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Enhanced osteopontin expression in a murine model of allergen-induced airway remodelling

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Clinical and Experimental Allergy

Summary

Background Airway remodelling is a central pathophysiological feature of chronic asthma. A wide variety of cytokines and growth factors are likely to be involved in the development of airway remodelling. Osteopontin (OPN) is a cytokine with pro-fibrotic properties; however, its role in airway remodelling in asthma has not been explored.

Objective To determine the expression and cellular sources of OPN in a murine model of chronic allergen-induced airway remodelling.

Methods BALB/c mice were sensitized and exposed to ovalbumin (OVA) or saline inhalations for 5 weeks and killed 24 h after the last inhalation. The following parameters of inflammation and remodelling were assessed: differential cell counts in bronchoalveolar lavage (BAL) fluid lung collagen content (colorimetric biochemical assay) and peribronchial smooth muscle content (immunohistochemistry, followed by image analysis). OPN expression in BAL and lung tissue was determined by PCR and ELISA. The cellular source and distribution of OPN were evaluated by immunohistochemistry and immunofluorescence.

Results OPN expression is up-regulated in lung tissue and in BAL fluid of OVA-treated mice and correlates with collagen content and peribronchial smooth muscle area. In addition, OPN significantly increases collagen deposition *in vitro* in a murine lung cell line. Cells producing OPN include the airway epithelium and cells of the submucosal inflammatory infiltrate (T cells, eosinophils, and macrophages). Positive staining for OPN was also observed in bronchial tissue from human asthmatic subjects.

Conclusion OPN expression in the lungs is increased in a murine model of allergen-induced chronic airway remodelling, suggesting a role for this cytokine in airway remodelling in asthma.

Keywords allergen-induced airway remodelling, asthma, osteopontin

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Introduction

Asthma is characterized by chronic airway inflammation as well as alteration of structural components of the airways. These changes, collectively referred to as 'airway remodelling', include damage and shedding of the airway epithelium, fibroblast proliferation and differentiation to myofibroblasts, increase of airway smooth muscle mass, reticular basement membrane thickening due to extracellular matrix (ECM) deposition, mucus gland hypertrophy, and neovascularity [1–3].

Several mediators are involved in airway remodelling in asthma. The most recognized is TGF- β 1, a cytokine that

stimulates fibroblast proliferation and differentiation, and enhances ECM production [4, 5]. 'Classical' T-helper type 2 (Th2) cytokines (IL-4, IL-5, and IL-13), inflammatory cytokines (TNF- α , IL-1, IL-6 and IL-11), growth factors [platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and endothelin-1], colony-stimulating factors [granulocyte macrophage colony-stimulating factor (GM-CSF)], and chemokines (CCL11/eotaxin) may all have a role in airway remodelling [6, 7].

Osteopontin (OPN) is a phosphorylated acidic glycoprotein that contains the arginine-glycine-aspartate (RGD) integrin-binding domain common to matrix proteins. It can function as both an ECM molecule and a cytokine [8].

Epithelial cells, macrophages, T cells and fibroblasts have been shown to express OPN [9–11]. By acting through a number of receptors, including CD44 isoforms, α_v (β_1 , β_3 , or β_5), and (α_4 , α_5 , α_8 , or α_9) β_1 integrins [8, 12], OPN can mediate interactions between cells and ECM, recruit T cells, stimulate Th1 cytokine expression, and promote angiogenesis [13, 14]. OPN also regulates tissue repair via alteration of active TGF- β 1 and matrix metalloproteinase (MMP) expression [15, 16].

OPN is up-regulated in a variety of pulmonary diseases and is likely to play a role in their pathogenesis [17]. It is involved in the formation of pulmonary granulomas in sarcoidosis via stimulation of the expression of Th1-derived cytokines such as IFN- γ , IL-12, and CD40 ligand [17, 18]. OPN is also overexpressed in a murine model of bleomycin-induced lung fibrosis and in human idiopathic pulmonary fibrosis, in which a pro-fibrotic role for OPN has been proposed [19–21]. Studies in animals demonstrate that OPN expression is critical for the development of lung metastasis [22], although this has not been demonstrated in humans. In contrast to the plethora of data linking OPN with fibrotic lung diseases, no studies have explored the role of OPN in asthma.

We hypothesized that OPN may play a role in the pathogenesis of asthma, and particularly in the remodelling seen in this disease. In this study, we report the up-regulation of OPN in a murine model of chronic allergen-induced airway remodelling. OPN expression significantly correlates with the structural changes observed in the model. We also characterize the cellular sources of OPN in mice exposed to ovalbumin (OVA). In addition, we detected OPN expression in bronchial tissue of human-asthmatic subjects. Thus, our data suggest that OPN may play a role in airway remodelling in asthma.

Methods

Allergen challenge protocol

Female BALB/c mice (Harlan Laboratories Ltd, Jerusalem, Israel), aged 6–8 weeks, were housed under specific pathogen-free (SPF) conditions and were provided with adequate food and water. Animals were sensitized with intraperitoneal OVA [10 μ g OVA/1 mg Al (OH) $_3$ in 0.5 mL 0.9% saline (Sigma, St Louis, MO, USA)], or with saline on days 1 and 10. Mice were then challenged with inhaled saline or OVA (2% weight/volume diluted in 0.9% saline: 4 mL/inhalation) three times a week for 5 or 10 weeks, starting on day 15. For OVA and saline challenges, mice were transferred into a perspex 30 $\times$ 40 $\times$ 40 cm box and exposed to saline or OVA administered by means of a micromist nebulizer (Hudson RCI, Temecula, CA, USA) feeding directly into the box at a flow rate of 7 L/min for 20 min. The box has additional holes for air circulation. Mice were killed 24 h after the last challenge. The groups

contained six treated (OVA) and six control animals (SAL). The Hebrew University – Haddasah Medical School Ethics Committee approved all experimental animal protocols.

