The Role of Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1 in Cryptogenic Organizing Pneumonia*

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Background: The processes observed in interstitial lung disease are associated with the production, deposition, and proteolysis of the extracellular matrix (ECM), which may lead to irreversible pulmonary structural remodeling or to appropriate repair without fibrosis. Matrix metalloproteinases (MMPs) and a tissue inhibitor of metalloproteinases (TIMPs) are known to regulate remodeling of the ECM and thus to be important in the process of lung fibrosis. Pulmonary structures are extensively remodeled in usual interstitial pneumonia (UIP), whereas severe architectural remodeling is minimally present in cryptogenic organizing pneumonia (COP). However, not much is known about the roles of MMP-9 and/or TIMP-1 in COP.

Methods: Levels of MMP-9, TIMP-1 and the molar ratio of MMP-9/TIMP-1 in BAL fluids were investigated in 11 patients with UIP, in 8 patients with COP, and in 10 control subjects. We checked the levels of MMP-9 and TIMP-1 by means of enzyme immunoassay, and the hydrolytic activity of MMP-9 in BAL fluids was measured by gelatin zymography. Further, we evaluated the expression of MMP-9 and TIMP-1 in lung tissue by immunohistochemistry.

Results: The concentrations of MMP-9 and TIMP-1 were significantly increased in patients with UIP and were even higher in patients with COP compared with control subjects. The levels of MMP-9 and TIMP-1 were significantly higher in patients with COP than in patients with UIP. The molar ratio of MMP-9/TIMP-1 was significantly higher in patients with COP than in control subjects. In patients with COP, the concentration of MMP-9 significantly correlated with the number of neutrophils and lymphocytes. Zymographic analysis revealed that the activity of the 92-kd pro-MMP-9 was increased in patients with UIP and was even higher in patients with COP, compared with control subjects.

Conclusions: These data suggest that overproduction of MMP-9 and TIMP-1, and an imbalance between MMP-9 and TIMP-1, may have a role as diagnostic references in COP.

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Key words: cryptogenic organizing pneumonia; lymphocyte; matrix metalloproteinase-9; neutrophil; tissue inhibitor of metalloproteinase-1; usual interstitial pneumonia

Abbreviations: COP = cryptogenic organizing pneumonia; DLco = diffusion capacity of the lung for carbon monoxide; ECM = extracellular matrix; IPF = idiopathic pulmonary fibrosis; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase; TLC = total lung capacity; UIP = usual interstitial pneumonia

The processes observed in interstitial lung disease are associated with the production, deposition, and proteolysis of the extracellular matrix (ECM), which may lead to irreversible pulmonary structural remodeling or to appropriate repair without fibrosis. The clinical findings of cryptogenic organizing pneumonia (COP) are rather stereotyped.1,2 The onset of symptoms is subacute in most patients, with a flu-like syndrome and/or fever, persistent and nonproductive cough, exertional dyspnea, malaise, anorexia, and weight loss. COP is characterized by intra-alveolar and bronchiolar intraluminal buds of granulation tissue consisting of loose collagen-embedding fibroblasts and myofibroblasts.3,4 Pulmonary structures are extensively remodeled in usual interstitial pneumonia (UIP), whereas severe architectural remodeling is minimally present in COP. COP responds to...
corticosteroid therapy, the prognosis is fairly good and histologic changes are reversible, and the disease is curable.

Recently, matrix metalloproteinases (MMPs) and specific tissue inhibitors of metalloproteinases (TIMPs) have been shown to participate in the parenchymal destruction and repair processes that result in ECM remodeling.\(^7\)–\(^12\) The balance between MMPs and their inhibitors is critical in tissue repair and remodeling, and its homeostasis plays an important role in the breakdown and deposition of ECM in the airway wall. The major sources of MMP-9 in the airways are macrophages and eosinophils. In addition, this enzyme may also be released by neutrophils. MMP-9 induces the migration of eosinophils and neutrophils across the basement membrane.\(^13\)–\(^14\)

Not much is known about the roles of MMP-9 and/or TIMP-1 in COP. In this study, we hypothesized that the pathophysiology of pulmonary parenchymal remodeling differs between UIP and COP, and that this may influence the different natural histories of these distinct diseases. We assessed the levels of MMP-9 and TIMP-1, and the molar ratio of MMP-9/TIMP-1, in BAL fluids using gelatin zymography and enzyme immunoassay to demonstrate their relationship to remodeling processes in patients with COP compared with patients with UIP and normal control subjects.

