High plasma concentrations of osteopontin in patients with interstitial pneumonia

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Summary Osteopontin (OPN) produced by alveolar macrophages functions as a fibrogenic cytokine in the development of bleomycin (BLM)-induced murine pulmonary fibrosis, and OPN mRNA is expressed on lung tissues from patients with idiopathic pulmonary fibrosis (IPF). The present study investigates plasma OPN levels in human interstitial pneumonia (IP) and their relationships with disease severity by analyzing the correlation between plasma OPN concentrations and pulmonary functions. The concentrations of OPN in plasma were measured in 17 patients with IP, in 9 with sarcoidosis and in 20 healthy controls using an antigen-capture enzyme-linked immunosorbent assay. The concentrations of OPN in plasma were significantly higher in IP patients than in those with sarcoidosis or in controls. Based on a Receiver Operating Characteristic curve analysis, cut-off points between 300 and 380 ng/ml discriminated between IP and control subjects with 100% sensitivity and 100% specificity. In such case, the sensitivity for sarcoidosis decreased (55.5–33.3%) in cut-offs with 100% specificity. Plasma OPN levels inversely and closely correlated with arterial oxygen tension (PaO2) in patients with IP. Immunohistochemically, OPN was localized predominantly in macrophages and airway epithelium. These findings suggest that plasma OPN levels were found to be associated with the presence of IP, and that OPN play an important role in the development of IP.

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Introduction

Osteopontin (OPN, also known as Eta-1) is a phosphorylated acidic glycoprotein secreted by a variety of cells including osteoclasts, activated T cells and activated macrophages.1 OPN contains an
arginine–glycine–aspartate (RGD)-binding motif that binds to the integrin family of adhesion molecules\(^2,3\) and it functions as a proinflammatory cytokine that causes cellular adhesion and chemotaxis of different cell types, such as inflammatory leukocytes and vascular smooth muscle cells.\(^4,5\) In this context, some investigators have identified a biological role of OPN in the pathogenesis of various diseases. For example, OPN is expressed by infiltrating macrophages in experimental cardiac injury and myocardial infarction,\(^6\) and it is also involved in the pathogenesis of glomerulonephritis as well as in the angiogenesis of cancer cells.\(^8,9\)

The potential role of OPN in the pathogenesis of pulmonary granulomatous diseases such as tuberculosis, silicosis and sarcoidosis is of interest. Macrophages and T cells produce abundant amounts of OPN during the granuloma formation associated with tuberculosis, Mycobacterium avium-intracellulare infection, silicosis and sarcoidosis.\(^10–12\) Furthermore, OPN is involved in lung injury. We demonstrated that OPN mRNA is overexpressed on alveolar macrophages in the lungs of transgenic mice that express tumor necrosis factor (TNF)-\(\alpha\) in type II pneumocytes, leading to pulmonary alveolitis and progressive fibrosis.\(^13,14\) In addition, OPN produced by alveolar macrophages functions as a fibrogenic cytokine that promote migration, adhesion, and proliferation of fibroblasts in the development of bleomycin (BLM)-induced murine pulmonary fibrosis.\(^15\) In human, mRNA from lung biopsies of patients with idiopathic pulmonary fibrosis (IPF) was subjected to cDNA microarray analysis, and similar to murine lung fibrosis induced by bleomycin, OPN was among the most prominently expressed cytokines when compared to normal lung.\(^16\) Collectively these findings suggest that OPN play a key role in the development of pulmonary alveolitis leading to fibrosis.

In this study we have measured plasma OPN levels in patients with interstitial pneumonia (IP) and examined their relationship with disease severity to clarify its role in the pathogenesis of this disease.