Lung pathology

Animals were anaesthetized using 150 μ L pentobarbitone sodium (200 mg/mL, CTS Chemical Ind., Tel Aviv, Israel) and a plastic cannula was placed in the trachea via a small transverse incision made in the neck. The trachea was exposed by blunt dissection and the cannula was tied in position. Bronchoalveolar lavage (BAL) was performed using four aliquots of 1 mL warmed 0.9% saline. Animals were then killed by abdominal aortic transection and exsanguination. Thoracotomy was performed and the right main bronchus was tied. The right lung was removed and snap frozen in liquid nitrogen for RNA analysis (lower lobe) and for tissue ELISA or collagen content determination (upper lobe). The left lung was fixed by intrabronchial infusion with 1 mL 4% formaldehyde (Biolab, Jerusalem, Israel), and then immersed in a fixative for an additional 24 h. Transverse sections of the left lung were embedded in paraffin, and sequential 4–6 μ m sections were stained with haematoxylin and eosin or used for immunostaining.

Paraffin-embedded sections (4–6 μ m) of endobronchial biopsies from asthmatic patients were obtained from pathology archives and used for immunostaining. Patients had long-standing (>15 years) moderate to severe asthma and were receiving high doses of inhaled corticosteroids ($n = 3$).

Bronchoalveolar lavage cell counts

BAL fluid was centrifuged for 10 min at 180 g and the supernatants were stored at -20°C for ELISA. Cell pellets were resuspended in 1 mL phosphate-buffered saline (PBS) and aliquots of 100 μ L were used for cytopspin preparations using a Shandon cytopspin 3 (Thermo Fisher Scientific, Waltham, MA, USA), and stained using Diffquik stain (Dade Diagnostica, Munich, Germany). A total of 200 cells were counted and classified for each slide. Total BAL cells were counted using a Newbauer Chamber (Marienfeld, Lauda-Königshofen, Germany). Results are expressed as number of cells per millilitre of BAL.

Polymerase chain reaction for osteopontin

Total RNA from right-lung lower lobes was extracted using TRI reagent (Sigma). Initial reverse transcription and qualitative PCR were performed using standard conditions, according to the manufacturer's recommendations (Promega, Madison, WI, USA). The primers were as follows:

Forward primer: 5'-TGT GTC CTC TGA AGA AAA GGA TGAC-3'

Reverse primer: 5'-TCT GTG GCA TCA GGA TAC TGT TCA-3'

Quantitative real-time PCR analysis was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR mixture contained 4 µL cDNA, 10 µL of 2 × Taqman Universal PCR master mix (Applied Biosystems), 1 µL of 20 × mix of unlabelled primers, and a 5'FAM-labelled fluorescent probe in a total volume of 20 µL. Primer pairs and probe were assay on demand. Gene expression products were designed by Applied Biosystems. The house-keeping gene 18S RNA was used as the internal control for normalization of the samples and DEPC water served as the negative control to verify the specificity of the reaction. Probe sequences for the genes analysed are as follows:

OPN: 5'-CGAGAAGCTTTACAGCCTGCACCCA-3'

18S: 5'-TGGAGGGCAAGTCTGGTGCCAGCAG-3'

For each primer pair, a dilution curve of a positive cDNA sample was included to calculate the efficiency of the amplification. Universal thermal cycling parameters were used for all reactions, with an initial denaturing step at 95 °C for 15 s, followed by annealing/extension at 60 °C for 1 min. Forty cycles were performed and each sample was amplified in duplicate. The threshold cycle (C_t) was recorded for each sample to reflect the mRNA expression level and relative quantification was determined using ΔC_t calculated as $C_{t(\text{target gene})} - C_{t(18S \text{ gene})}$. Product is expressed as fold change of gene expression according to the formula $2^{-\Delta C_t}$.

Enzyme-linked immunosorbent assay for osteopontin

ELISA for OPN was performed on BAL fluid and lung tissue homogenates using a Duoset ELISA kit (R&D systems, Minneapolis, MN, USA) following the manufacturer's recommendations.

For ELISA determinations from tissue homogenates, lung upper lobes stored at -80 °C were placed in ELISA buffer composed of bovine serum albumin 1% (Sigma), phenol red 0.005% (Sigma), Tween 20 0.05% (JT Baker, Phillipsburg, NJ, USA), and sodium azide (NaN_3) 0.1% (BDH, Poole, UK), pH 7.4, at a 100 mg lung tissue/0.5 mL buffer ratio. Tissue was then homogenized, transferred to an Eppendorf tube, and centrifuged at 20 800 g for 10 min. ELISA was performed using the supernatants.

Immunohistochemistry

Immunohistochemical staining was performed on 4–6 µm paraffined sections of murine or human lung tissue following formaldehyde fixation. The sections were deparaffinized with a HistoChoice clearing agent (Sigma) or with xylene (Gadot Lab Supplies Ltd, Netanya, Israel). The slides were then dried with ethanol (JT Baker) and washed. Antigen retrieval was performed in citrate buffer 0.01 M, pH = 6. The slides were then placed in 0.2% Triton (BDH).

