Open Journal of Respiratory Diseases, 2014, 4, 101-109
Published Online August 2014 in SciRes. http://www.scirp.org/journal/ojrd
http://dx.doi.org/10.4236/ojrd.2014.43014

The Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Idiopathic Interstitisal Pneumonia

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Received 5 June 2014; revised 6 July 2014; accepted 4 August 2014

Abstract

Background: Idiopathic interstitial pneumonia is characterized by fibroblast proliferation and extracellular matrix (ECM) accumulation. Matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) have been shown to regulate remodeling of the ECM, which indicates that they are important factors in the process of lung fibrosis. Therefore, we evaluated the expression of MMPs and TIMPs in tissues obtained from patients with idiopathic interstitial pneumonia and control tissues. Methods: Thirty-seven patients who were diagnosed with IIP (22: IPF, 13: NSIP, 2: COP) and 5 controls were enrolled in this study. The MMP-2 and -9 activity in lung tissue obtained from these patients was analyzed using gelatin zymography and the levels of TIMP-1 and -2 were measured by western blotting. We also evaluated the expression of MMP-2 and -9, as well as that of TIMP-1 and -2 in lung tissue using immunohistochemistry. Results: The levels of MMP-2 and MMP-9 were significantly increased in patients with IPF compared to those with NSIP and COP. The activities of TIMP-1 and -2 were also higher in patients with IPF than NSIP/COP patients and control subjects. There were no significant differences observed in the activities of MMPs and TIMPs obtained from patients with NSIP/COP and control subjects. The immunohistochemical analysis showed that TIMP-2 and MMP-2 were strongly stained at the fibroblasts of the fibroblastic foci in patients with IPF. Conclusions: These results suggest that over-expression of gelatinases and TIMPs in patients with IPF are important factors in the irreversible fibrosis that is associated with lung parenchyma.

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How to cite this paper: Shin, J.Y., Kim, Y.J., Kyung, S.Y., Park, J.-W., Ha, S.Y. and Jeong, S.H. (2014) The Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Idiopathic Interstitisal Pneumonia. *Open Journal of Respiratory Diseases*, **4**, 101-109. http://dx.doi.org/10.4236/ojrd.2014.43014

Keywords

Idiopathic Interstitial Pneumonia, Idiopathic Pulmonary Fibrosis, Matrix Metalloproteinases, Tissue Inhibitor of Matalloproteinases, Nonspecific Interstitial Pneumonia, Cryptogenic Organizing Pneumonia

1. Introduction

Idiopathic interstitial pneumonia (IIP) is comprised of a group of interstitial pneumonias of unknown etiology that is divided into seven disease entities, including idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP) and cryptogenic organizing pneumonia (COP), on the basis of clinical, radiological and pathological findings [1]. Idiopathic pulmonary fibrosis (IPF), which is one of the more common types of IIP, presents histologic lesions associated with usual intersitial pneumonia (UIP) [2] [3]. The pathologic features of IPF are patchy fibrosis, prominent fibroblast foci, scant interstitial inflammation, and honeycombing change [3]. Nonspecific interstitial pneumonia (NSIP) is characterized by homogenous interstitial inflammation and fibrosis, rare fibroblast foci, and occasional organizing pneumonia [3]. Cryptogenic organizing pneumonia (COP) shows prominent interstitial inflammation and intralumenal organizing fibrosis in distal air spaces [3]. Although there is interstitial fibrosis associated with inflammation in IPF, NSIP and COP, their clinical courses are very different [1]-[4]. IPF has a very poor prognosis, with patients surviving an average of only 2.5 - 3.5 years from time of diagnosis [1]-[3], whereas NSIP and COP both have a more favorable prognosis and response to treatment than IPF [3]-[5]. Generally, patients with NSIP and COP show relatively preserved lung architecture, reversible changes of histologic abnormalities and recovery of clinical symptoms. However, patients with IPF show a deterioration of clinical symptoms, ultimately resulting in respiratory failure due to irreversible progressive interstitial fibrosis [3]. Although the pathogenesis of IPF is still unclear, matrix metalloproteinases (MMP) and tissue inhibitors of matalloproteinases (TIMP) are known to be important factors that affect the progress of IPF [6]. MMPs are proteolytic enzymes known to regulate the extracellular matrix (ECM) turnover, therefore it has been suggested that they play an important role in lung diseases associated with tissue remodeling [7] [8]. It has also been suggested that, in cases of IPF, over-expression of TIMP inhibits degradation of the extracellular matrix (ECM), which induces excess deposition of ECM [6]. In addition, gelatinases, such as MMP-2 and -9, might play an important role in the disruption of the basement membrane, which in turn induce migration of the fibroblasts [6]. It is well known that the activities of TIMPs and gelatinases are high in patients with IPF, however it is still unclear if the expression of MMPs and TIMPs in cases of IIP is associated with a favorable prognosis, such as occurring in cases of NSIP and COP. Therefore, this study was conducted to determine if the expression of MMPs and TIMPs differed between patients with IPF and patients with other IIPs such as NSIP and COP, and if so, if this difference influenced the poor clinical prognosis of patients with IPF. To accomplish this, we evaluated the activities of MMP-2 and -9, as well as those of TIMP-1 and -2 using lung tissue obtained from patients with IPF, NSIP and COP.