**Materials and Methods**

**Subjects**

Study subjects included 11 patients with UIP, 8 patients with COP, and 10 normal control subjects (Table 1). The diagnosis of interstitial lung disease was based on the presence of progressive breathlessness, bilateral dry crackles, widespread bilateral infiltrates in chest radiographs, and pulmonary function test results showing a reduced total lung capacity (TLC), reduced FVC, decreased single-breath diffusion capacity of the lung for carbon monoxide (DLCO), and resting hypoxemia. Histologic confirmation was done by open-lung biopsy. The pathologic criteria were as follows: UIP is characterized by patch scarring of the lung parenchyma with intervening normal or nearly normal alveoli. The most fibrotic zones show honeycomb formation with complete destruction of the architecture, and in these regions, inflammation, mucostasis, and metaplastic epithelial changes may all be prominent (Fig 1, left, A). COP is characterized by intra-alveolar and bronchiolar intraluminal buds of granulation tissue consisting of loose collagen-embedding fibroblasts and myofibroblasts, and the presence in the alveolar lumen of numerous foamy macrophages (Fig 1, right, B). Patients were excluded from the study if they had collagen vascular disorder or a clinical history of environmental exposure, hypersensitivity pneumonitis, drug-induced pulmonary disease, or chronic pulmonary infections. None of the subjects were current or previous smokers. We successfully retrieved BAL fluids from all subjects enrolled in this study, before the treatment of the disease. This study was approved by the Ethics Committee of Chonbuk National University Medical School, and fully informed, written consent was obtained from each subject. After conducting a clinical evaluation including pulmonary function tests, we performed BAL and an open-lung biopsy in the most involved area using high-resolution CT. For proof of histology, three pathologists reviewed the slides, being only informed of the age and sex of each patient and the presence of unilateral or bilateral pulmonary disease determined by the chest posteroanterior radiograph and the high-resolution CT.

**BAL Procedure and Assay of Cell Counts**

The study subjects were pretreated with atropine (0.5 mg) and midazolam (2 mg) immediately before the BAL procedure, which was performed in the segmental bronchus of the right middle lobe. Fifty milliliters of warmed, normal saline solution was instilled into the bronchial tree five times followed by gentle suction with a negative pressure < 100 mm Hg, using a fiberoptic bronchoscope (Olympus B2–10; Olympus Optical; Tokyo, Japan). No complications occurred during the BAL procedures. The total volume of recovered fluids was measured. The aspirated fluid was collected into sterile siliconized containers at 4°C and then filtered through sterile gauze and separated into its fluid and cellular components by centrifugation at 400g for 10 min at 4°C. The number of total cells was determined using a hemocytometer. The cells were cytocentrifuged (Cytospin 2; Shandon Instruments; Runcorn, UK) and stained by Diff-Quik staining (International Reagents; Kobe, Japan) to perform the differential cell counts. Two independent investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations.

**Gelatin Zymography**

The hydrolytic activity of MMP-9 in BAL fluids was measured by gelatin zymography, as previously described.\(^15\)–\(^16\) Samples containing the same amount of protein (10 µg) were mixed with 5X sample buffer (0.4 mol/L Tris-HCl, pH 6.8, 5% sodium dodecyl sulfate, 20% glycerol, and 0.1% bromophenol blue) and were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis containing 0.1% gelatin. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 1 h and were then placed in the enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 mol/L NaCl, 5 mM CaCl\(_2\), and 0.02% Brij-35) for 24 h at 37°C. The gels were stained with 0.5% Coomassie brilliant blue-250 solution and destained with several changes of 30% methanol and 10% acetic acid. Zones of enzymatic activity were evident as clear bands against the blue background. Reference standards used were MMP-2 and MMP-9 (Chemicon International; Temecula, CA).