After rewashing, the sections were immersed in 0.3% H_2O_2 (Sigma) in methanol to quench endogenous peroxide and were then incubated with BeatTM blocking solutions A and B from a HistomouseTM-SP bulk kit (Zymed Laboratories, South San Francisco, CA, USA). For α -smooth muscle actin (α -SMA) immunostaining, a monoclonal anti-mouse α -SMA antibody (Sigma) was added. The primary antibody was subsequently detected using a HistomouseTM-SP broad-spectrum (AEC) detection kit (Zymed Laboratories) following the manufacturer's recommendations. For OPN immunostaining, a monoclonal anti-mouse OPN antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. The primary antibody was subsequently detected using a Dako detection kit (DakoCytomation, Glostrup, Denmark) following the manufacturer's recommendations. Finally, all slides were counterstained with haematoxylin (Zymed Laboratories).

Immunofluorescence

Co-staining was performed on 4–6 µm paraffinized sections of lung tissue. After deparaffinization, the slides were incubated with 2C11 (hamster anti-mouse CD3, a gift from Prof Zalman Ben-Sasson, The Lautenberg Center for General and Tumor Immunology, Hadassah-Hebrew University Medical School, Jerusalem, Israel), rat anti-mouse CCR3-Fluorescein (R&D Systems), or rat anti-mouse F4/80 (eBioscience, San Diego, CA, USA), and with mouse anti-OPN (Santa Cruz Biotechnology). Binding was detected by exposing the slides to Cy5 anti-mouse, FITC anti-Armenian hamster, or FITC anti-rat antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Tissue slides were examined using a 410 laser scanning confocal microscope (Zeiss, Carl Zeiss, AG, Oberkochen, Germany) attached to the Zeiss Axiovert 135M inverted microscope. All the pictures were taken from the sub-epithelial inflammatory infiltrate of the bronchial wall.

Quantification of peribronchial smooth muscle area

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

Determination of lung collagen content

Total lung collagen content from the right upper lung lobes was determined using the Sircol Collagen Assay kit (Biocolor Ltd, Belfast, Northern Ireland) according to the manufacturer's instructions.

Table 1. Differential cell counts in bronchoalveolar lavage fluid

	Number per mL ($\times 10^5$) (%)				
	Cells	Eosinophils	MΦs	Lymphocytes	PMNs
Saline	2.11 $\pm$ 0.26	0 $\pm$ 0 (0)	2.03 $\pm$ 0.24 (96.2)	0.08 $\pm$ 0.02 (3.8)	0 $\pm$ 0 (0)
OVA	23.87 $\pm$ 6.21*	10.27 $\pm$ 3.35 (43) [†]	7.65 $\pm$ 1.15 (32)	5.21 $\pm$ 1.61 (21.8)	0.74 $\pm$ 0.10 (3.2)

Total and differential cell counts were performed as described in 'Methods'. Data are mean $\pm$ SEM of six mice per group.

* $P < 0.001$; [†] $P < 0.01$.

MΦs, macrophages; PMNs, polymorphonuclear leucocytes; SEM, standard error of the mean; OVA, ovalbumin.

Determination of collagen deposition by cultured mouse lung fibroblasts

MLg, a mouse normal lung fibroblast cell line, was obtained from ATCC (Rockville, MD, USA). MLg cells were seeded in 24-well plates and incubated in minimum essential medium (MEM, Biological Industries Ltd, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) at 37 °C in 5% CO₂. When cells reached semi-confluence (~80%), they were exposed to increasing concentrations of OPN (Sigma) (5–500 ng/mL) for 48 h. Collagen deposited in each well was stained using the Sircol Collagen Assay kit (Biocolor Ltd) according to the manufacturer's instructions. The amount of collagen was assessed by spectrophotometry at 540 nm. Results were expressed as fold increase over untreated cells.

Data analysis

The mean and standard error of mean values are given for each group. Comparison between saline- and OVA-treated groups was performed using the non-parametric Mann-Whitney test. For experiments performed with MLg cells, parametric ANOVA, followed by the Tukey-Kramer multiple comparison tests were used. $P < 0.05$ was considered to be statistically significant.

Results

Chronic ovalbumin challenge induces accumulation of inflammatory cells in bronchoalveolar lavage fluid and structural changes in the airways

To examine airway inflammation and remodelling after chronic allergen challenge, mice were sensitized and challenged with either OVA or saline. The number of inflammatory cells in BAL fluid, the lung collagen content, and the area of the peribronchial smooth muscle layer were evaluated.

As expected, OVA challenge induced a significant increase in the total number of inflammatory cells and in the number of eosinophils in BAL fluid of mice exposed to

