

Osteopontin Deficiency Protects against Airway Remodeling and Hyperresponsiveness in Chronic Asthma

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Rationale: Osteopontin (OPN) is a cytokine that is upregulated in epithelial cells and macrophages in the lungs of mice during chronic allergen challenge and airway remodeling and also in lungs of patients with asthma. However, it remains unclear whether OPN has an *in vivo* effect on lung remodeling in allergic asthma. Based on its ability to induce smooth muscle and fibroblast proliferation and migration we hypothesize that OPN regulates lung remodeling and also affects subsequent airway hyperresponsiveness (AHR).

Objectives: Study the role of OPN in airway remodeling using OPN-knockout (KO) mice and a reversal approach administering recombinant mouse OPN (rOPN) in KO mice before challenge.

Methods: A chronic allergen-challenge model of airway remodeling with OPN KO mice, KO mice treated with rOPN, and human bronchial smooth muscle were used.

Measurements and Main Results: OPN deficiency protected mice against ova-induced AHR, which was associated with lower collagen and mucus production, gob-5 mRNA expression, submucosal cell area infiltration, and proliferation. Administration of rOPN to KO mice, just at the final five allergen challenges, exacerbated AHR and all the remodeling characteristics measured. In addition, rOPN increased the expression of IL-13 and pro-matrix metalloproteinase-9 in the lungs. Moreover, we demonstrated that rOPN induces proliferation of human BSM through binding to its $\alpha_v\beta_3$ integrin receptor and activation of PI3K/Akt downstream signaling pathway.

Conclusions: We conclude that OPN deficiency protects against remodeling and AHR. Thus our data reveal OPN as a novel therapeutic target for airway remodeling and associated AHR in chronic asthma.

Keywords: osteopontin; human smooth muscle cells; remodeling asthma

Chronic allergic asthma is characterized by persistent airway hyperresponsiveness (AHR) and structural changes in the lungs, such as extracellular matrix (ECM) deposition, increased airway smooth muscle (ASM) mass, and goblet cell hyperplasia (1–4), collectively known as airway remodeling. AHR is defined by exaggerated airway narrowing caused by nonspecific irritants or agonists that can usually be reversed by bronchodilators (1). AHR is associated with increased lung infiltration by inflamma-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Osteopontin (OPN) is a cytokine that is up-regulated in epithelial cells and macrophages of patients with asthma. Remodeling properties of OPN in various models of fibrosis were associated with induction of fibroblast proliferation and migration.

What This Study Adds to the Field

OPN absence prevented lung function worsening and remodeling in a model of chronic allergen-induced airway remodeling. Recombinant OPN administration confirmed its profibrotic role, pointing to OPN as a novel therapeutic target in asthma remodeling.

tory cells, mainly eosinophils and lymphocytes, and elevated levels of IL-13 (2, 4). Nevertheless, some patients develop persistent AHR even after prolonged antiinflammatory steroid therapy (5, 6). The persistent AHR seen in these patients is associated with remodeled airway wall structure and composition leading to a change in mechanical properties (1–4). However, the precise mechanisms responsible for cellular hyperplasia, hypertrophy, and altered matrix deposition in lung remodeling are far from resolved.

Osteopontin (OPN) is a cytokine that has been associated with tissue remodeling in various models of fibrosis (7, 8) by inducing migration and proliferation of murine fibroblasts and stimulating production of collagen (9). In addition, OPN promotes proliferation and migration of murine smooth muscle cells (10, 11). OPN is also expressed in several murine immune cell types, such as macrophages, T cells, B cells, and mast cells (12, 13). In patients with asthma, OPN is upregulated in bronchial epithelial cells and macrophages (14). We have previously shown that OPN suppresses *in vivo* acute allergic airway inflammation and disease in mice (14). Upregulation of OPN in models of chronic allergen challenge has been observed and mirrors findings seen in patients with asthma (15). The authors associated the increased ECM deposition in chronic asthma with the capacity of OPN to induce *in vitro* collagen production in a murine lung fibroblast cell line (15). However, it remains unclear whether OPN has an *in vivo* effect on lung remodeling.

Herein, we investigated the *in vivo* effects of OPN in lung remodeling using a well-established model of chronic allergic airway disease involving multiple allergen challenges (16, 17). We demonstrated that OPN participates in the induction of lung remodeling and AHR. These effects were prevented in the absence of OPN. Furthermore, we validated and extended our findings by studying the effects of OPN in human bronchial

(Received in original form July 14, 2008; accepted in final form February 13, 2009)

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Supported by the Thorax Foundation (Athens, Greece) and by an unrestricted GSK grant (#1012-2007). V.P. and G.X. are supported by Hellenic Ministries of Health and Education and by the General Secretariat of Research and Technology (03ED750, V.P.).

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This article has an online supplement, which is available from the issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 179, pp 894–902, 2009

Originally Published in Press as DOI: 10.1164/rccm.200807-1081OC on February 20, 2009
Internet address: www.atsjournals.org

smooth muscle (BSM) cells *in vitro*, and identified the signaling pathway of OPN-induced human BSM cell proliferation. Some of the results of these studies have been previously reported in the form of an abstract (18).