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**Table 1—Demographic and Physiologic Profiles of Subjects Studied**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>UIP</th>
<th>COP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, No.</td>
<td>10</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Age, yr</td>
<td>53.9 ± 8.28</td>
<td>58.3 ± 8.01</td>
<td>53.4 ± 8.70</td>
</tr>
<tr>
<td>Male/female sex, No.</td>
<td>5/5</td>
<td>6/5</td>
<td>4/4</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>106.3 ± 3.40</td>
<td>69.5 ± 3.62†</td>
<td>72.6 ± 3.96†</td>
</tr>
<tr>
<td>FEV(_1)/FVC, %</td>
<td>91.6 ± 2.22</td>
<td>83.4 ± 2.50†</td>
<td>76.8 ± 4.59†</td>
</tr>
<tr>
<td>TLC, % predicted</td>
<td>92.4 ± 2.27</td>
<td>71.5 ± 4.06†</td>
<td>78.3 ± 3.77†</td>
</tr>
<tr>
<td>DLCO, % predicted</td>
<td>94.0 ± 2.67</td>
<td>42.7 ± 3.55†</td>
<td>80.4 ± 4.41†</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD unless otherwise indicated.

\(†p < 0.01\) vs normal control subjects.

\(†p < 0.01\) vs UIP.
Measurement of MMP-9 and TIMP-1

Levels of MMP-9 and TIMP-1 were quantitated by enzyme immunoassay according to the protocol of the manufacturer (Fuji Chemical Industries; Toyama, Japan). The detection limits were, respectively, 3.1 ng/mL for MMP-9 and 1.2 ng/mL for TIMP-1; values are reported as nanograms per milliliter.

Histology and Immunohistochemistry

Lung tissues were fixed with 10% (volume/volume) neutral-buffered formalin. Paraffin tissue blocks were sectioned at 4 μm and were stained with hematoxylin-eosin. Immunohistochemical studies were carried out on paraffin sections using the avidin-biotinylated peroxidase complex method. Briefly, the sections were first treated with a blocking protein to minimize nonspecific binding of other reagents, followed by incubation overnight at 4°C with primary antibodies for a mouse antihuman MMP-9 or a mouse antihuman TIMP-1 (Chemicon International). Incubation with a biotinylated secondary antibody solution for 1 h and then with a avidin-biotin-peroxidase complex for 1 h were carried out. Peroxidase activity was visualized using 3-amino 9-ethylcarbazole as the substrate. The sections were counterstained with hematoxylin. As controls, sections were incubated with nonimmunized mouse Ig (IgG) instead of specific monoclonal antibodies, and were processed according to the same procedure. The distribution and intensity of the immunohistochemical positive reactions were evaluated by three independent observers without knowledge of the kinds of tissues and antibodies used.

Statistical Analysis

All values are reported as means ± SD. The Mann-Whitney U test was used for between group comparisons. Spearman rank correlation was calculated to assess the correlation between data. Comparisons of the general characteristics of each group were made through analysis of variance and Sheffe’s test. Statistical significance was assumed for p < 0.05.