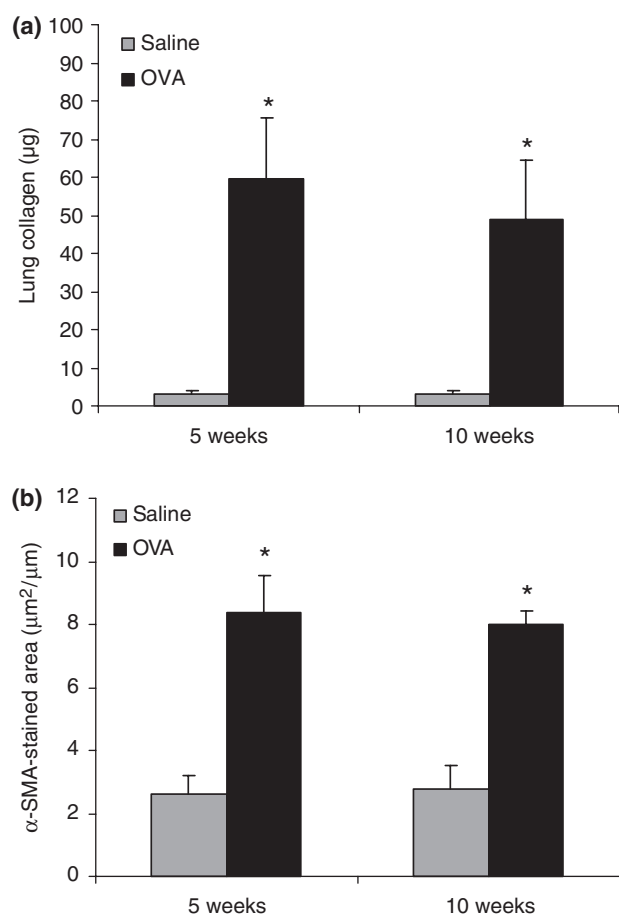


Fig. 1. Chronic ovalbumin (OVA) challenge induces structural changes in the lung. (a) After 5 and 10 weeks of inhalations, lung collagen content was significantly increased in ovalbumin (OVA)-treated mice in comparison with saline-treated controls, and (b) peribronchial α -smooth muscle actin (α -SMA)-immunostained area (per μm basement membrane) was significantly increased in OVA-treated mice in comparison with saline-treated controls. Saline vs. OVA, * $P < 0.05$; $n = 6$ for all groups, data are presented as mean $\pm$ standard error of mean.

the allergen for 5 weeks when compared with saline-treated control mice (Table 1). Histological examination of lung sections of the mice stained with haematoxylin

and eosin showed inflammatory cell infiltrates in perivascular and peribronchial regions (data not shown). No significant differences were observed in the number of inflammatory cells in BAL fluid after 10 weeks of exposure to OVA (as compared with 5 weeks, data not shown).

Increased subepithelial deposition of ECM proteins, especially collagens, is recognized as a prominent feature of airway remodelling in asthmatic airways [1–3]. We quantified the total collagen content in lungs of OVA- and saline-treated mice using a biochemical colorimetric assay. Total collagen content was significantly increased in lungs of animals exposed to OVA for 5 weeks (OVA collagen $59.48 \pm 16.28 \mu\text{g}$ vs. saline collagen $2.97 \pm 0.82 \mu\text{g}$, $P < 0.05$, Fig. 1a).

Another prominent feature of airway remodelling in asthma is airway smooth-muscle cell hypertrophy and hyperplasia [1–3]. Using α -SMA immunostaining and image processing software, we quantified the area of the peribronchial smooth muscle layer in saline- and OVA-treated mice. The area of the peribronchial smooth muscle layer of lungs of OVA-treated mice was significantly increased after exposure to OVA for 5 weeks (OVA $8.38 \pm 1.18 \mu\text{m}^2/\mu\text{m}$ bronchial perimeter, vs. saline $2.63 \pm 0.55 \mu\text{m}^2/\mu\text{m}$ bronchial perimeter, $P < 0.05$) (Fig. 1b).

Similar amounts were obtained for both remodelling parameters determined in mice exposed to OVA for 10 weeks (Fig. 1).

Chronic ovalbumin challenge up-regulates osteopontin in bronchoalveolar lavage fluid and in lung tissue homogenates

We investigated the expression of OPN at both mRNA and protein levels in lung tissue homogenates and in BAL fluid of mice exposed to OVA and saline (controls) for 5 weeks. PCR experiments showed a stronger intensity in the OPN mRNA bands of lung tissue extracts derived from OVA-treated mice as compared with controls (Fig. 2a). Quantitative real-time PCR experiments revealed a significant increase of OPN mRNA expression in lung tissue homogenates of OVA-treated mice when compared with controls (OVA 16.94 ± 5.93 vs. saline 1.84 ± 0.90 , $P < 0.01$, Fig. 2b). The increment of OPN mRNA expression was accompanied by an increase in the amount of the immunoreactive OPN protein in lung tissue homogenates as assessed by ELISA (saline $1.4 \pm 0.2 \text{ ng/mL}$ vs. OVA $8.3 \pm 1.3 \text{ ng/mL}$, $P < 0.01$, Fig. 2c). In addition, the concentration of soluble OPN in BAL fluid of OVA-treated