MATERIAL AND METHODS

Animals

Osteopontin knockout (KO) mice, backcrossed onto C57BL/6 background (14, 19), were used in all experiments. Mice were housed at the Experimental Surgery Unit of Evangelismos Hospital. All procedures were approved by the Veterinary Administration Bureau of the Prefecture of Athens, Greece and the Institutional Animal Care and Use Guidelines, which are in accordance with European Union Directive for animal research. All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Allergen-induced Chronic Airway Remodeling

KO mice and wild-type (WT) littermates were sensitized intraperitoneally with ovalbumin (ova) in alum (Days 0, 12) (16). Control mice received the same volume of phosphate-buffered saline (PBS) in alum as previously described (alum) (16). Acute airway inflammation was induced by six challenges with aerosolized (5%) ova (Days 18–23). Chronic allergic airway remodeling was induced when mice were subsequently exposed to aerosolized ova challenges three times a week from Days 26 to 54. Mouse recombinant OPN (rOPN) (or PBS) was administered (2.5 µg/mouse *i.p.*) 2 to 3 hours before the final five challenges from Days 44 to 54 (*see* Figure E1 in the online supplement). This dose has been previously shown to be the optimum *in vivo* effect (14).

Assessment of Airway Hyperresponsiveness Using Forced Oscillation Technique

Mice were anesthetized, tracheostomized, paralyzed, and ventilated with Flexivent (SCIREQ, Montreal, Canada) as previously described (20). After baseline measurements of respiratory impedance, aerosolized methacholine or saline was delivered (Aeroneb; SCIREQ) for 10 seconds. Afterward, a 2-second forced oscillation perturbation (1–20 Hz) was performed. Measurements of respiratory impedance were interpreted using the constant phase model (21). Lung function was investigated by assessing airway Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H). Model parameters were expressed as % ratio of the baseline value (room air).

Extracellular Matrix Deposition and Goblet Cell Hyperplasia Analyses

Paraffin lung sections (4 µm) were stained with picro-sirius red (16) and periodic acid-Schiff (PAS) (20) to determine the extent of total ECM deposition and goblet cell hyperplasia, respectively. A semi-quantitative score was calculated for both stains (16, 20). Total collagen in lung homogenates was quantified using Sircol Collagen-Assay kit (Biocolor, Newtownabbey, Northern Ireland) (16, 22). RNA expression of the mucus marker Gob-5 was quantified by real-time polymerase chain reaction (20, 23) (*see* online supplement).

Quantitation of Lung Remodeling Mediator

Lung lobes were homogenized in 10 vols (wt/vol) of Hanks' balanced salt solution containing protease inhibitors (20), centrifuged, supernatant collected, and IL-13, pro-matrix metalloproteinase (MMP)-9, and transforming growth factor (TGF-β) quantified using ELISA (R&D Systems, St. Louis, MO).

Immunohistochemistry

Percentage of positive submucosal cells for the Ki-67 cell proliferation marker was calculated from the total number of cells in three high-power fields (×400). Submucosal area was measured as a percentage of the bronchus area using ImageJ (NIH, Bethesda, MD) software (*see* online supplement).

Human Bronchial Smooth Muscle Cell Proliferation

Human BSM cells (Cambrex, Walkersville, MD) were treated with increasing concentrations of human rOPN (R&D Systems, Minneapolis,

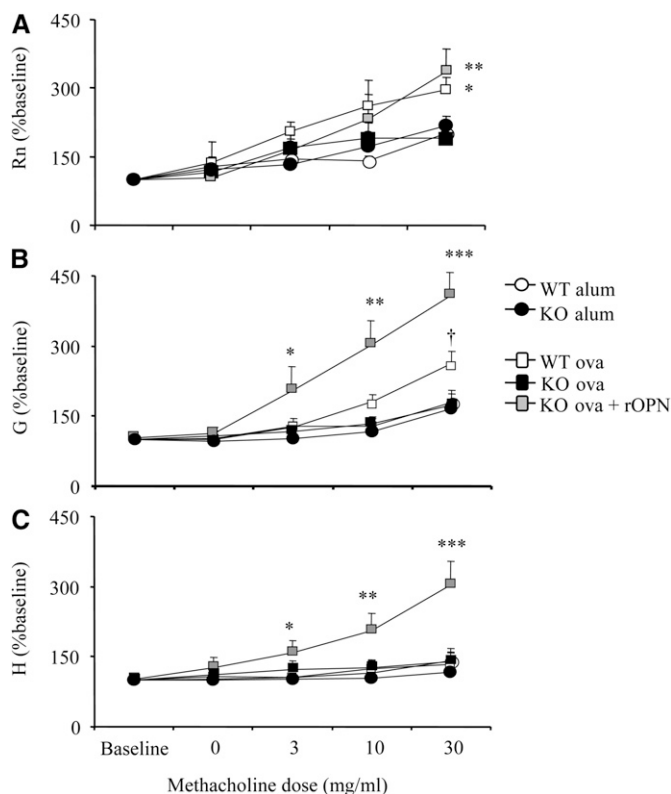


Figure 1. Osteopontin (OPN) deficiency in chronic asthma reduces airway hyperresponsiveness. Airway reactivity to increasing amounts of methacholine was assessed day 55 in wild-type (WT) and knockout (KO) mice or KO mice treated with recombinant OPN sensitized with ova and alum groups as described in the METHODS section. (A) Newtonian resistance (Rn) (* $P < 0.05$ for alum groups; * $P = 0.025$ for KO-ova; ** $P = 0.05$ for alum groups; ** $P = 0.05$ for KO-ova), (B) tissue damping (G) (* $P < 0.05$ for alum groups; ** $P = 0.024$ for WT-alum; ** $P < 0.01$ for KO-alum and KO-ova; *** $P < 0.05$ for alum groups; *** $P = 0.016$ for KO-alum; *** $P = 0.011$ for KO-ova; † $P < 0.05$ for alum groups; † $P = 0.05$ for KO-ova), and (C) tissue elastance (H) (* $P < 0.05$ for alum groups; * $P = 0.0014$ for WT-ova; ** $P < 0.014$ for WT-alum and -ova; ** $P = 0.017$ for KO-alum and -ova; *** $P = 0.002$ for alum and ova groups). Afterward mice were killed and tissue collected. Results are presented as means $\pm$ SEM. $n = 8$ –12 mice per group.

