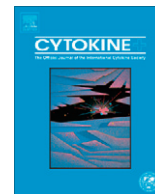


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Increased levels of osteopontin in sputum supernatant of smoking asthmatics

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ABSTRACT

Smoking may modify the inflammatory pattern of the asthmatic airways. Osteopontin (OPN) has been associated with inflammation and fibrosis. In asthma, sputum levels of OPN are elevated and have been related to the underlying severity and to mediators expressing remodeling and inflammation.

To evaluate the levels of OPN in sputum supernatants of asthmatic patients and to investigate the possible role of smoking as well as associations with mediators and cells involved in the inflammatory and remodeling process.

We studied 103 asthma patients (49 smokers) and 40 healthy subjects (20 smokers) who underwent lung function tests, bronchial hyperresponsiveness to methacholine, and sputum induction for cell count identification and measurement of OPN, TGF- β 1, IL-8, IL-13 and ECP in sputum supernatants. The concentrations of all mediators were measured using enzyme immunoassays.

OPN levels (pg/ml) were significantly higher in smoking asthmatics compared to non-smoking asthmatics, and both non-smoking and smoking controls [median (interquartile ranges) 1120 (651, 1817) vs. 197 (118, 341) vs. 50 (42, 70) vs. 102 (77, 110) pg/ml, respectively; $p < 0.001$]. Regression analysis provided significant associations between OPN and sputum neutrophils, IL-8 and TGF- β 1, the most significant being the one with TGF- β 1. These associations were present only in smoking asthmatics.

Smoking habit significantly affects sputum OPN levels in asthma. The associations of OPN with sputum neutrophils, TGF- β 1 and IL-8 in smoking asthmatics suggest a possible role for OPN in the neutrophilic inflammation and remodeling process in this phenotype of asthma.

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1. Introduction

Asthma is a heterogeneous, chronic inflammatory airway disease that results in variable airflow obstruction in response to a variety of stimuli [1]. Prevalence rates of cigarette smoking among asthmatic patients are similar to those in the general population [2]. Smoking asthmatics present an accelerated decline in lung function, more severe asthma symptoms, increased rates of hospital based care and increased mortality following hospital admission due to a potentially fatal episode of asthma, compared to non-smoking asthmatics [3,4]. Airway inflammation in asthmatic smokers, as evaluated by induced sputum cell counts, is characterized by increased numbers of neutrophils and increased IL-8 levels, but decreased numbers of eosinophils, compared to non-smoking asthmatics [5,6]. These effects have been associated with reduced

efficacy of currently available treatments, especially inhaled corticosteroids (ICS), in smoking asthmatics [7,8].

Osteopontin (OPN) is a phosphorylated acidic glycoprotein that can function as both an extracellular matrix (ECM) molecule and a cytokine [9]. Its origin is not well clarified but epithelial cells, macrophages, T cells and fibroblasts have been shown to express OPN [10–12]. Moreover, IFN- γ has been shown to stimulate OPN mRNA and protein expression from monocytoid cells [13]. OPN has previously been found to be associated with tissue remodeling, since it promotes proliferation and migration of murine smooth muscle cells and fibroblasts and stimulates collagen production [9,14]. Moreover, OPN can mediate interactions between cells and ECM, recruit T cells, stimulate Th1 cytokine expression and promote angiogenesis [15,16].

We have recently shown that sputum OPN levels are higher in non-smokers with severe refractory asthma (SRA) compared to less severe forms of the disease and healthy subjects [17]. In the same study, sputum OPN was positively associated with sputum levels of cysteinyl leukotrienes, interleukin (IL)-13 and transforming growth factor β 1 (TGF- β 1) in patients with SRA but not in those

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with milder forms of the disease [17]. Furthermore, Schneider et al., demonstrated increased OPN expression in the distal airways of patients with COPD that was associated with airway obstruction, suggesting that OPN may represent a possible therapeutic target for COPD patients with high tobacco-consumption rates [18].