Methods

Study subjects

We studied 11 male and 6 female patients with IP of mean (± SD) age 59 (± 16) years, whose conditions included idiopathic pulmonary fibrosis (IPF; \(n = 8\)), idiopathic nonspecific interstitial pneumonia (NSIP; \(n = 2\)), cryptogenic organizing pneumonia (COP; \(n = 2\)), giant cell interstitial pneumonia (GIP; \(n = 1\)), collagen vascular disease-associated IP (CVD-IP; \(n = 3\)); each patient had rheumatoid arthritis, mixed connective tissue disease or dermatomyositis) and drug-induced IP (\(n = 1\)). Each patient with NSIP or COP was receiving therapy with 10 mg of corticosteroid and none of the remaining patients had received corticosteroids or other immunomodulators at the time of peripheral blood collection. We also studied 5 male and 4 female patients of mean age 53 (± 15) years with sarcoidosis at stage I in 3 patients, II in 5 and III in 1. None of these patients were medicated with corticosteroids or other immunomodulators at the time of plasma sample collection. Patients with cancer, those suspected of having a malignancy or those with cardiovascular diseases were excluded from the study since elevated OPN levels in peripheral blood samples of these diseases have been demonstrated.\(^17,18\) Malignancies were undetectable in any of the patients throughout the study. Plasma samples were also collected from 10 healthy male and 10 healthy female volunteers (mean age, 58 (± 16) years) with no history of IP or other interstitial lung diseases, and whose chest radiographs showed no evidence of respiratory diseases. All participants provided written, informed consent to participate in the study.

Study design

Peripheral blood was collected into tubes containing EDTA, and plasma samples were stored at −80°C. Serum samples were simultaneously obtained to measure KL-6, surfactant protein (SP)-A and SP-D. Pulmonary function test was tested and blood gas was analyzed at the time of or within one month before and after peripheral blood sampling.

OPN, KL-6, SP-A and SP-D levels

Plasma OPN concentrations were measured by an antigen-capture enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Human OPN assay kit, Immuno-Biological Laboratories Co., Gunma, Japan). This ELISA kit was recently developed based on the method reported by Kon et al.\(^19\) The sensitivity of the assay was 5 ng/ml. We measured levels of each marker using specific kits according to the protocols provided by the manufacturers. KL-6 concentrations were measured using a sandwich-type electrochemiluminescence immunoassay (ECLIA) kit (Picolumi KL-6; Sanko Junyaku Co., Tokyo, Japan). SP-A and SP-D levels were measured using sandwich-type enzyme
immunoassay (EIA) kits (SP-A test-F; Kokusai Shiyaku Co., Hyogo, Japan and SP-D kit Yamasa; Yamasa Shoyu Co., Tokyo, Japan, respectively). Serum cut off values were established at 500 U/ml for KL-6 43.8 ng/ml for SP-A, 110 ng/ml for SP-D. All assays were performed in duplicate.

**Immunohistochemistry of lung tissues**

Immunohistochemical analysis of OPN in the lungs of patients with IPF or with NSIP was performed. Surgical lung biopsy specimens were obtained from two IPF patients and two NSIP patients. Control specimens were obtained from the normal parts of lungs removed for lung cancer. Briefly, after deparaffinization and rehydration tissue sections (5 μm thick) were treated with pronase for 5 min (DAKO Corporation, Carpinteria, CA), followed by 3% hydrogen peroxide in methanol for 20 min to inactivate endogenous peroxidases. Lung sections were incubated with non-specific staining blocking reagent (DAKO) for 10 min, followed by overnight incubation at 4°C with primary monoclonal anti-OPN antibody (MPIIIB10; developmental studies, hybridoma bank, University of Iowa, Iowa City, IA) at a concentration of 1:300 in a moist chamber. The immunoreaction product was detected with the ENVISION system (DAKO) and with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. The primary antibody was replaced by normal mouse IgG1 for negative control slides.