MN) in the presence of complete medium as previously described (8, 24) and proliferation was determined using Cell-Proliferation Assay (Promega, Madison, WI) (*see* online supplement).

Western Blotting

Protein lysates from human BSM cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted with anti-Akt and anti-phospho-Akt (Cell Signaling Technology, Beverly, MA) (*see* online supplement).

Statistical Analysis

Results are presented as means $\pm$ SEM. Comparisons were made either by *t* test or by analysis of variance followed by the Tukey's *post hoc* test using SPSS software. Differences were considered significant when *P* was less than 0.05.

RESULTS

Osteopontin Deficiency Attenuates Airway Hyperresponsiveness

Mice sensitized (ova/alum) and chronically challenged with ova had significantly increased OPN levels (4.42 ng/ml, approxi-

mately twofold) in their lung homogenates as compared with alum control mice (2.03 ng/ml).

As AHR is a hallmark clinical manifestation of allergic asthma, we assessed the effects of OPN on ova-induced AHR by studying Rn, G, and H. WT mice sensitized and challenged with ova (WT-ova) had significantly increased Rn (Figure 1A) compared with alum control groups. KO mice sensitized with ova (KO-ova) had significantly decreased Rn compared with WT-ova. The deficiency in OPN was sufficient to bring Rn close to the responses seen in alum groups (Figure 1A). Using a reverse approach, we administered rOPN *in vivo* during the five final challenges (Figure E1). Administration of rOPN to KO-ova mice was sufficient to increase Rn to levels similar to WT-ova mice. AHR was also studied in terms of G, which was elevated in WT-ova mice compared with alum control groups. OPN deficiency in ova-sensitized mice significantly reduced G (Figure 1B). Nevertheless, treatment with rOPN significantly increased G above the responses seen in the other groups. H response was not significantly different among any of the groups

studied (Figure 1C). However, treatment with rOPN induced a significant increase in H compared with the other groups (Figure 1C). Thus, deficiency of OPN attenuated and protected against AHR, whereas rOPN increased AHR.

Osteopontin Deficiency Protects against Extracellular Matrix Deposition and Mucus Production

AHR is often associated with lung fibrosis and remodeling. We next examined the effect of OPN deficiency on structural changes in the airways. Larger deposition of ECM, with dense subepithelial fibrotic response and thicker fibrils of collagen, were found in WT-ova mice compared with alum control mice (Figures 2A and 2B). KO-ova mice were protected against extensive ECM matrix deposition and subepithelial fibrosis. In contrast, treatment of KO-ova mice with rOPN induced significantly increased subepithelial fibrosis and ECM deposition (Figure 2B). ECM profile was confirmed by measuring total collagen in lung homogenates (17). Prolonged allergen challenge of WT-ova mice resulted in significantly increased collagen