2. Materials and Methods

2.1. Subjects

The study subjects consisted of 22 patients with IPF, 13 patients with NSIP, 2 patients with COP, and 5 control subjects. Additionally, histologically normal lung tissues were obtained by lobectomy or pneumonectomy from 5 patients diagnosed with lung cancer to be used as controls. The diagnosis of IIP was based on clinical, radiological and functional findings, as well as pathologic findings obtained by open lung biopsy. None of the patients had been treated with steroids or any immunosuppressive agents at the time of the open lung biopsy. To confirm the pathology, two pathologists independently reviewed the slides. Patients with a collagen vascular disorder or with a clinical history of environmental exposure, hypersensitivity pneumonitis, drug-induced pulmonary disease or chronic pulmonary infection were excluded from this study. We retrospectively reviewed medical records, including laboratory data regarding patients that were enrolled in this study. All subjects gave appropriate informed consent and the study design was approved by the Ethics Committee of Gachon University

of Medicine and Science.

2.2. Gelatin Zymography

Gelatin zymography was used to measure the gelatinolytic activity of MMP-2 and -9 in lung tissue. Proteins were extracted from lung tissue, and then separated by 10% sodium dodecylsulfate-polyacryllamide gel electrophoresis on a gel containing 0.1% gelatin at constant voltage of 60 V for 5 hours. After electrophoresis, the gel was incubated in 2.5% triton X-100 for 30 minutes, and then incubated in enzyme buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100) overnight at 37˚C. The gel was then stained with 0.5% Coomassie blue. After destaining in a solution of methanol and acetic acid, zones of enzyme activity were detected as regions of clear bands against the blue background. The levels of the 66 kDa active form of MMP-2 (aMMP-2), the 72 kDa proform of MMP-2 (pMMP-2), the 85 kDa active form of MMP-9 (aMMP-9) and the 92 kDa proform of MMP-9 (pMMP-9) were assessed and densitometry was performed for quantitative analysis. MMP-2 and MMP-9 were used as reference standards (Chemicon International; Temecula, CA).

2.3. Western Blot Analysis

Nonspecific staining was blocked by incubating the lung tissue for 90 minutes in TBS containing 5% nonfat dry milk. Next, the tissue was incubated with rabbit polyclonal antiserum for TIMP-1 and -2 diluted in TBS, and then incubated for 90 minutes with biotinylated goat antirabbit immunoglobulin G diluted 1:1000 as the secondary antibody (Santa Cruz Biotechnology, Inc.; Santa Cruz, USA). The protein bands were then evaluated using the ECL Plus Western blotting detection system with subsequent exposure to X-ray film, and then densitometry was performed to estimate the relative amount of protein. β-actin was used as a positive control to determine the sensitivity and specificity of the antibodies.

2.4. Immunohistochemistry

The obtained tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and then stained with hematoxylin, eosin and Masson trichrom to enable histopathologic diagnosis. Immunohistochemical studies were performed on 4 μm paraffin sections. Tissue sections were attached to slides covered with poly-L-Lysine, deparaffinized with cylene, rehydrated using alcohol, and then the endogeneous peroxide enzyme was blocked with 3% H_2O_2 for 10 minutes, followed by washing with tris buffer solution (TBS) for 5 minutes. Antigen retrieval was carried out in 10 mM citrate buffer solution and processed in a microwave oven for 5 minutes. The sections were then treated with a blocking protein, normal goat serum, to minimize binding of nonspecific proteins. Next the sections were incubated overnight at 4˚C with primary antibodies for MMP-2, MMP-9, TIMP-1 and TIMP-2 (Fuji Chemical Industries; Toyama, Japan). The sections were then incubated with a biotinylated secondary antibody solution for 30 minutes, and then treated with streptavidin peroxidase reagent at room temperature for 20 minutes. The color of the sections was revealed using diaminobenzidine and the sections were counterstained with Mayer's hematoxylin.