RESULTS

The mean age and sex of patients with UIP and COP and of control subjects in this study were similar (Table 1). Values for FVC, FEV1/FVC, TLC, and DLCO were significantly lower in patients with COP and in patients with UIP than in control subjects (all p < 0.01). The values for FEV1/FVC, DLCO, and TLC were also significantly different between patients with COP and patients with UIP (all p < 0.01). The recovery rates of BAL fluids in patients with COP, in patients with UIP, and in control subjects were similar (Table 2). The total number of cells in BAL fluids was significantly greater in patients with COP and in patients with UIP than in control subjects (both p < 0.01). The

Table 2—Cellular Profiles in BAL Fluids of Patients and Control Subjects*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control (n = 10)</th>
<th>UIP (n = 11)</th>
<th>COP (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery rate, %</td>
<td>56.3 ± 6.54</td>
<td>51.3 ± 5.18</td>
<td>52.7 ± 7.02</td>
</tr>
<tr>
<td>Total cell number, × 10⁴/mL</td>
<td>16.2 ± 1.93</td>
<td>37.8 ± 5.84†</td>
<td>43.4 ± 4.63†</td>
</tr>
<tr>
<td>Macrophages, × 10⁴/mL</td>
<td>14.7 ± 1.75</td>
<td>26.7 ± 3.96†</td>
<td>20.7 ± 3.79†</td>
</tr>
<tr>
<td>Lymphocytes, × 10⁴/mL</td>
<td>1.16 ± 0.28</td>
<td>4.21 ± 1.05†</td>
<td>16.02 ± 3.36†</td>
</tr>
<tr>
<td>Neutrophils, × 10⁴/mL</td>
<td>0.28 ± 0.11</td>
<td>4.48 ± 1.08†</td>
<td>5.24 ± 1.15†</td>
</tr>
<tr>
<td>Eosinophils, × 10⁴/mL</td>
<td>0.09 ± 0.08</td>
<td>2.45 ± 0.96†</td>
<td>1.37 ± 0.33†</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
†p < 0.01 vs normal control subjects.
‡p < 0.01 vs UIP.

FIGURE 1. Histology of lung tissue from patients with UIP (left, A), or COP (right, B). UIP shows zones of dense fibrosis and fibrosis alternating with nearly normal lung. COP shows typical fibroblast plugs filling bronchioles and alveolar spaces (hematoxylin-eosin, original × 100).
number of lymphocytes and neutrophils in BAL fluids was higher in patients with COP and in patients with UIP than in control subjects (both \( p < 0.01 \)), the number of eosinophils was higher in patients with UIP than in control subjects \( ( p < 0.01 ) \). The number of lymphocytes was significantly higher in patients with COP than in patients with UIP \( ( p < 0.01 ) \).

The mean concentration of MMP-9 was significantly increased in patients with UIP \( (7.52 \pm 2.54 \text{ ng/mL}) \) and was even higher in patients with COP \( (21.6 \pm 5.83 \text{ ng/mL}) \) compared with control subjects \( (2.48 \pm 1.51 \text{ ng/mL}) \) [both \( p < 0.01 \)]. The level of MMP-9 was significantly higher in patients with COP than in patients with UIP \( (p < 0.01; \text{Fig 2, top A}) \). In patients with COP, the concentration of MMP-9 was significantly correlated with the number of neutrophils and lymphocytes (both \( p < 0.01 \); Fig 3, top A and bottom C). The concentration of TIMP-1 was significantly increased in patients with UIP \( (11.7 \pm 4.60 \text{ ng/mL}) \) and was even higher in patients with COP \( (25.0 \pm 5.91 \text{ ng/mL}) \) compared with control subjects \( (5.01 \pm 2.07 \text{ ng/mL}) \) [both \( p < 0.01 \)]. The level of TIMP-1 was significantly higher in patients with COP than in patients with UIP \( (p < 0.01; \text{Fig 2, center B}) \). In patients with COP, the molar ratio of MMP-9/TIMP-1 was significantly correlated with the number of neutrophils \( (p = 0.047; \text{Fig 3, center B}) \).

Consistent with the results obtained from the enzyme immunoassay, zymographic analysis revealed that the activity of the 92-kd pro-MMP-9 was increased in patients with UIP and was even higher in patients with COP compared with control subjects (Fig 4). The activity of the 72-kd pro-MMP-2 was increased in patients with UIP and was even higher in patients with COP compared with control subjects (Fig 4).