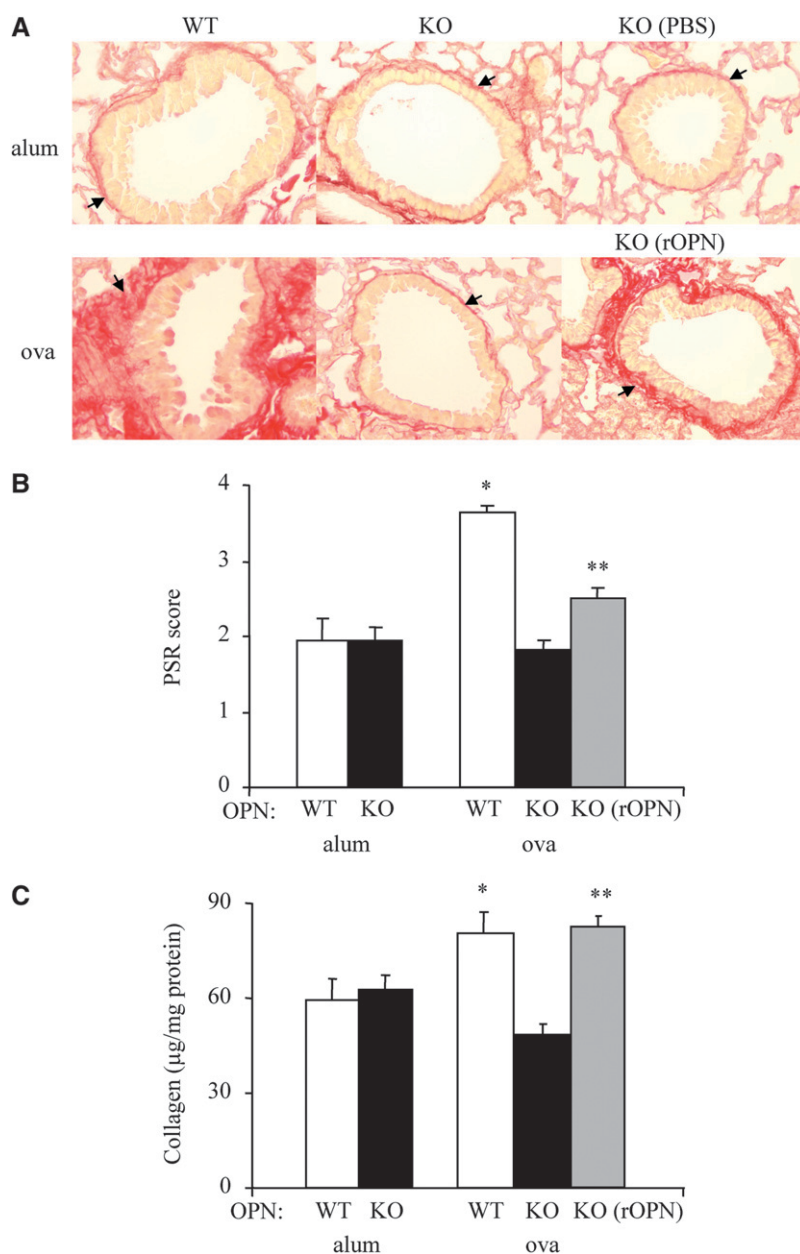


Figure 2. Osteopontin (OPN) deficiency in chronic asthma reduces extracellular matrix deposition. Wild-type (WT) and knockout (KO) mice or KO mice treated with recombinant OPN sensitized with ova and alum groups were studied for deposition of extracellular matrix. (A) Lung paraffin sections were stained with picro-sirius red (original magnification $\times 60$) and (B) scored for extracellular matrix deposition by a blinded observer ($*P = 0.0001$ for all mice groups; $**P = 0.049$ for KO-ova). (C) Total lung collagen was measured using Sircol assay ($*P < 0.05$ for alum groups; $*P = 0.002$ for KO-ova; $**P = 0.002$ for KO[rOPN]-ova). Data are presented as mean $\pm$ SEM; $n = 8-12$.

deposition in the airways compared with alum control mice (Figure 2C) (17). In accordance with the above results, KO-ova mice had significantly less deposition of collagen. Administration of rOPN to KO-ova mice increased significantly the level of collagen deposition to levels similar to those of WT-ova mice, indicating that collagen is probably the ECM responsible for the PSR score.

Airway obstruction can also be induced by mucus plugging, wherein the excess of mucus is mainly due to goblet cell metaplasia and glandular enlargement (16, 17). The number of goblet cells positively stained for mucin with PAS was significantly increased in the airway epithelium of WT-ova compared with alum mice (Figures 3A and 3B). OPN deficiency protected KO-ova mice from goblet cell hyperplasia (Figure 3A and B). However, this protective effect was significantly reversed by rOPN treatment of KO-ova mice. These observations were confirmed by the level of gob-5 mRNA expression (Figure 3C), whose overexpression is associated with mucus production (25). In agreement with the PAS results, absence of OPN in KO-ova mice significantly decreased the expression of gob-5 and

treatment with rOPN had the opposite effect. Therefore deficiency of OPN protects against ECM deposition and goblet cell hyperplasia with mucus production.

Mediators of Lung Remodeling Are Reduced in the Absence of Osteopontin

The level of the profibrotic cytokine IL-13 was significantly increased in lung homogenates of WT mice during chronic airway remodeling compared with alum control mice (Figure 4A). Deficiency of OPN significantly decreased the level of IL-13 in lung homogenates. Interestingly, administration of rOPN significantly increased production of IL-13, which, however, was not accompanied by a significant increase in airway inflammation (data not shown) (23, 26). Furthermore, OPN modulated the production of the respiratory protease MMP-9 (Figure 4B). The expression of pro-MMP-9 was significantly increased in lungs of mice during chronic allergen challenge compared with alum control mice. OPN deficiency significantly decreased the levels of pro-MMP-9 expression to levels similar to those of control mice. However, administration of rOPN significantly