3. Statical Analysis

All values were expressed as the means ± the standard deviation (SD). An ANOVA test was used to compare the group comparisons and a p value of <0.05 was considered to be statistically significant.

4. Results

4.1. Patient Population

The mean age, sex and physiologic profiles of patients with IPF and NSIP/COP in this study are presented in **Table 1**. The initial FVC and DLco values were not significantly different between patients with IPF and those with NSIP/COP. However, the % change of FVC was significantly different between the IPF and NSIP/COP groups ($p < 0.05$), and the % change of DLco was significantly lower in patients with IPF than in patients with NSIP/COP ($0.05 < p < 0.1$). During the follow-up periods, two patients with IPF expired due to pneumonia and acute exacerbation of IPF (median follow-up periods, 30 months), however none of the patients in the NSIP or COP groups died during the follow up period.

Table 1. Demographic and physiologic profiles of subjects studied.

Variables	IPF (n = 22)	NSIP (n = 13)	COP (n = 2)	p value
Age, yr	57.5 ± 9.3	51.2 ± 7.9	55.5 ± 20.5	
Male/Female, No	14/8	1/12	1/1	
FVC, % predicted				
Initial	77.6 ± 21.0	61.0 ± 17.2	59.0 ± 0.2	
Follow-up	82.2 ± 23.2	79.0 ± 21.0	104.0 ± 0.2	
% change	3.3 ± 5.3	19.0 ± 16.9	45 ± 0.0	0.040
DLCO, % predicted				
Initial	72.0 ± 27.5	62.0 ± 20.3	68.0 ± 0.3	
Follow-up	77.1 ± 17.5	90.0 ± 26.4	95.0 ± 0.0	
% change	4.0 ± 3.8	27.0 ± 23.3	53.0 ± 0.0	0.063
FEV1/FVC, %	81.2 ± 8.4	86.7 ± 7.4	87.0 ± 12.7	
Follow-up period	30.0 ± 24.8	57.6 ± 24.1	38.4 ± 5.1	

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

4.2. Gelatin Zymography

Densitometric analysis of gelatinolytic bands corresponding to pMMP-2, aMMP-2, pMMP-9 and aMMP-9 are shown in **Figure 1**. The activity of aMMP-2 was significantly higher in patients with IPF (139.8 ± 10.5 OD/mm^2) than in patients with NSIP or COP (113.6 ± 7.0, 104.9 ± 0.1 OD/mm^2, $p < 0.05$, both) and the control group (98.5 ± 7.3 OD/mm^2) ($p < 0.05$). The level of pMMP-2 was also higher in patients with IPF (140.0 ± 6.8 OD/mm^2) than in patients with NSIP and COP (113.4 ± 3.6, 108.1 ± 0.7 OD/mm^2, $p < 0.001$, both) and the control subjects (99.9 ± 9.2 OD/mm^2, $p < 0.05$). The activity of aMMP-9 in patients with IPF (151.6 ± 13.8 OD/mm^2) was significantly higher than that of patients with NSIP and COP (110.9 ± 14.2, 104.6 ± 1.2 OD/mm^2, $p < 0.05$, both), and there was no difference observed between patients with IPF and control subjects (149.7 ± 0.8 OD/mm^2) when aMMP-9 activity was considered. The level of pMMP-9 was also significantly higher in patients with IPF (141.2 ± 14.2 OD/mm^2) than in patients with NSIP and COP (104.9 ± 2.9, 103.2 ± 4.6 OD/mm^2, $p < 0.05$, both), however it was not significantly different than that of the control subjects (133.7 ± 0.1 OD/mm^2). However, the levels of pMMP-9 in parents with COP were significantly lower than those of the control subjects ($p < 0.05$).