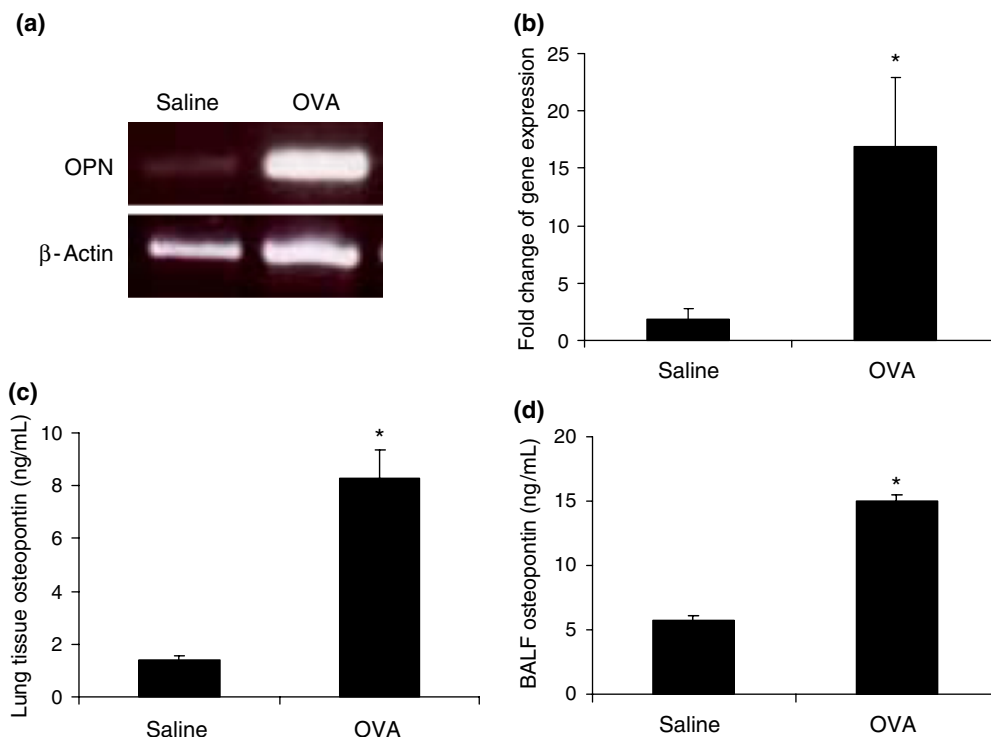


Fig. 2. Osteopontin (OPN) is up-regulated in a murine model of chronic inflammation and remodelling. In comparison with saline-treated controls, OPN mRNA, as determined by PCR (a, b), and lung tissue OPN protein expression, as determined by ELISA (c), are significantly augmented in mice exposed to ovalbumin (OVA) for 5 weeks. Levels of soluble OPN (d) in bronchoalveolar fluid (BALF) of mice exposed to OVA for 5 weeks are also significantly increased in comparison with saline-treated controls. * $P < 0.01$; $n = 6$ for all groups, data are presented as mean $\pm$ standard error of mean.

mice after 5 weeks of inhalations was also significantly increased when compared with saline controls (saline 5.8 ± 0.3 ng/mL vs. OVA 15.0 ± 0.5 ng/mL, $P < 0.01$, Fig. 2d). The results of OPN mRNA and protein expression obtained for mice exposed to OVA for 10 weeks were similar to those obtained after 5 weeks (data not shown).

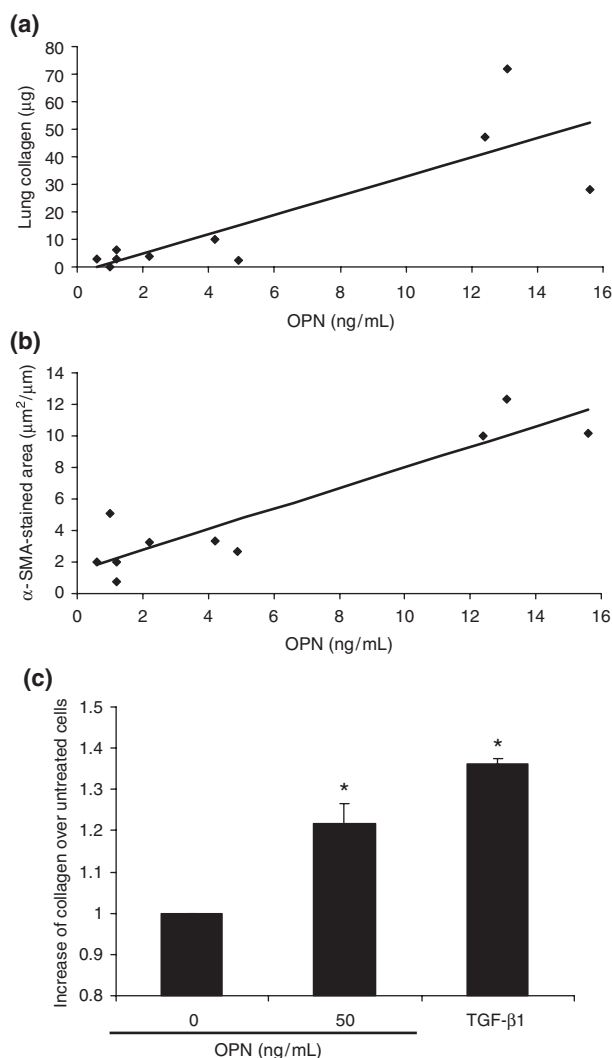


Fig. 3. Correlation between osteopontin (OPN) levels and structural changes in the lungs, and effect of OPN on mouse lung fibroblast collagen deposition. OPN levels from bronchoalveolar lavage (BAL) fluid for each individual mouse were plotted against the amount of lung collagen, and against the area of peribronchial α -smooth muscle actin measured for the same animal. Significant correlations were found between: (a) OPN levels in BAL fluid and lung collagen content (correlation coefficient = 0.83, $P < 0.01$); and (b) OPN levels in BAL fluid and airway smooth muscle area (correlation coefficient = 0.91, $P < 0.001$). (c) Collagen deposition was significantly increased in MLg cells exposed to OPN for 48 h, as determined by Sircoll colorimetric assay. The data are expressed as mean $\pm$ standard error of mean from three independent experiments (* $P < 0.05$).

Osteopontin expression in bronchoalveolar lavage fluid correlates with structural changes and osteopontin stimulates collagen deposition in mouse lung fibroblasts

After we observed enhanced OPN levels in our model of allergen-induced airway remodelling, we determined whether levels of OPN correlate with structural changes in the lungs. Lung collagen content (Fig. 3a) and airway smooth muscle area per micrometre of bronchi (Fig. 3b) for each individual mouse significantly correlated with OPN levels in BAL fluid (correlation coefficients 0.83, $P < 0.01$ and 0.91, $P < 0.001$, respectively).

To determine whether OPN has a direct pro-fibrogenic effect, we investigated whether OPN can directly stimulate collagen deposition in mouse lung fibroblasts. We observed a modest but consistent increment of collagen deposition on cell monolayers after 48 h of exposure to OPN. Collagen deposition was increased by 21% at 50 ng/mL (Fig. 3c). An increase in dose did not lead to a further increase of collagen synthesis. TGF- β 1 (10 ng/mL), a pro-fibrotic cytokine known to induce collagen synthesis in fibroblasts, was used as a positive control.