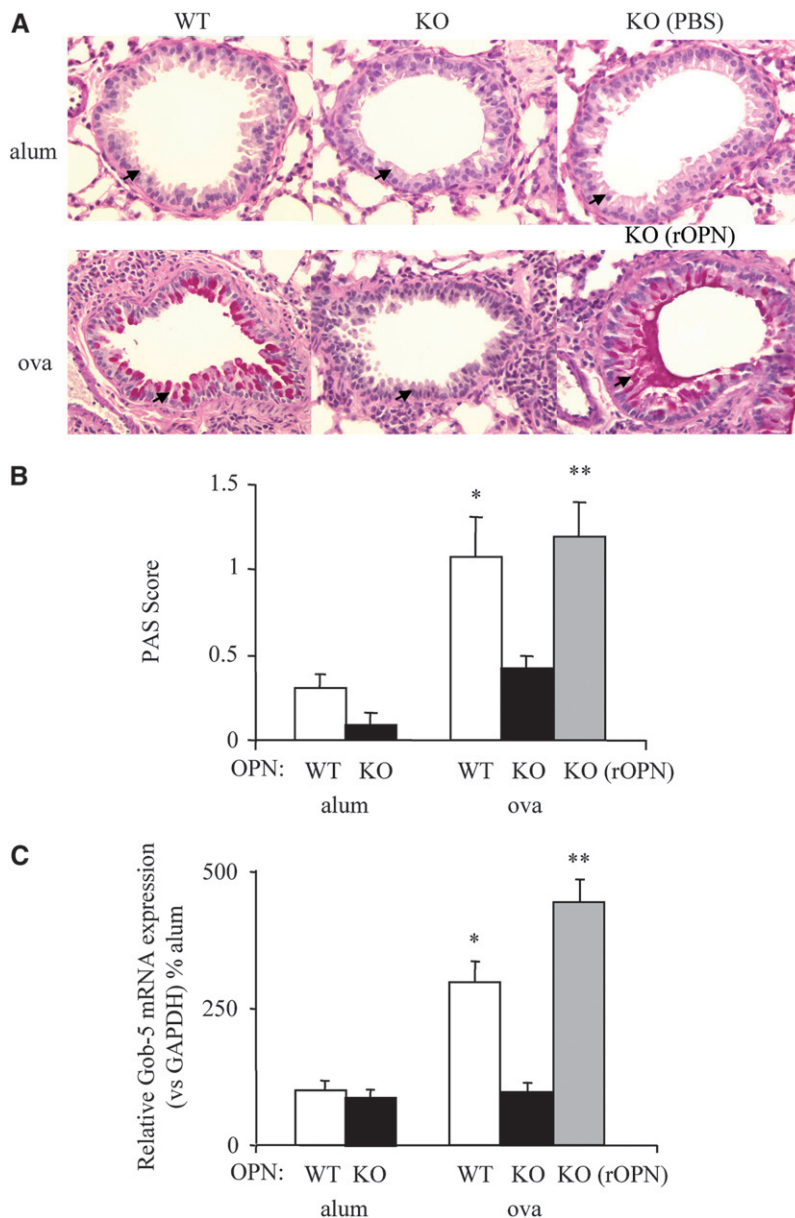


Figure 3. Goblet cell hyperplasia and mucus production are reduced in absence of osteopontin (OPN). (A) Representative photomicrographs of stained paraffin lung sections with periodic acid-Schiff (PAS) from wild-type (WT) and knockout (KO) mice or KO mice receiving recombinant OPN sensitized and challenged ova and respective alum groups are shown (original magnification $\times 60$). (B) PAS-stained sections were assessed by blinded observer and scored (* $P = 0.04$ for WT-alum; * $P = 0.004$ KO-alum; * $P = 0.016$ for KO-ova; ** $P = 0.017$ for KO-ova). (C) Gob-5 mRNA level in lung homogenates was analyzed with real-time polymerase chain reaction as described in METHODS (* $P = 0.014$ for alum groups; * $P = 0.021$ for KO-ova; ** $P = 0.019$ for KO-ova). Data are presented as mean $\pm$ SEM; $n = 8-12$.

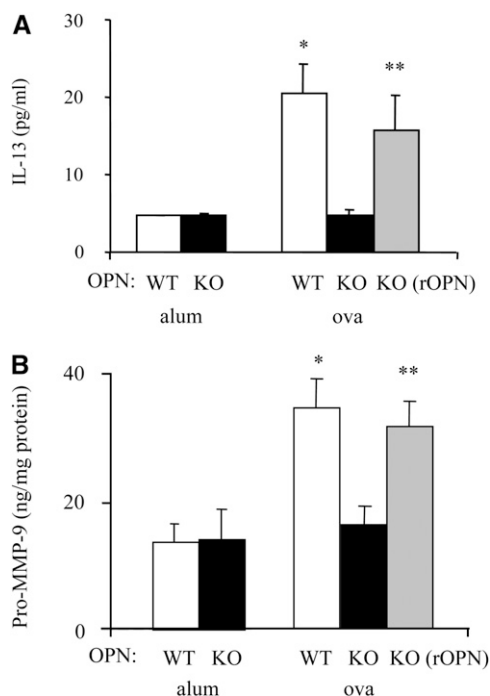


Figure 4. IL-13 and matrix metalloproteinase (MMP)-9 are the possible effector mechanisms for osteopontin protection against lung remodeling and airway hyperresponsiveness. Lung homogenates from wild-type (WT) and knockout (KO) mice treated with ova and alum were analyzed for (A) the expression of IL-13 (* $P = 0.011$ for alum groups; * $P = 0.003$ for KO-ova; ** $P = 0.04$ for KO-ova), and (B) pro-MMP-9 (* $P < 0.005$ for alum groups; * $P = 0.003$ for KO-ova; ** $P = 0.014$ for KO-ova) using ELISA. Data are presented as mean $\pm$ SEM of evaluation of $n = 8-12$.

increased production of pro-MMP-9. TGF- β levels in lung homogenates were not significantly affected by OPN (data not shown).

Osteopontin Deficiency Restricts Smooth Muscle Mass

Airway smooth muscle mass has been associated with AHR (1, 27, 28). In our model of chronic allergen airway remodeling significant increased numbers of Ki-67+ submucosal cells were observed in the WT-ova mice as compared with alum control mice (Figures 5A and 5B). Interestingly, these changes were significantly reduced in KO-ova mice, whereas treatment with rOPN increased the number of Ki-67+ submucosal cells (Figures 5A and 5B). These results were confirmed by measurement of the submucosal area (Figure 5C), which was significantly increased in WT-ova mice as compared with alum groups. Absence of OPN significantly reduced the submucosal area. rOPN increased significantly the submucosal area to levels similar to those of WT-ova mice. Therefore, deficiency of OPN decreased smooth muscle mass, and this protective effect was reversed by rOPN.