The aim of the present study was to evaluate the levels of OPN in sputum supernatants of smoking asthmatic patients in comparison to non-smoking asthmatics and healthy controls.

We hypothesized that sputum OPN would be elevated in patients with smoking compared to non-smoking asthma and that it would be associated with inflammatory cells and mediators involved in the ongoing airway inflammation and remodeling process in this specific asthma phenotype.

2. Materials and methods

2.1. Subjects

Patients were recruited from an open cohort of patients with asthma who are followed up in the asthma clinics of the 1st and 2nd Respiratory Medicine University Departments in Athens. The diagnosis of asthma was established according to the Global Initiative for Asthma (GINA) guidelines [1]. Patients with asthma with a smoking habit >10 pack-years and non-smoking asthmatics were included. Patients with COPD or any other respiratory disease, as well as patients with gastroesophageal reflux disease, malignancies, heart, renal, liver or collagen disease, were also excluded. Patients with respiratory tract infections or asthma exacerbations in the 8 weeks before inclusion to the study, as well as patients with severe refractory asthma were also excluded [19]. Asthma was well-controlled on study inclusion in all asthmatic patients, as evaluated by GINA guidelines and the Asthma Control Test (ACT) [20]. Forty healthy non-atopic subjects (20 current smokers with a smoking habit >10 pack-years and 20 never smokers) served as a control group. The study was approved by the ethics committees of the two hospitals and all subjects provided informed consent.

2.2. Study design

On day 1 all subjects underwent medical history and physical examination by a dedicated respiratory physician, lung function measurements, measurement of body mass index (BMI), bronchial hyperresponsiveness (BHR) assessment and skin prick tests. On the same day, sputum induction was performed. All measurements were performed between 8.00 am and 10.00 am. All smokers refrained from smoking for at least 2 h, under supervision of one of the study coordinators.

2.3. Induced sputum

Sputum was induced as previously described [21] using all necessary modifications according to asthma severity [22]. Sputum was processed using selected plugs as previously described [23,24]. Dithiothreitol was added in a volume equal to four times the weight of the sputum specimen and it was further diluted with phosphate buffered saline in a volume equal to the sputum plus dithiothreitol. Total cell counts were performed on a haemocytometer using Trypan blue stain. Slides were prepared by cytospin (Shandon, Runcorn, UK) and were stained with May-Grunwald and Giemsa for differential cell counts. Cell counting was performed by an observer blind to the clinical characteristics of the subjects. At least 500 inflammatory cells were counted in each sample. A sample was considered adequate when the patient was able to expectorate at least 2 ml of sputum and the slides

contained <10% squamous cells on differential cell counting. The total cell count (expressed as the number of cells $\times 10^6$) and the percentage of sputum inflammatory cells was used for analysis. Sputum supernatants were kept at -70°C for further measurement of OPN, IL-8, IL-13, eosinophilic cationic protein (ECP) and TGF- $\beta 1$.

2.4. Lung function and bronchial hyperresponsiveness (BHR)

FEV1 and FVC were measured using a commercially available system (Master Screen Body, Jaeger, Hoechberg, Germany), according to the American Thoracic Society (ATS) guidelines [25]. BHR was measured according to the ATS guidelines using a commercially available system (APS; Viasys Healthcare, Jaeger), and was expressed as PD20 to methacholine [26].

2.5. Exhaled nitric oxide measurements

Fraction of exhaled nitric oxide (FeNO) was measured using a portable nitric oxide analyser (NIOX MINO Airway Inflammation Monitor; Aerocrine, Solna, Sweden) as previously described [27,28].

2.6. Atopic status

A positive skin prick test to any of 20 common aeroallergens (including mites, grass and tree pollens, fungi and fur of domestic animals) was considered as confirmation that the subject was atopic.