Distribution of osteopontin in lung tissue of both OVA- and saline-treated mice

We examined the distribution and the sources of OPN in lung tissue of saline- and OVA-treated mice using immunohistochemistry and immunofluorescence.

Airway epithelial cells stained positive for OPN in animals exposed to OVA (Figs 4b–d). Positive immunostaining for OPN was detected in cells of the peribronchial and perivascular inflammatory infiltrate in these animals. OPN was also expressed in clusters of alveolar macrophages present in the lung parenchyma of OVA-treated animals. OPN was also detected in airway epithelial cells of saline-treated mice (Fig. 4a).

To identify which cells of the inflammatory infiltrate express OPN, we co-stained tissue sections with OPN (Fig. 5, row 1) together with markers for T cells (anti-CD3), eosinophils (anti-CCR3), and macrophages (anti-F4/80) (Fig. 5, row 2). Positive double staining was observed for T cells, eosinophils, and macrophages (Fig. 5, row 3).

Expression of osteopontin in bronchial tissue of asthmatic patients

We determined whether OPN is expressed in bronchial tissue of asthmatic patients using immunohistochemistry. OPN is strongly expressed in bronchial epithelial cells and in some subepithelial inflammatory cells of asthmatic individuals, while no OPN expression was found in tissue sections of non-asthmatic control subjects (Fig. 6).

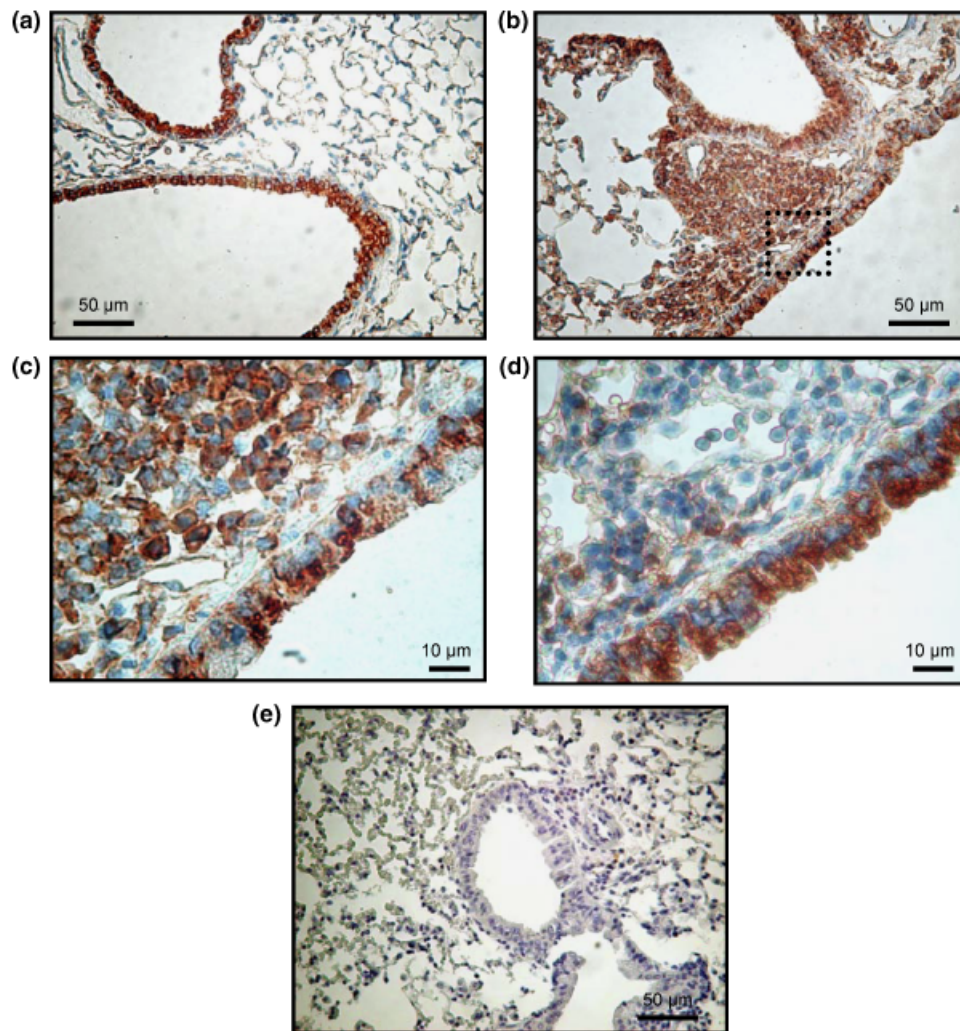


Fig. 4. Distribution of osteopontin (OPN) in lungs of saline- and ovalbumin (OVA)-treated mice. Positive OPN immunostaining was observed in the airway epithelium of saline-treated mice (a). Lung sections of mice exposed to OVA for 1 (d) and 5 (b, c) weeks exhibited strong staining in airway epithelial cells and in cells located in the peribronchial and perivascular inflammatory infiltrate. No staining was detected in lung sections incubated with an irrelevant isotype-matched IgG antibody (e).

Discussion

Using a murine model of allergen-induced chronic airway remodelling, we show that OPN is up-regulated at both RNA and protein levels in the lungs of mice exposed to OVA. The cellular source of OPN in lungs of OVA-treated mice was also characterized, showing that OPN is mainly expressed in the airway epithelium, and also in T cells, macrophages, and eosinophils located in the peribronchial inflammatory infiltrate.