**Statistical analysis**

All values were expressed as mean ± SD values. Data were statistically analyzed using StatView-J 5.0 software (Abacus Concept, Inc., Berkeley, CA). Differences between groups were examined using the ANOVA test with a post-hoc analysis (Fisher’s PLSD test). Correlations between two defined parameters were determined using the Spearman’s rank correlation analysis. A P-value below 0.05 was considered significant.

**Results**

**Plasma OPN concentrations**

We compared plasma OPN concentrations among patients with IP, patients with sarcoidosis and healthy controls. Fig. 1 shows that plasma OPN concentrations in patients with IP were significantly higher than in controls and in patients with sarcoidosis (945.5 ± 256.0 ng/ml vs 156.7 ± 52.2 and 321.2 ± 146.8 ng/ml; P < 0.0001, respectively). Plasma OPN concentrations of the latter group were also significantly higher than in the controls (P < 0.05). No significant difference was observed in the levels of plasma OPN between IPF and other IP (data not shown). Based on a Receiver Operating Characteristic (ROC) curve analysis (Fig. 2), cut-off points between 300 and 380 ng/ml discriminated between IP and control subjects with 100% sensitivity and 100% specificity. In such case, the sensitivity for sarcoidosis decreased (55.5–33.3%)
Plasma OPN levels did not significantly differ between males and females or between smokers and nonsmokers in the control and patient groups. In addition, OPN levels did not increase with age (data not shown).

Plasma OPN concentrations inversely and closely correlated with the value of PaO2, but not with either %VC or %TLCO in patients with IP (Fig. 3 and Table 1). A correlation analysis of the levels of KL-6, SP-A, and SP-D (markers of disease activity in interstitial lung diseases) with these clinical parameters revealed significant correlations only between KL-6 and %VC (Table 1). Plasma OPN levels and the serum concentrations of KL-6, SP-A, or SP-D did not significantly correlate in patients with IP (data not shown).

**Immunostaining of OPN**

Immunohistochemical study in two patients with IPF using anti-OPN antibody demonstrated high levels of immunoreactivity for OPN both in the alveolar and interstitial cells of macrophage morphology in contrast to no expression in lymphocytes (Fig. 4a, b). A strong OPN signal was also detected in airway epithelium (Fig. 4a). Similar staining for OPN was observed in sections from two patients with NSIP (Fig. 4d, e). No staining was seen with the isotype control (Fig. 4c, f). In control lung tissue alveolar macrophages and airway epithelium also exhibited OPN staining; however, this was less than that seen in IPF and NSIP.

**Discussion**

The role of OPN on pulmonary fibrosis or IP has been demonstrated in an experimental mouse model. OPN gene and protein were expressed mainly on alveolar macrophages in the mouse lung after the administration of BLM15 or in the TNF-α transgenic mouse lung13,14 in parallel with the infiltration of inflammatory cells and thickening of alveolar walls and with subsequent pulmonary fibrosis. OPN is expressed during inflammatory alveolitis, yet levels continue to increase and peak at stages of fibrosis when inflammatory infiltrates have receded.13–15,20 A study in vitro also demonstrated the cell adhesion and migration of fibroblasts mediated by OPN.15 These observations suggest that OPN is involved in the pathogenesis of lung injury.

However, the relationship between OPN and IP in humans has not been fully evaluated. The significantly higher plasma levels of OPN in patients with IP relative to those in patients with sarcoidosis or healthy controls support this supposition. In addition, when the cut-off value was set between 300 and 380 ng/ml based on a ROC curve, the levels of OPN in plasma reliably distinguished IP case patients from control subjects. Thus, plasma OPN levels were found to be associated with the presence of IP.

High plasma OPN levels significantly correlated with arterial oxygen tension, a clinical parameter indicative of disease severity. The present immunohistochemical study demonstrated that a strong

![Figure 3](image-url)  

**Figure 3** Correlation between plasma OPN concentrations and arterial oxygen tension in patients with IP (n = 15). Each symbol indicates one patient. Plasma OPN concentrations inversely correlated with PaO2 values (r = –0.734, P = 0.0080).