Recombinant Osteopontin Activates Proliferation of Human Bronchial Smooth Muscle Cells *In Vitro*

The results described so far suggest a profibrotic role of OPN *in vivo*. To validate our findings in humans, we studied the effects of rOPN on human BSM cell proliferation *in vitro*. rOPN added to human BSM cultures induced a dose-dependent increase in human BSM cell proliferation, with highest effects at 100 μ g/ml (Figure 6A). Lower concentrations of rOPN did not induce human BSM proliferation (data not shown). The primary receptor for OPN in smooth muscle cells is the $\alpha_v\beta_3$

integrin (13). To study whether the effect of rOPN on human BSM cell proliferation was mediated by $\alpha_v\beta_3$ integrin receptor, we pretreated cells with anti- $\alpha_v\beta_3$ integrin (or immunoglobulin control). The proliferation induced by rOPN was studied in the presence of anti- $\alpha_v\beta_3$ (or IgG control) to observe whether $\alpha_v\beta_3$ integrin receptor mediates the response in human BSM. The proliferative response induced by rOPN was significantly abrogated by the addition of anti- $\alpha_v\beta_3$ antibody compared with Ig control (Figure 6A).

The proliferative effect caused by rOPN also induced Akt phosphorylation, which was detected within 5 minutes, reaching a maximum between 15 and 30 minutes (Figures 6B and 6C). These results indicate that proliferation of human BSM cells is stimulated by rOPN binding to $\alpha_v\beta_3$ integrin receptor and leading to activation of Akt.

DISCUSSION

Patients with moderate to severe asthma have markedly increased expression of OPN in epithelial and immune cells (14). In the present study, we demonstrated that OPN is upregulated in lungs of mice in chronic models of allergen-induced airway remodeling as described by others (15), suggesting a role for this cytokine in lung remodeling in asthma. We also demonstrated that OPN has an *in vivo* effect on AHR and remodeling. Absence of OPN protected mice from the development of AHR and widespread structural changes characteristic of lung remodeling, such as ECM deposition, goblet cell hyperplasia, mucus production, and submucosal smooth muscle cell area. These effects were reversed by *in vivo* administration of rOPN in KO mice at the final stages of allergen challenge. Therefore, we propose that during chronic allergen challenge OPN is mainly responsible for AHR and initiates lung remodeling by inducing collagen deposition, goblet cell hyperplasia with mucus production, and increased submucosal smooth muscle cell area.

Airway remodeling is postulated to be a determinant of AHR and accelerated loss of lung function in patients with asthma (2, 5, 29-31). AHR was studied with forced oscillation technique, which enabled us to study AHR in terms of Rn, G, and H. Absence of OPN protected against Rn, whereas rOPN administration promoted an increase in Rn back to similar levels to those observed in WT-ova mice. This effect was concomitant with increased mass of airway smooth muscle cells (increased submucosal Ki-67+ proliferation cells), and increased ECM deposition. Increased airway smooth muscle mass, even without a change in contractile phenotype, enhances AHR as shown by An and colleagues (1). Our data indicate that rOPN administration *in vivo* in KO mice was responsible for inducing similar remodeling events in the central airways as those observed in WT-ova mice.

The rOPN treatment increased G as well as ECM deposition with particular increase in collagen. This indicates a specific contribution of collagen deposition on remodeling and ultimately AHR (4). Therefore, OPN promoted a strong lung mechanical response probably by affecting the amount, composition, and location of ECM in the lung parenchyma (27, 28). The increase in ECM deposition with rOPN treatment was predictable in view of the fact that OPN has a strong profibrotic role (7, 32). OPN has the ability of inducing collagen production in a mouse lung fibroblast cell line (15). Moreover, other studies have shown that in KO mice collagen deposition is decreased (7, 9, 19, 32) as a result of decreased migration and proliferation of fibroblasts (7), which are collagen-producing cells.

Despite the increase in Rn and G, H did not increase in WT-ova mice in our studies. This is different from recent results shown in an acute model of airway inflammation using BALB/c