We observed OPN-positive staining in the airways of mice exposed to OVA for only 1 week, while changes in collagen and smooth muscle deposition were not present at this time. Both in our model and in previously published studies, at least 4 weeks of allergen exposure were necessary before these structural changes of the airways became evident [6, 23–26]. Thus, OPN expression clearly

predates the increase in collagen and smooth muscle. Furthermore, we found a significant correlation between OPN levels in BAL fluid and the structural changes observed in our model. Taken together, these findings suggest that OPN may contribute to the fibrotic changes occurring after chronic allergen challenge.

OPN has multiple pro-fibrogenic properties including the promotion of migration, adhesion, and proliferation of human and murine fibroblasts [20, 27]. A direct link has been established between OPN expression and bleomycin-induced pulmonary fibrosis in mice as well as between OPN and human idiopathic pulmonary fibrosis [19, 20, 27]. OPN-null mice treated with bleomycin have reduced collagen, active TGF- β 1, and MMP-2 activity [15]. The pro-fibrotic effects of OPN are mediated via CD44 and/or α_v integrin receptors, as demonstrated in studies using blocking antibodies [20, 27].

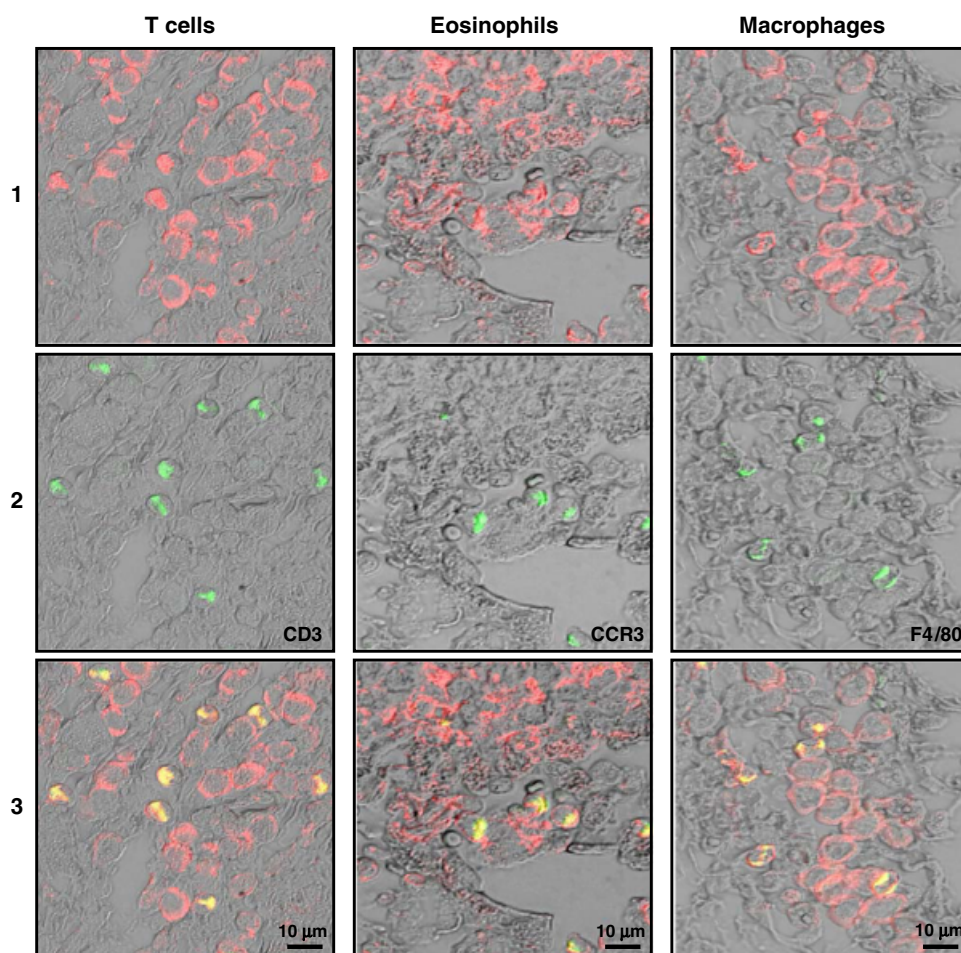


Fig. 5. Osteopontin (OPN) is expressed in T cells, eosinophils and macrophages located in the subepithelial inflammatory infiltrate. Immunofluorescent double staining was performed for OPN and specific cellular immunomarkers in lung sections of ovalbumin-treated mice. All the pictures were taken from the subepithelial inflammatory infiltrate of the bronchial wall. Images in row 1 show staining for OPN. Images in row 2 show staining for CD3 (T cells), CCR3 (eosinophils) and F4/80 (macrophages). Yellow-stained areas in row 3 show an overlay of rows 1 and 2.

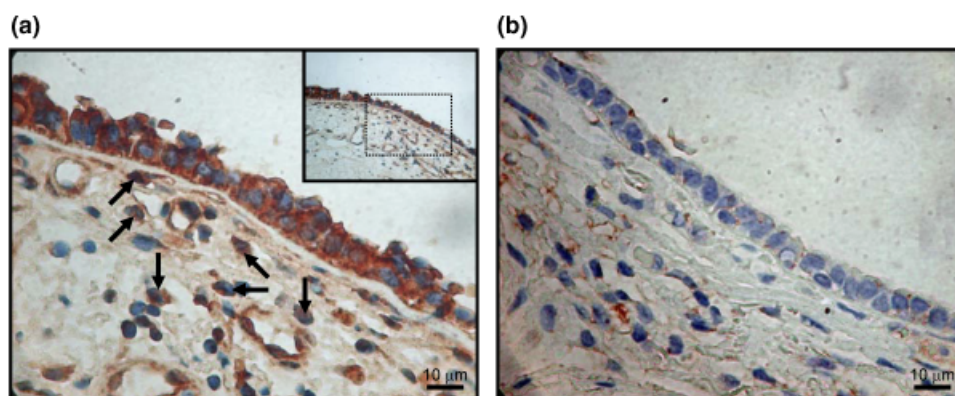


Fig. 6. Osteopontin (OPN) expression in a human bronchial epithelium from an asthmatic patient. Immunostaining of bronchial sections from an asthmatic patient revealed positive staining for OPN especially in epithelial cells but also in some subepithelial inflammatory cells (arrows) (a). No staining was detected in sections of control subjects (b).