4.3. Western Blotting of TIMP-1 and -2 (Figure 2)

The levels of TIMP-1 were significantly higher in patients with IPF (559.8 ± 35.3 OD/mm^2) than in patients with NSIP and COP (410.9 ± 41.8, 371.9 ± 48.0 OD/mm^2, $p < 0.05$, both) and the control subjects (471.7 ± 30.9, $p < 0.05$). The levels of TIMP-1 in patients with COP were significantly lower than those of the control subjects ($p < 0.05$). The levels of TIMP-2 were also significantly higher in patients with IPF (500.5 ± 50.8 OD/mm^2) than in patients with NSIP and COP (352.5 ± 36.7, 341.5 ± 52.9 OD/mm^2, $p < 0.05$, both) and the control subjects (395.7 ± 46.3 OD/mm^2, $p < 0.05$).

4.4. Immunohistochemistry

1) IPF

TIMP-1 was weakly detected in alveolar epithelial cells and interstitial fibroblasts and myofibroblasts (**Figure 3(A)**). TIMP-2 was expressed intensely only in the interstitial fibroblasts of the fibroblastic foci (**Figure 3(D)**). MMP-2 was expressed at low levels by alveolar epithelial cells and alveolar macrophages (**Figure 3(G)**), however, fibroblasts were strongly stained for MMP-2. MMP-9 was widely expressed in alveolar epithelial cells and alveolar macrophages, as well as in fibroblasts and neutrophils (**Figure 3(J)**), with fibroblasts and neutrophils exhibiting particularly strong staining for MMP-9.

2) NSIP/COP

TIMP-1 was weakly expressed in the alveolar epithelial cells and alveolar macrophages of patients with NSIP (**Figure 3(B)**), and the fibroblasts only stained weakly for TIMP-2 in these patients (**Figure 3(E)**). However,

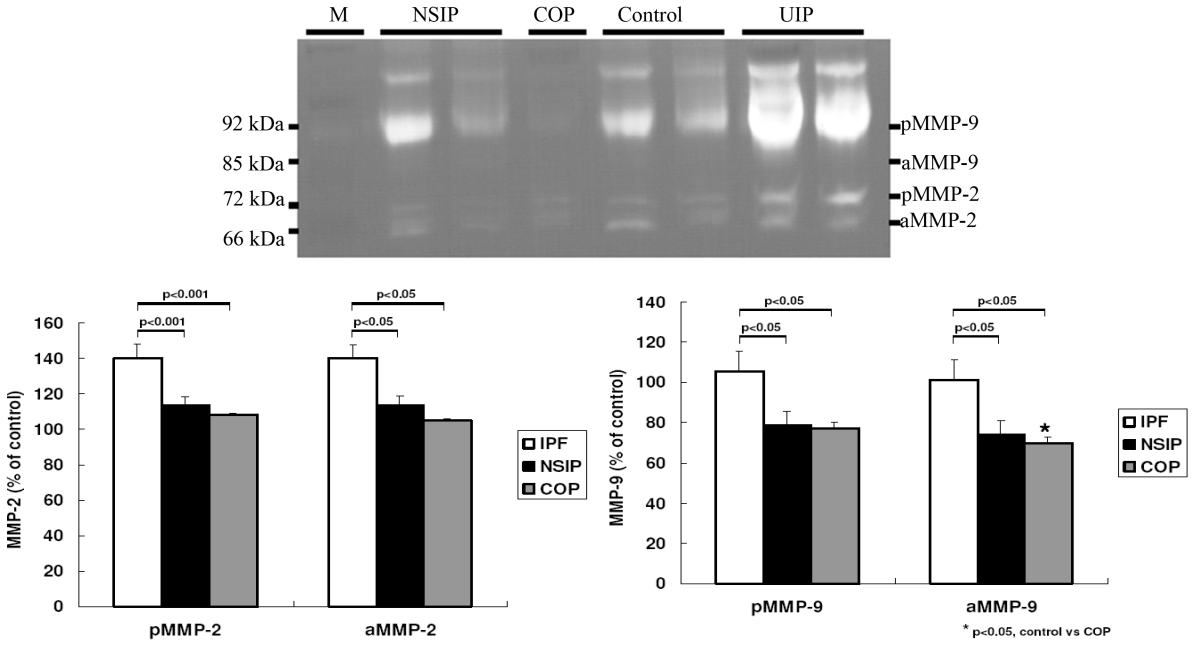

Figure 1. Gelatin zymography and densitometric analysis of MMP-2 and -9 in lung tissue obtained from IPF, NSIP, COP and control subjects. Intense lytic bands corresponding to MMP-2 and -9 are visible in samples obtained from IPF patients. The bands corresponding to MMP-2 and -9 in NSIP, COP and control samples show less expression than those obtained from IPF patients. The mean concentration of pMMP-2 and aMMP-2 in IPF patients is higher than that of NSIP, COP and control subjects. The level of pMMP-9 and aMMP-9 in IPF patients is higher than of NSIP/COP patients. Additionally, the level of aMMP-9 is higher in the control samples than in the NSIP and COP patients.