2.7. Mediator assays

ECP was measured using Unicap ECP kit (Pharmacia Diagnostics; Uppsala, Sweden) with a detection limit of 0.5 ng/ml. IL-13 and IL-8 were measured using an ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) with detection limits of 32 pg/ml and 3.5 pg/ml, respectively. TGF- $\beta 1$ and human OPN were measured by ELISA kits (R&D Systems) with lower detection limits of 50 pg/ml and 6 pg/ml, respectively. All values were expressed as pg/ml. The intra-assay and interassay variability were assessed according to the manufacturers' instructions for all the mediators measured and were within acceptable coefficient of variation (%). For OPN, the intra-assay and interassay variabilities were 4% and 6.5%, respectively. The recovery and linearity of the assays after appropriate spiking experiments produced samples with values within the dynamic range of the assay.

2.8. Statistical analysis

Normally distributed data are presented as mean $\pm$ standard deviation (SD), whereas skewed data are presented as median (interquartile ranges, IQR). Normality of distribution was checked with Kolmogorov–Smirnov test. Statistical comparisons between groups were performed with one way analysis of variance (ANOVA) for normally distributed data and Kruskal–Wallis tests for skewed data, accompanied by appropriate post hoc tests for multiple comparisons (Bonferroni and Dunn's, respectively). Differences in numerical variables between two groups were evaluated with unpaired t-tests or Mann–Whitney U-tests for normally and skewed data respectively, whereas comparisons of proportions were performed using chi-square tests. In order to examine the associations between OPN and sputum cells and mediators (ECP, IL-8, IL-13, TGF- $\beta 1$), linear regression analysis was performed using OPN as the dependent variable, after proper adjustments for age, gender, BMI, atopy, duration of the disease, smoking habit (in pack years) and treatment regimens. OPN levels were not normally

distributed and were log-transformed for this analysis. Data were interpreted as standardized coefficients with 95% confidence intervals. Associations between OPN and lung function tests as well as BHR were evaluated with Spearman's rank or Pearson's correlation coefficients according to normality of data. *p*-Values < 0.05 (2-sided) were considered statistically significant. Statistical analysis was performed using SPSS 16.0 (Chicago, Illinois, USA) and Graph Pad Prism 5 (GraphPad Software, California, USA).

3. Results

3.1. Demographic characteristics

We studied 103 asthmatic patients (49 smokers) and 40 healthy subjects (20 smokers). The demographic characteristics of the study population are summarized in Table 1. The mean age of smoking asthma patients was 48 ± 11 years. There was no difference in age, gender and BMI between the four groups. Additionally, smoking asthma and non-smoking asthma patients did not differ in FEV₁ and BHR (PD15).

3.2. Inflammatory variables in induced sputum

Patients' inflammatory variables are summarized in Table 2. OPN levels (pg/ml) were significantly higher in smoking asthmatics (SA) compared to non-smoking asthmatics (NSA), and both non-smoking and smoking controls [median (IQR) 1120 (651, 1817) vs. 197 (118, 341) vs. 50 (42, 70) vs. 102 (77, 110) pg/ml, respectively; *p* < 0.001; Table 2, Fig. 1]. Additionally, TGF- β 1, ECP, IL-8 and IL-13 levels were also significantly higher in SA compared to the other study groups (Table 2).

3.3. Associations of OPN

Regression analysis after proper adjustments provided significant associations between log OPN levels and sputum neutrophils, IL-8 and TGF- β 1 (Table 3). Stepwise regression analysis showed that TGF- β 1 represented the strongest association with OPN

($R^2 = 0.71$, *p* < 0.001). These associations were observed only in SA and not in NSA. No significant associations were observed in the control subjects (data not shown). Furthermore, no significant associations between OPN and lung function tests or BHR were observed in any of the study groups (data not shown).

4. Discussion

The major finding of the present study is that OPN levels in sputum supernatants are significantly higher in smoking asthmatics compared to non-smoking asthmatics, and both non-smoking and smoking healthy subjects. Sputum OPN levels were positively associated with sputum neutrophils, IL-8 and TGF- β 1 in smoking asthmatics, but not in non-smoking asthmatics. This is the first study to our knowledge that has evaluated the levels of OPN in the sputum of smoking asthmatics and its associations with biomarkers of airway inflammation and remodeling.