<table>
<thead>
<tr>
<th></th>
<th>PaO2 (Torr)</th>
<th>% VC</th>
<th>% TLCO</th>
</tr>
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<tbody>
<tr>
<td>Osteopontin</td>
<td>R = –0.734 (P = 0.0080)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>KL-6</td>
<td>NS</td>
<td>R = –0.648 (P = 0.0289)</td>
<td>NS</td>
</tr>
<tr>
<td>SP-A</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>SP-D</td>
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PaO2, arterial oxygen tension; VC, vital capacity; TLCO, carbon monoxide transfer factor; NS, not significant.
OPN signal was detected in macrophages and airway epithelium in human IP, while not in lymphocytes. Normal lung did not show significant staining for OPN. This was similar to the findings previously reported by Nau et al. that OPN staining of airway epithelium is seen in inflamed but not normal lung. Additionally, using cDNA microarray analysis OPN mRNA from lung biopsies of patients with IPF was among the most prominently expressed cytokines when compared to normal lung removed for lung cancer. These studies suggest the local production of OPN in the lung of human IP. OPN mediates the migration, proliferation and adhesion of fibroblasts in the alveolar paace, which are essential for the initial process of pulmonary fibrosis. Ultrastructural studies during the early stages of pulmonary fibrosis have shown that fenestrations develop in the alveolar capillary endothelial cells and that gaps form in the epithelial basement membrane. Thus, the high plasma OPN levels identified in the present study might relate to leakage that is accelerated as a result of damage to the air–blood barrier in lung parenchyma, and the inverse relationship between plasma OPN concentrations and PaO2 might reflect the initial or early stage of the fibrotic process in the lung. Collectively our finding suggests that similar to murine lung fibrosis, OPN play an important role in the development of IP.

Plasma OPN concentrations in patients with sarcoidosis were also significantly higher compared with controls, but to a lesser extent than those of patients with IP. Sarcoid granulomas exhibit a characteristic pattern of strong lymphocyte-associated OPN staining, and the late fibrotic granulomas exhibit significant less OPN expression, which was limited to multinucleated giant cells. These data demonstrate that OPN expression is more pronounced in the early cellular granulomas of sarcoidosis and that this pattern of expression is
distinct from that seen in IP, indicating that plasma OPN levels in sarcoidosis may vary with the stage. KL-6, SP-A and D are biomarkers of IP. However, the present study found no relationships between these markers and pulmonary function tests including PaO₂ except for KL-6 and %VC. These findings confirmed that these three markers are not correlated with pulmonary function indicators in patients with IPF, including PaO₂, %VC and %TLCO and that serum levels of neither SP-A nor SP-D correlated with %VC or %TLCO. Additionally, our study could not find any correlation between plasma OPN levels and serum concentrations of KL-6, SP-A or SP-D (data not shown), probably because of differences in the origins of these markers. KL-6 derives from damaged or regenerating type II pneumocytes in IPF, whereas SP-A and D are produced by two types of epithelial cells in the peripheral airway, namely Clara cells and alveolar type II cells. These findings suggest that serum concentrations of KL-6, SP-A, and SP-D rather reflect the later stage (remodeling stage) of the process of fibrosis.

In conclusion, our study might be the first to show that plasma concentrations of OPN are increased in patients with IP and inversely correlated with arterial oxygen tension. OPN expression was also evident in macrophages and airway epithelium. Our findings suggest that the plasma OPN levels were found to be associated with the presence of IP, and that OPN play an important role in the development of IP. However, as our study included several types of IP and recent several reports have demonstrated the high OPN levels in cancer and in pulmonary tuberculosis, further evaluations are necessary to elucidate the potential of OPN in the clinical management of IP.

References

11. Takahashi H, Fujishima T, Koba H, et al. Serum surfactant proteins A and D are biomarkers of IP. 22,23