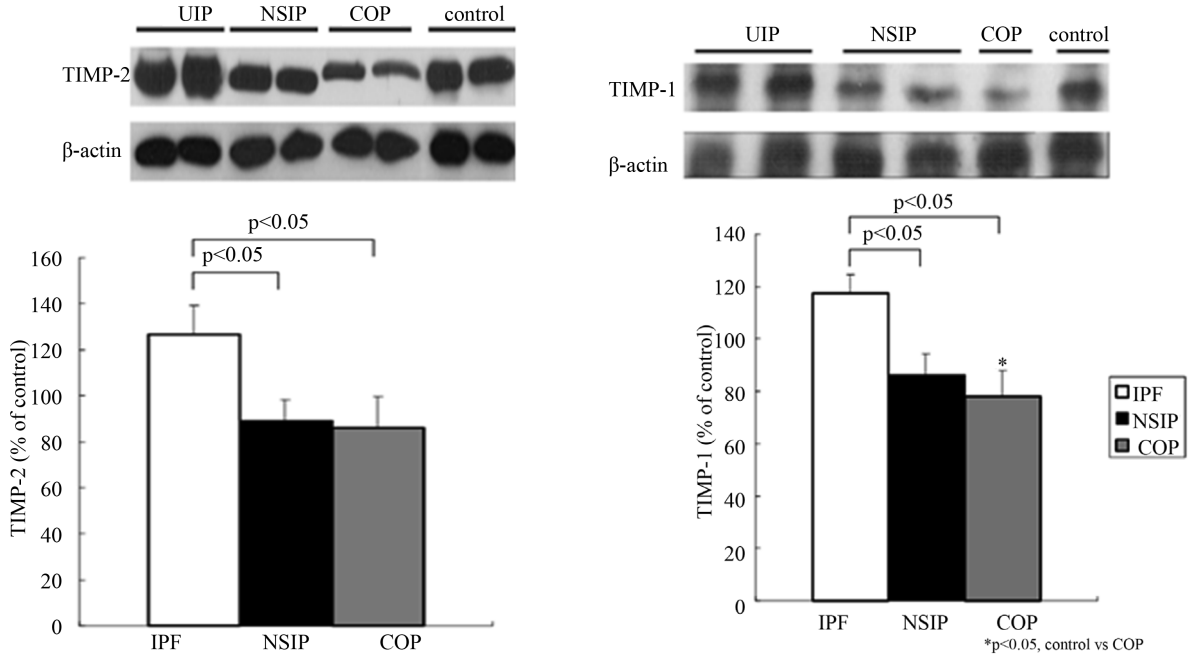

Figure 2. Western blotting for TIMP-1 and -2 in lung tissue obtained from IPF, NSIP, COP and control subjects. Intense bands of 22 kDa (TIMP-2), 30 kDa (TIMP-1) and 40 kDa (ß-actin) were detected. Bands corresponding to TIMP-1 and TIMP-2 are more intense in IPF patients than NSIP/COP patients and control subjects. The concentrations of TIMP-1 and TIMP-2 in IPF patients are higher than in NSIP/COP and control subjects. Additionally, TIMP-1 expression is greater in control samples than in COP patients.