Previous studies have found increased levels of OPN in the induced sputum and BAL fluid of patients with asthma [29,30]. Sputum OPN levels were recently shown to be higher in patients with severe refractory asthma compared to less severe forms of the disease [17]. Smoking asthma often represents a more severe form of the disease compared to well-controlled non-smoking asthma and elevated OPN levels in SA compared to NSA may provide evidence for a possible contribution of OPN to the observed difference in disease severity.

OPN levels have been previously associated with pulmonary disorders related to smoking habit. Prasse et al., assessed the essential role of OPN in smoking-related interstitial lung diseases [31]. In that study, BAL cells from patients with pulmonary Langerhans cell histiocytosis (PLCH) or desquamative interstitial pneumonitis (DIP) spontaneously produced abundant amounts of OPN, whereas BAL cells from healthy smokers produced 15-fold less OPN and those from non-smoking healthy volunteers produced no OPN, suggesting a possible pathogenic role of increased OPN production in both PLCH and DIP [31]. Furthermore, Schneider et al., assessed the contribution of adenosine and OPN to the development of COPD, the most common smoking-related disease, in a

Table 1
Subjects characteristics.

	Non-smoking asthma (n = 54)	Smoking asthma (n = 49)	Non-smoking controls (n = 20)	Smoking controls (n = 20)	<i>p</i> - value
Age	50 ± 10	48 ± 11	52 ± 10	55 ± 9	0.745
Gender F/M	31/23	26/23	11/9	12/8	0.65
Atopy	31/54	28/21	0	0	0.555
Pack years	0	50 (31–55)	0	43 (38–51)	0.288
Asthma duration (y)	36 (30–42)	33 (27–38)	N/A	N/A	0.15
BMI (kg/m ²)	26 ± 3	28 ± 4	26 ± 4	27 ± 4	0.207
FeNO ppb	19 (14–25)*, #, \$	12 (10–17)\$	12 (10–13)	9 (6–11)	0.003
BHR PD15 mg	0.16 (0.10–0.23)*, #, \$	0.16 (0.08–0.32)*, #, \$	>2	>2	0.451
FEV1% pred	86 (78–98)*, #, \$	82 (76–95)*, #, \$	94 (85–98)	95 (83–97)	0.002
FEV1/FVC	74 ± 9*, #, \$	72 ± 8*, #, \$	87 ± 6	88 ± 8	0.003
ACT score	23 ± 1.3	22 ± 1.2	N/A	N/A	0.328
<i>Treatment regimens</i>					
ICS	54	49	N/A	N/A	
(mean dose of budesonide or equivalent/ μg)	510	490			
LABA	34	28			
LTRA	10	11			

Normally distributed data are presented as mean ± standard deviation (SD); skewed data are presented as median (interquartile ranges). Bold letters indicate significant *p* values among the four groups.

Abbreviations: M = Male, F = Female, BMI = Body mass index, FeNO = fraction of exhaled nitric oxide, BHR = Bronchial hyperresponsiveness, FEV₁ = Forced expiratory volume in one second, FVC = Forced vital capacity, DLCO = Diffusion capacity, FRC = Functional Residual capacity, ICS = inhaled corticosteroids, LABA = Long acting β_2 agonists, LTRA: Leukotriene receptor antagonists. N/A = not applicable.

* Statistically significant difference compared to smoking asthma.

Statistically significant difference compared to non-smoking healthy subjects.

\$ Statistically significant difference compared to smoking healthy subjects.

Table 2
Inflammatory variables of study subjects.