Collagen deposition is recognized as a prominent feature of airway remodelling [1–3]. In this study, we observed a significant correlation between lung collagen production and OPN levels in BAL fluid. We also show that OPN stimulates collagen protein deposition in a murine lung fibroblast cell line. These findings suggest that OPN contributes to the increase of lung collagen seen in our model of experimental asthma. Previous *in vitro* studies have shown that OPN stimulates $\alpha 1$ type I collagen mRNA expression in human lung fibroblasts [20]. These data are consistent with our findings; however, in their study, the authors did not measure collagen protein levels. Although the OPN-induced increase we observed in collagen protein levels was modest, this was only slightly less than the effect obtained after exposure to TGF- $\beta 1$, used as a positive control.

In the present study, we examined the association of OPN with lung collagen production and peribronchial smooth muscle area. OPN may induce additional structural changes, such as neovascularity, which has been observed in asthmatic biopsies and correlates with asthma severity [28–30]. OPN has been shown to promote angiogenesis *in vitro* and *in vivo* [31, 32]. We have previously shown that OPN may be involved in eosinophil-mediated angiogenesis [33], raising the possibility that OPN contribute to the angiogenesis observed in asthmatic airways.

We observed that OPN is strongly expressed in epithelial cells of OVA-treated mice. These findings add further evidence that airway epithelial cells contribute not only to the inflammatory process in asthma, but may also be involved in remodelling. Airway epithelial cells are also an important source of other pro-fibrotic mediators such as TGF- $\beta 1$, PDGF, GM-CSF, and EGF [6]. OPN can stimulate epithelial cell migration and proliferation via CD44 and α_v integrin receptors [20]. Interestingly, expression of CD44, one of the receptors that bind OPN, is enhanced in the epithelium of asthmatics [34]. Thus, OPN produced by epithelial cells may also function in an autocrine feedback manner, leading to proliferation, migration, and alteration in the structure of the epithelial layer seen in asthmatic airways. We also observed positive staining for OPN in bronchial epithelial cells of human asthmatic individuals; however, no other data linking OPN to human asthma have been published to date and further studies will be important to elucidate this issue.

We observed that T cells, macrophages and eosinophils present in the inflammatory infiltrate in the lungs of OVA-treated mice express OPN. These results are consistent with previous *in vitro* and *in vivo* studies showing that OPN is expressed by inflammatory cells, including T cells and macrophages [8, 10, 35]. These inflammatory cells also possess other 'pro-remodelling' properties. The CD4⁺ subpopulation of T cells increases airway smooth muscle cell mass [36]. Eosinophils and macrophages promote the activation of fibroblasts [37–39]. We have previously

demonstrated that OPN is expressed by human peripheral blood eosinophils and contributes to eosinophil-induced angiogenesis [33]. This study is the first to demonstrate that OPN is expressed by eosinophils in a murine model of chronic experimental asthma. Our finding that eosinophils present in OVA-treated mice express OPN adds further evidence of a role for eosinophils, not only as pro-inflammatory, but also as pro-fibrogenic cells in asthma.

In addition to the pro-fibrogenic functions of OPN, this molecule also mediates Th1 cytokine expression. OPN stimulates T cells to produce IFN- γ , and induces macrophage IL-12 production via its interaction with $\alpha_v\beta_3$ integrin [8, 13, 18]. OPN is up-regulated in several murine models of 'classical' Th1 inflammatory diseases that are associated with tissue fibrosis, such as experimental autoimmune uveitis and acute respiratory distress syndrome [40, 41]. A role for OPN in asthma therefore appears to be counterintuitive, as this disease has traditionally been considered to be a Th2-dominant disease. It has been shown, however, that OPN is up-regulated in tear fluids of patients with allergic conjunctival diseases [42], suggesting that OPN may have a role in Th2-derived diseases.

Recently, the concept that asthma is a Th2-mediated disease has been challenged. It has been suggested that asthma may be caused by a combined Th1/Th2 immune response [43]. 'Classical' Th1 cytokines such as IFN- γ , IL-2 and IL-18 are involved in asthma pathogenesis [44]. Elevated levels of IFN- γ , IL-2, and IL-18 in serum, BAL fluids, or lung cells of asthmatic patients have been reported [45–48]. It is therefore not surprising that OPN may also contribute to the pathogenesis of asthma both as a 'so-called' Th1 cytokine and as a pro-fibrogenic mediator.

In summary, this study is the first to establish a link between OPN and allergen-induced airway remodelling. We demonstrate the up-regulation of OPN and characterize the structural and inflammatory cells that produce OPN in a murine model of allergen-induced airway remodelling. Expression of OPN correlates with the structural changes observed in our chronic model, suggesting that OPN may be causally linked to remodelling. Studies using OPN-knockout mice and using human asthmatic lung tissue will be helpful to further clarify the involvement of OPN in allergic airway remodelling and asthma.

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