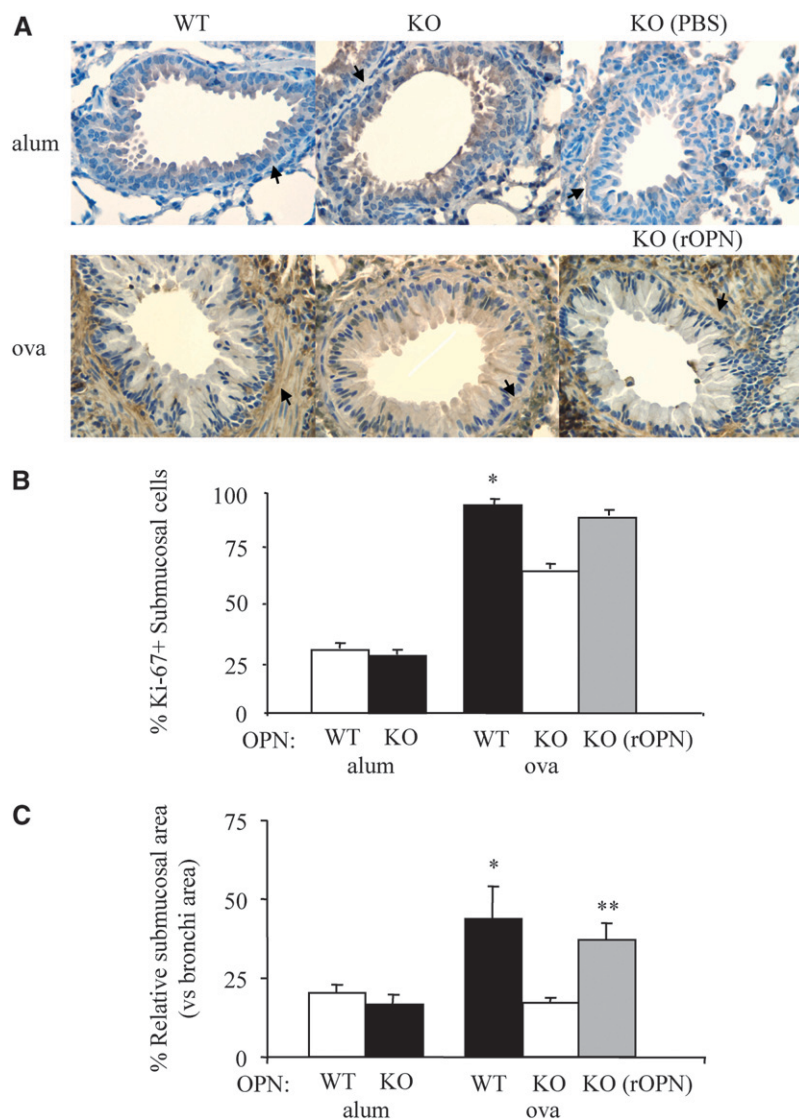


Figure 5. Proliferating submucosal cells and area are decreased in absence of osteopontin (OPN). (A) Representative photomicrographs of immunofluorescence reactivity of lung sections using antibody against Ki-67 (original magnification, $\times 60$) were used to study proliferation on lung sections of wild-type (WT) and knockout (KO) mice or KO mice receiving recombinant OPN sensitized and challenged ova or alum. (B) Percentage of Ki-67-positive submucosal cells. For each section, data were obtained using three high-power fields ($\times 400$) ($*P = 0.009$ from alum groups; $*P = 0.05$ from KO-ova). (C) Percentage of submucosal area relative to bronchi area measured using ImageJ program ($*P < 0.005$ for alum groups; $*P = 0.003$ for KO-ova; $**P < 0.05$ for alum groups; $**P = 0.026$ for KO-ova). Data are presented as mean $\pm$ SEM; $n = 8$ –12.

mice (33), in which the authors used a model of acute allergic airway inflammation with mice receiving one ova challenge. These discrepancies are mainly due to the different animal models used (34) and the different mouse strains (35). In our study, we have used a model of chronic allergic airway remodeling wherein mice after ova/alum sensitization received 18 ova aerosol challenges over a 55-day period. In addition, our OPN KO mice were backcrossed onto the C57BL/6 background, which have different AHR responses (35) as compared with Balb/c mice used in the Wagers and colleagues study (33). Moreover, studies using chronic models demonstrated that increased deposition of collagen is accompanied by progressive decrease in AHR (36) indicating that thickening/stiffening of the airway may be protective against peripheral airway closure.

Increased ECM deposition leading to increased lung stiffness could be responsible for the increase in H seen in rOPN mice (33). However, growing evidence indicates that exaggerated airway closure also contributes significantly to increase in change in H (ΔH) (37). The consequent airway wall thickening can also increase the airway closure causing an increase in rOPN mice ΔH without affecting the narrowing of the central airways (Figures 1A and 1C) (33, 34). In addition, an increased propensity for closure could be either due to excessive mucus production (Figures 3A and 3B) or to an increased surface

tension due to production of fibrin or other plasma proteins. These conditions would narrow the airway lumen accentuating the tendency for liquid bridging to take place as the airways narrow, with some airways proceeding to full closure leading to increased H (37, 38). In addition, rOPN administration could have induced other pathological changes in the airways, such as angiogenesis and increased migration and/or proliferation of ASM and fibroblasts (9–11). The effects may have stimulated contractile elements in the lung parenchyma or surface tension into the lumen increasing ΔH and/or affecting the lung mechanics (34, 39). This result was accompanied by upregulation of gob-5. Interestingly, *in vivo* gob-5 transgene and knockout approach demonstrated an important role of gob-5 in the development of murine AHR (25). In addition, *in vitro* overexpression of gob-5 resulted in increased mRNA expression of another mucin gene, MUC5AC (25). The mechanism by which OPN deficiency restricts epithelial cell metaplasia remains elusive. However, increased PAS score and gob-5 mRNA expression is induced by overexpression of IL-13 (23, 40), the expression of which was regulated by OPN.

IL-13 is a potent stimulator of pulmonary remodeling as it induces lung and alveolar enlargement as well as mucus metaplasia, AHR, and fibrosis with increased collagen production (3, 22, 41). Moreover, an elegant work by Wilson and colleagues

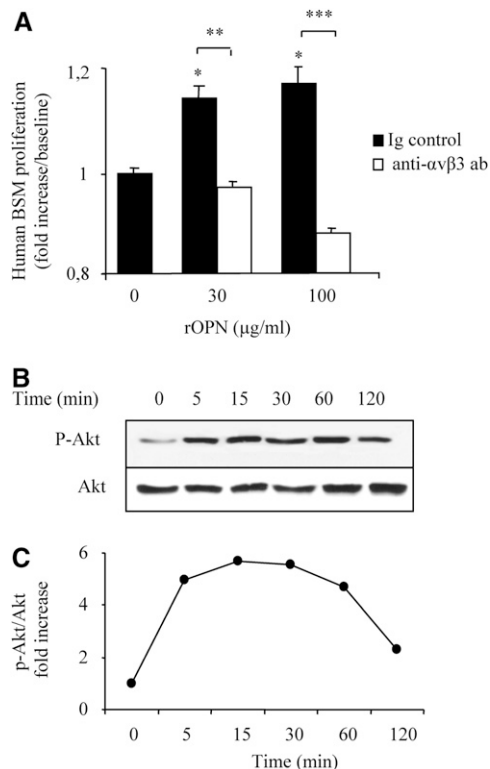


Figure 6. Human bronchial smooth muscle (BSM) proliferation is enhanced by osteopontin (OPN). Cell proliferation activity was assessed by use of CellTiter 96 AQueous One Solution (Promega, Madison, WI). (A) Human BSM cells were treated with increasing concentration of recombinant human OPN (rOPN) in the culture medium and with either antibody against $\alpha_v\beta_3$ or immunoglobulin control (* $P = 0.013$ for 0 $\mu\text{g/ml}$ rOPN; ** $P = 0.007$; *** $P = 0.0001$). Phosphorylation of Akt in human BSM cells after rOPN (30 $\mu\text{g/ml}$) treatment was studied by Western blot at various time periods using an anti-phospho-specific Akt antibody (P-Akt, upper panel) or an anti-Akt antibody (Akt, lower panel). (B) Representative Western blot and (C) course of relative phosphorylation of Akt during OPN treatment are depicted. Values are means $\pm$ SEM; $n = 10$.