Variables	Non-smoking asthma (n = 54)	Smoking asthma (n = 49)	Non-smoking controls (n = 20)	Smoking controls (n = 20)	p-Value
Cells × 10 ⁶ /ml	1.5 (0.8–2.6) ^{#,§}	1.7 (1.0–3.4) ^{#,§}	0.8 (0.6–1.1)	1 (0.6–1.3)	<0.001
Eosinophils (%)	3 (2–5) ^{*,#}	6 (3–9) ^{#,§}	0.5 (0–1)	0.2 (0–1)	0.005
Neutrophils (%)	28 (20–39) ^{*,#}	44 (36–57) ^{#,§}	23 (17–25) [§]	31 (24–47)	<0.001
Macrophages (%)	69 (57–74) [*]	50 (38–60) ^{#,§}	64 (56–70)	64 (49–74)	0.005
Lymphocytes (%)	1 (0–2.5)	1 (0–2)	0.25 (0–1)	0.4 (0–1)	0.520
OPN (pg/ml)	197 (118–341) ^{*,#}	1120 (651–1817) ^{#,§}	50 (42–70) [§]	102 (77–110)	<0.001
TGF-β1 (pg/ml)	1654 (1234–1998) ^{*,#}	1854 (989–2499) ^{#,§}	850 (645–1005)	788 (690–980)	0.003
ECP (pg/ml)	1045 (700–1650) ^{*,#}	1500 (900–2100) ^{#,§}	334 (221–487)	321 (213–450)	0.020
IL-8 (pg/ml)	1595 (1070–3200) ^{*,#}	5093 (3760–6200) ^{#,§}	432 (314–556) [§]	870 (750–1380)	<0.001
IL-13 (pg/ml)	85 (67–108) ^{*,#}	156 (98–211) ^{#,§}	34 (33–38)	49 (42–63)	<0.001

Data are presented as median (interquartile ranges). Bold letters indicate significant p values referring to differences among the four groups.

Abbreviations: OPN = Osteopontin, TGF-β1 = Transforming growth factor β1, ECP = Eosinophilic cationic protein, IL-8 = Interleukin-8, IL-13 = Interleukin-13.

* Statistically significant difference compared to smoking asthma.

Statistically significant difference compared to non smoking healthy subjects.

§ Statistically significant difference compared to smoking healthy subjects.

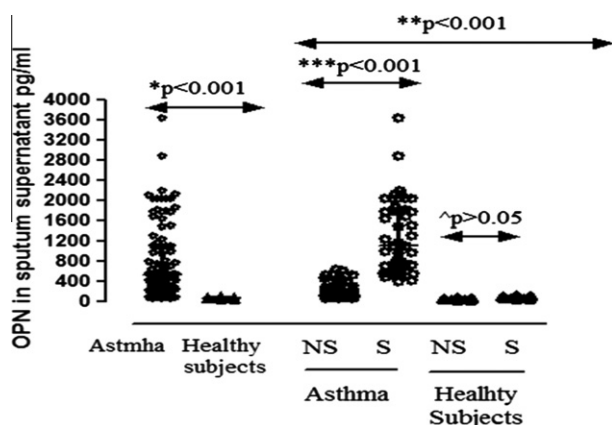


Fig. 1. Osteopontin (OPN) levels (pg/ml) in sputum supernatant in patients with asthma (All, non-smoking-NS and smoking-S) and healthy subjects (All, non-smoking-NS and smoking-S). **p* < 0.001 in favor of asthma, ***p* < 0.001-values indicate differences across the 4 groups, ****p* < 0.001 in favor of smoking asthma, ^*p* > 0.05 indicate no significant difference. Values are presented as median interquartile ranges.

Table 3
Regression analysis between OPN, inflammatory cells and mediators in patients with SA.

Variables	Beta standardized coefficient (95% CI)	Adjusted R ²	p-Value
Total cells × 10 ⁶ /ml	0.03 (−63, 93)	0.005	0.703
Eosinophils (%)	−0.18 (−84, 27)	0.001	0.302
Neutrophils (%)	0.62 (−6, 70)	0.390	0.020
Macrophages (%)	0.12 (−35, 47)	−0.007	0.702
Lymphocytes (%)	−0.29 (−56, 29)	−0.001	0.514
IL-8 (pg/ml)	0.28 (0.006, 0.23)	0.210	0.039
ECP (pg/ml)	0.09 (−0.04, 0.11)	0.007	0.340
IL-13 (pg/ml)	0.11 (−1, 3.3)	0.040	0.280
TGF-β1 (pg/ml)	0.5 (0.2, 0.7)	0.710	<0.001

Regression analysis was performed after proper adjustments for age, gender, BMI, duration of the disease, atopy and treatment regimens. Bold numbers indicate significant association.