Immunohistochemical analysis revealed that MMP-9 was expressed by epithelial cells, alveolar macrophages, lymphocytes, and neutrophils in UIP (Fig 5, top left A). TIMP-1 expression was detected in epithelial cells, alveolar macrophages, lymphocytes, and neutrophils in lung tissue from patients with UIP (Fig 5, bottom left C). In COP, MMP-9 was expressed at high levels in lymphocytes, neutrophils, and macrophages (Fig 5, top right B). TIMP-1 expression was detected in epithelial cells and macrophages in lung tissue from patients with COP (Fig 5, bottom right D).

**Discussion**

In this study, we have demonstrated that levels of MMP-9 and TIMP-1 were significantly increased in patients with UIP and were even higher in patients with COP compared with control subjects. The levels of MMP-9 and TIMP-1 were significantly higher in patients with COP than in patients with UIP. The molar ratio of MMP-9/TIMP-1 was significantly higher in patients with COP than in control subjects.

MMPs are capable of degrading various compo-
MMP-9 overexpression in patients with UIP contributes more to lung matrix remodeling and the repair process leading to fibrosis than it does to degradation. The level of MMP-9 in the air spaces may relate to the degree of severity of inflammation of lung disease.21,22 COP is characterized by intra-alveolar and bronchiolar intraluminal buds of granulation tissue consisting of loose collagen-embedding fibroblasts and myofibroblasts.3-4 Pulmonary structures are extensively remodeled in UIP, whereas severe architectural remodeling is only minimally present in COP, which responds to corticosteroid therapy. For COP, the prognosis is fairly good,3-5 and histologic changes are reversible and the disease is curable.6 Therefore, the pathophysiology of pulmonary parenchymal remodeling differs between UIP and COP, and this may influence the natural histories of these distinct diseases. In this study, overproduction of MMP-9 may be more important in the pathogenesis of COP than of UIP.

The cellular profiles of BAL fluids accurately reflect the cellularity of peripheral parts of the lung on diffuse interstitial lung disease.23,24 The cellularity of lung lesions was estimated by numerical analysis of inflammatory cells in the BAL fluids. In this study, the total number of cells was significantly greater in patients with COP and in patients with UIP than in control subjects. The number of lymphocytes and neutrophils in BAL fluids was higher in patients with COP and in patients with UIP than in control subjects. The number of lymphocytes was significantly higher in patients with COP than in patients with UIP. In patients with COP, the concentration of MMP-9 was significantly correlated with the number of neutrophils and lymphocytes. The major sources of MMP-9 in the airways are macrophages, eosinophils, and neutrophils.25,26 In addition, several studies27-28 reported that lymphocytes are important cellular sources of MMP-9. It was reported that elevated levels of MMP-9 correlate with the number of neutrophils in BAL fluids from patients with ARDS.9,10 In this study, we have demonstrated that MMP-9 is expressed at high levels by lymphocytes and by neutrophils using immunohistochemistry. These results suggest that increased numbers of neutrophils and lymphocytes lead to the increased release of MMP-9 in COP. The molar ratio of MMP-9/TIMP-1 correlated significantly with the number of neutrophils (p = 0.047, r = 0.714). This is a marginally acceptable correlation, most likely due to the small sample size. These data suggest that the number of neutrophils may be more closely related to levels of MMP-9 than to levels of TIMP-1. To further clarify this matter, studies with a much larger patient sample size will be necessary.