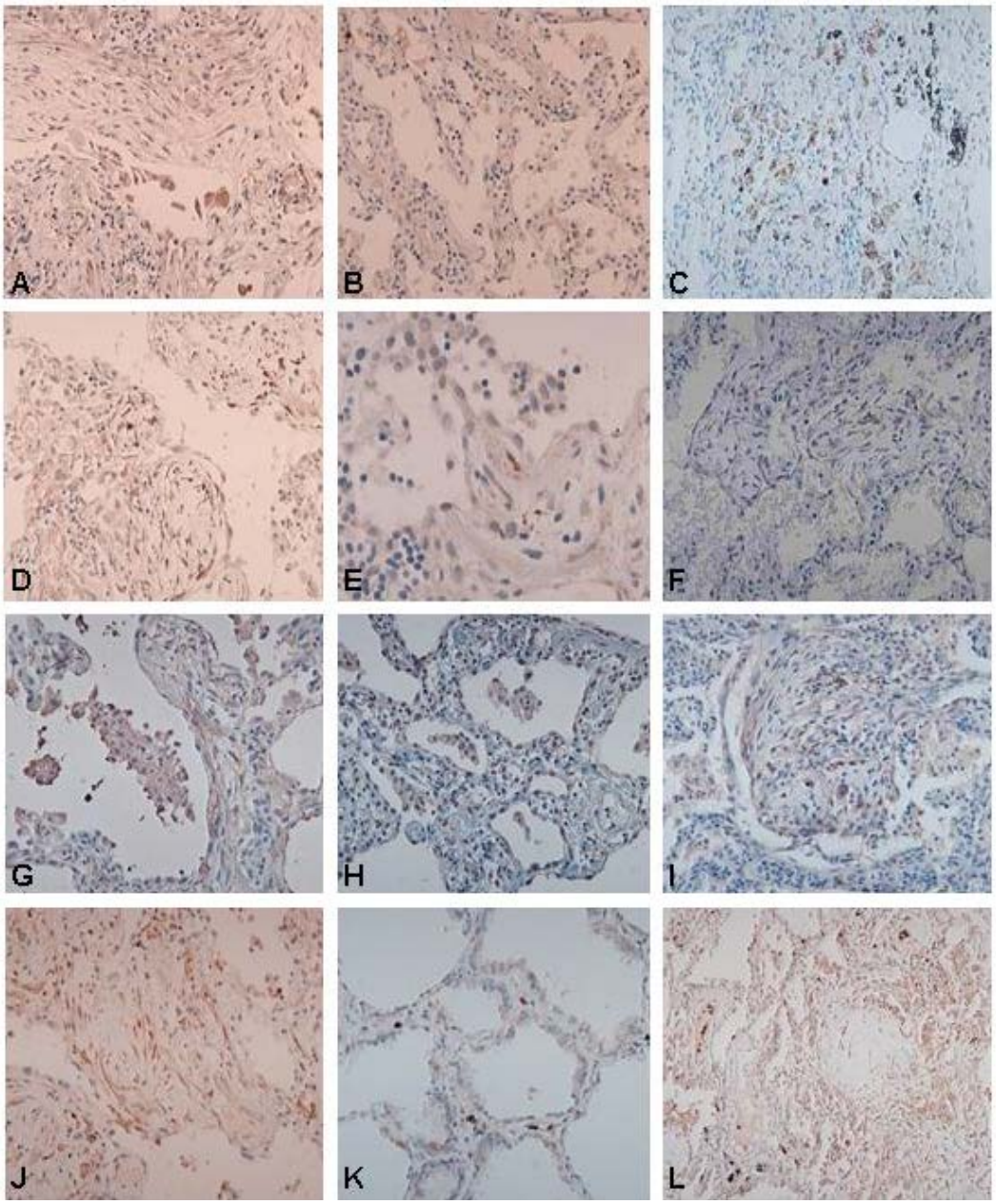

Figure 3. Immunohistochemical detection of TIMP-1 in lung tissue obtained from patients with IPF (A), NSIP (B), COP (C); TIMP-2 in lung tissue obtained from patients with IPF (D), NSIP (E), COP (F); MMP-2 in lung tissue obtained from patients with IPF (G), NSIP (H), COP (I); MMP-9 in lung tissue obtained from patients with IPF (J), NSIP (K), COP (L). Macrophages and myofibroblasts show a weak to moderate reaction for TIMP-1 (A). Only a small amount of immunoreactivity for TIMP-1 is seen in the epithelial cells lining the alveolar space. Macrophages show only a minimal increase in reactivity (B). Alveolar epithelial cells and alveolar macrophages show a weak to moderate reaction for TIMP-1 (C). TIMP-2 is intensely stained in the interstitial cells located in the subepithelial fibroblasts/myofibroblast foci (D). Most areas of tissue obtained from patients with NSIP (E) and COP (F) are negative for TIMP-2. Rare foci of myofibroblastic cells in tissue obtained from patients with NSIP (E) and COP (F) show a positive reaction of TIMP-2. MMP-2 staining in samples obtained from patients with IPF (G), NSIP (H) and COP (I) is seen in the interstitial cells located in the subepithelial fibroblast/ myofibroblast foci. Fibroblasts/myofibroblasts in samples obtained from IPF patients show a moderate reaction for MMP-9 (J). Additionally, moderate to strong MMP-9 staining of tissues obtained from patients with IPF (J), NSIP (K) and COP (L) is observed in the cellular components, such as neutrophils and lymphocytes. All panels, original × 100.

