and other cytokines. Neovascularity is another common feature of airway remodeling (2–4). OPN promotes angiogenesis *in vitro* and *in vivo* (43), although others have observed an inhibitory effect (44). We have previously shown that OPN (together with VEGF) accounts for eosinophil-mediated angiogenesis (45). In the present study, we found that the elevation in VEGF levels after OVA challenge is abrogated in OPN^{-/-} mice, thus adding further evidence for a contribution of OPN to angiogenesis in airway remodeling.

To date, there is no effective treatment for airway remodeling. In this study, we demonstrate that OPN may be an attractive therapeutic target to attenuate this process. In addition to its pathologic role observed in chronic asthma, OPN plays a role in normal bone remodeling (46) and wound healing (11, 25), raising the possibility that neutralizing OPN may have deleterious effects on these physiologic processes. Nevertheless, to date there is no evidence to support this concern, and OPN^{-/-} mice are phenotypically normal, with normal growth, external morphology, fertility, and behavior compared with WT mice (25).

In summary, we demonstrate that OPN plays a key role in the development of multiple aspects of allergen-induced airway remodeling in mice. Our study provides evidence for a complex pro-fibrotic role for OPN on lung fibroblasts after chronic

allergen exposure *in vivo*. OPN may modulate lung fibroblast phenotype and activation via a direct effect on these cells or via an indirect effect that involves altered airway inflammation and the expression of fibrotic mediators such as TGF- β 1, MMP-2, and Th2 cytokines (IL-13 and IL-4), which in turn alter fibroblast behavior.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Anita Kol and Golan Buznak (graphic designer) for their technical assistance. The authors are grateful to Prof. Philip Lazarovici and Shimon Lecht (Dept. of Pharmacology, School of Pharmacy, Hebrew University of Jerusalem) for their assistance in performing zymography experiments.

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