![Figure 3. Correlation between the neutrophil count and the concentration of MMP-9 (top, A), and MMP-9/TIMP-1 molar ratio (center, B), and correlation between the lymphocyte count and the concentration of MMP-9 (bottom, C) in patients with COP. Statistical analysis was performed using Spearman rank test.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21977/)
Suga et al. found that idiopathic pulmonary fibrosis (IPF)-UIP cases showed predominant expression of MMP-9, whereas bronchiolitis obliterans organizing pneumonia cases showed predominant MMP-2 expression in BAL fluids and in tissues. In BAL fluids samples from rapidly progressive IPF-UIP cases, neutrophil-derived MMP-9 activity, as well as MMP-9 active form, were characteristically detected. Furthermore, the MMP-9 activity correlated significantly with an increase of neutrophils in BAL fluids, whereas the MMP-2 activity associated with bronchiolitis obliterans organizing pneumonia corre-

Figure 4. Analysis of BAL fluids by gelatin zymography in control subjects (lane 2), UIP patients (lane 3), and COP patients (lane 4). Lane S contains standards (lane 5): the proform of MMP-9 (pMMP 9), the proform of MMP-2 (pMMP 2), and the active form of MMP-2 (aMMP 2). Molecular weight markers (M) [lane 1] were used to estimate molecular masses. Gelatinolytic bands of 92-kd pro-MMP-9 (pMMP-9) and 72-kd pro-MMP-2 (pMMP-2) are detected.

Figure 5. Immunohistochemical staining for MMP-9 and TIMP-1 in lung tissue from patients with UIP (top left, A; bottom left, C) and COP (top right, B; bottom right, D). In UIP, MMP-9 is expressed by epithelial cells, alveolar macrophages, lymphocytes, and neutrophils (left, A). TIMP-1 expression is detected in epithelial cells, alveolar macrophages, lymphocytes, and neutrophils (left, C). In COP, MMP-9 is expressed at high levels in lymphocytes, neutrophils, and macrophages (right, B). TIMP-1 expression is detected in epithelial cells and macrophages (right, D) [all images original × 100].
related with an increase of lymphocytes. Our data differ from those reported by Suga et al.29 In our study, the level of MMP-9 was significantly higher in patients with COP than in patients with UIP. We considered several circumstances that may be responsible for these different results. First, none of the subjects in our study were current or previous smokers. However, of the 26 UIP patients in the study reported by Suga et al., 16 were smokers, and it is known that cigarette smoking can increase the release of MMP-9.30 Second, in the study reported by Suga et al.,29 the IPF-UIP patients were classified into two groups, a rapidly progressive IPF-UIP (R) group and a slowly progressive IPF-UIP (S) group. The IPF-UIP (S) samples showed significantly lower MMP-9 activities than did the IPF-UIP (R) samples. In our study, the UIP patients were not classified into two groups. Consistent with the results obtained in the study reported by Suga et al.,29 the zymographic analysis in our study revealed that the activity of pro-MMP-2 was increased in patients with COP.

The capacity to generate high levels of MMP-9 during the course of interstitial lung disease is accompanied by the generation of elevated levels of TIMP-1, although to a lesser extent.20 The increased concentrations of TIMP-1 found in COP and UIP can result from the effects of several mediators released during the development of airway inflammation in this disease. A high concentration of TIMP-1 can lead to an increased deposition of ECM components, and may increase myofibroblast proliferation acting through its cell growth promoting activity.31 In this study, the concentration of TIMP-1 was significantly increased in patients with UIP and was even higher in patients with COP compared with control subjects. The level of TIMP-1 was significantly higher in patients with COP than in patients with UIP.

Vignola et al.15 suggest that an imbalance between MMP-9 and TIMP-1 may have a role in the pathogenesis of ECM remodeling and airflow obstruction. The evaluation of MMPs and of their inhibitors has also been proposed in other studies,32,33 suggesting that they may be useful markers of airway inflammation and remodeling. In this study, the molar ratio of MMP-9/TIMP-1 was significantly higher in patients with COP than in control subjects. These results suggest that an imbalance between MMP-9 and TIMP-1 may play a role in the pathogenesis of COP. In conclusion, these data suggest that overproduction of MMP-9 and TIMP-1, and an imbalance between MMP-9 and TIMP-1, in BAL fluids may have a role as diagnostic references in COP.

REFERENCES


Clinical Investigations