Abbreviations: OPN = Osteopontin, TGF-β1 = Transforming growth factor, ECP = Eosinophilic cationic protein, IL-8 = Interleukin-8, IL-13 = Interleukin-13, SA = Smoking asthma, CI = Confidence intervals.

model of COPD *Ada*−/− double knockout mice [18]. In that study, *Ada*−/− mice exhibited OPN-dependent neutrophilia, alveolar air-space enlargement, and increases in mediators of air-space enlargement. The authors of this study additionally showed that patients with COPD had increased OPN expression within distal

airways that was associated with clinically relevant airway obstruction [18].

In another recent study, the gene for OPN was highly expressed in the lungs of smoke-exposed mice and was required for the T(H)17 responses and the induction of emphysema *in vivo* [32]. Moreover, Lazar et al. suggested that cigarette smoking and nicotine may contribute to pancreatic ductal adenocarcinoma inflammation through induction of MCP-1, the latter possibly mediated by OPN [33]. Our results are compatible with the above findings, suggesting a possible role for OPN in the induction of neutrophilic inflammation in smoking asthmatics, through promotion of OPN expression by the smoking habit.

The extremely low OPN levels in non-smoking asthmatics and in healthy subjects may be attributed to the absence and/or inactivation of OPN-producing cells. In asthmatic patients this may be plausibly related to the effect of treatment, especially ICS, since all our asthmatics were well controlled at the time of sputum induction. In accordance with our findings, a previous study showed no OPN expression in tissue sections of a limited number of non-asthmatic control subjects [34]. Healthy smoking subjects showed higher OPN levels compared to healthy non-smokers but lower compared to non-smoking asthmatics, indicating that both smoking habit and asthma disease contribute to the determination of OPN levels.

In the present study, OPN was positively associated with sputum neutrophils, IL-8 and TGF-β1 only in the group of SA. All structural and immune cells in the lung can express and secrete TGF-β1 [35] and TGF-β1 has been implicated in airway remodeling in asthma and has been further up-regulated in severe forms of the disease [36]. Previous studies have shown that OPN contributes to tissue fibrosis through modulation of TGF-β1 [37,38]. An altered epithelial repair of the asthmatic airway epithelium was shown to be partially mediated through the epithelial mesenchymal transition induced by TGF-β1 [39]. The strong association between OPN and TGF-β1 observed in our study supports a possible indirect role of OPN in the process of airway remodeling in smoking asthmatics, through modulation of TGF-β1. This strong association between sputum OPN and TGF-β1 has also been reported in severe refractory asthma [17].

The association of OPN with sputum neutrophils observed in smoking asthmatics is compatible with studies showing a shift of the inflammatory cell pattern towards neutrophilia [5]. Moreover, the association of OPN with IL-8, which is the major chemoattractant for neutrophils, provides an explanation for the trafficking of neutrophils in the airways and the sputum of smoking asthmatics. OPN could also be directly associated with neutrophilic chemotaxis apart from IL-8. In a previous study in an experimental model of colitis, defective recruitment of neutrophils was observed in

OPN-null mice, while the addition of exogenous OPN was associated with a robust increase in peritoneal infiltration of neutrophils [40]. These findings support the importance of OPN in the recruitment of neutrophils and accordingly OPN may present a therapeutic target in neutrophilic-dependent asthma.

5. Conclusions

OPN levels are significantly elevated in the induced sputum of smoking asthmatics, compared to non-smoking asthmatics and healthy controls. The associations of OPN with sputum neutrophils, TGF- β 1 and IL-8 in smoking asthmatics suggest a possible role for OPN in the induction of neutrophilic inflammation and the airway remodeling process in this asthma phenotype.

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