(23) using IL-13 decoy receptor demonstrated that IL-13 promotes AHR, fibrosis, and goblet cell hyperplasia in a chronic model of allergic remodeling, independently of T_H2 inflammation. These findings support the notion that increased IL-13 levels may be a possible mechanism for the rOPN-induced effects on lung remodeling. The increased expression of IL-13 in lung homogenates of rOPN-treated mice is probably derived from activated airway epithelial cells as previously described (42) and/or from other resident cells, such as ASM or fibroblasts. In fact our studies showed that rOPN treatment increased ASM proliferation *in vitro* and this could have also lead to increased IL-13 production. Another mechanism that could explain the effect of OPN on allergic airway remodeling is increased expression of MMP-9. MMP-9 is one of the major proteinases involved in airway remodeling in patients with asthma (43, 44) and in mice during chronic allergen-induced remodeling (45). In our study we observed that OPN regulates pro-MMP-9 expression (7, 32, 46). However, IL-13 may have also affected the expression of MMP-9 (22, 40).

In our present study, rOPN was administered during the remodeling phase (Figure E1), when acute inflammation is considered to have receded and structural changes are shown to be prominent features of allergic airway disease (17, 47). In this phase, administration of rOPN induced structural changes in

the lung (Figures 2, 3, and 5) with a pronounced profibrotic effect without lung inflammation. In agreement, several studies of postinflammatory lung fibrosis demonstrated that OPN levels peak during the fibrotic phase when inflammatory infiltrates have receded (as reviewed by O'Regan [26]). In a previous study by our group (14) rOPN was administered during the acute phase of allergic airway disease, wherein it exerted antiinflammatory effects. The difference in the effects of rOPN is mainly due to different mouse models used and the timing of rOPN administration. Our two studies are complementary, showing the dual role of OPN in asthma: initially OPN suppresses allergic airway inflammation and subsequently it mediates tissue repair responses that lead to lung remodeling. A similar dual function has been attributed to other cytokines, such as TGF- β (48). We propose that in sensitized individuals the recruited inflammatory cells and resident epithelial cells become activated and secrete proinflammatory cytokines, whereas later they produce regulatory or antiinflammatory cytokines, such as TGF- β and OPN, to prevent excessive inflammation and damage and to induce healing. However, if expression of OPN is deregulated increased structural changes in the lungs occur leading to enhanced remodeling and decline in lung function.

The importance of ASM mass on AHR has been increasingly appreciated, as shown in studies in which ASM mass of patients with asthma was partially reduced through bronchial thermoplasty leading to amelioration of lung function (49). In the present study, absence of OPN reduced the percentage of proliferating submucosal cells and the submucosal area throughout the mouse lung. This protective effect was reversed by *in vivo* administration of rOPN. rOPN (at the concentration range studied) also induced increased *in vitro* proliferation of human BSM cells in the presence of complete medium (11). The lower levels of human BSM proliferation in the presence of $\alpha_v\beta_3$ anti-integrin antibody probably indicate that this is the main receptor used by OPN (50). Therefore, the OPN-induced proliferative mechanism was dependent on the integrin receptor $\alpha_v\beta_3$ (Figure 6A) (10, 51), which probably binds to the adhesive RGD motif of OPN (52). However, we also observed that $\alpha_v\beta_3$ anti-integrin antibody alone promoted a reduction in human BSM proliferation as compared with baseline values, which suggests that $\alpha_v\beta_3$ anti-integrin antibody inhibited proliferative effects of other proteins present in the complete medium (19).

OPN can mimic ECM signaling, via binding of its RGD motif to several integrins that affect a wide range of cellular processes, such as adhesion, migration, differentiation, survival, repair, and growth of smooth muscle cells *in vitro* (52–54). Furthermore, we demonstrated that rOPN activates Akt in human BSM cells (Figures 6B and 6C) (11), which is one of the downstream targets of phosphoinositide 3-kinase (PI3K). OPN has been reported to activate various kinases, such as PI3K (11), which is involved in the proliferation of human ASM in asthma (54). The PI3K/Akt signaling pathway promotes T_H2 -cell cytokine secretion and AHR (55). Our data demonstrated that rOPN induces proliferation of human BSM cells by activating the PI3K/Akt signaling pathway through the $\alpha_v\beta_3$ integrin receptor.

In conclusion, OPN deficiency protected from airway remodeling and AHR. Administration of rOPN *in vivo* reversed these effects and uncovered OPN as a novel therapeutic target for chronic pulmonary remodeling in human asthma.

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.

Acknowledgment: The authors thank L. Liaw (Maine Medical Center Research Institute) for permission to use the KO mice, A. Agapaki (BRFAA) for histology preparations, and James G. Martin for critical reading of the manuscript.

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