MMP-2 was widely expressed in alveolar epithelial cells, alveolar macrophages and fibroblasts obtained from NSIP patients (**Figure 3(H)**), and MMP-9 was expressed in alveolar epithelial cells, alveolar macrophage and neutrophils of NSIP patients (**Figure 3(K)**). The strong staining of the neutrophils for MMP-9 observed in samples collected from NSIP patients was similar to the results obtained when samples collected from IPF patients were evaluated, however MMP-9 was not expressed in the fibroblasts of NSIP patients, as was seen in IPF patients. The staining patterns of TIMP-1 and TIMP-2 in patients with COP were consistent with those of patients with NSIP (**Figure 3(C)**, **Figure 3(F)**). Additionally, MMP-2 expression was weakly detected in alveolar epithelial cells, alveolar macrophages and fibroblasts (**Figure 3(I)**) obtained from COP patients, whereas MMP-9 was strongly expressed by cellular components and neutrophils in these patients, and was moderately expressed by fibroblasts in these patients (**Figure 3(L)**).

5. Discussion

In this study, we demonstrated that the levels of MMP-2 and -9, as well as those of TIMP-1 and -2 were significantly increased in patients with IPF when compared with patients with NSIP/COP. Additionally, patients with IPF showed poor physiologic data when compared to the patients with NSIP/COP, and two of the patients with IPF expired during the follow up period.

In general, MMPs are expressed at low levels in normal adult tissues, however, their expression becomes elevated when wound healing, repair or remodeling processes are occurring in diseased tissues [7] [9]. Several studies have shown that the over-expression of MMPs appears to play an important role in the development of a number of pathological processes, including pulmonary fibrosis [7]-[9]. Although previous studies have reported that the expression of MMPs and TIMPs in patients with IIP is significantly higher than that of normal controls, within IIP, the relationship of the expression of MMPs and TIMPs in patients with IPF and those with NSIP/COP is not yet fully understood [6] [10]-[15]. Several publications have shown that the levels of MMP-2 are higher in patients with NSIP and COP than in patients with IPF [10] [12] [14], however, in our study, the level of MMP-2 was significantly higher in patients with IPF than in those with NSIP/COP and control subjects. Additionally, the activities of MMP-9 have been shown to be different in several studies. Some studies have reported that the levels of MMP-9 were greater in patients with COP compared to those with IPF, however, Suga *et al.* reported that patients with IPF showed higher MMP-9 activities than those with NSIP or COP [10] [12] [14]. In this study, the level of MMP-9 was also shown to be significantly higher in patients with IPF than those with NSIP/COP. Previous studies have also shown that gelatinases such as MMP-2 and -9 may play an important role in the disruption of basement membranes in IPF patients, which results in the fibroblasts migrating into the alveolar space through the disrupted basement membrane [6] [16] [17]. The findings of our study support those of the previous studies. Conversely, patients with NSIP/COP may have preserved pulmonary architecture that has not been subjected to basement membrane disruption because their gelatinase activities are not high. When immunohistochemistry was considered, the main locus of the MMP-2 was fibroblasts composed of interstitial tissues. In previous reports, the concentration of MMP-2 in the fibrotic tissues was found to be correlated with the degree of progression of pulmonary fibrosis [6] [12], which is consistent with the results of our study. Conversely, MMP-9 was observed in the neutrophils and lymphocytes of patients with IIP. The production of MMP-9 by inflammatory cells may induce activities in the BALF of patients with COP that exceed those reported for IPF patients in previous reports [8] [14].

In this study, the expression of TIMP-1 and -2 were significantly higher in IPF patients than in NSIP/COP and control subjects. There have been several reports regarding the high expression of TIMPs in patients with IPF [10] [14] [15] [18] [19]. Selman *et al*. reported that there was a higher expression of TIMPs than collagenases in IPF patients, which suggested that a nondegrading fibrillar collagen microenvironment was prevailing in IPF patients [15]. Additionally, Fukuda et al reported that increased TIMP-2 expression in the myofibroblasts of IPF patients may contribute to stable ECM deposition and irreversible pulmonary structural remodeling [10]. However, Choi *et al*. proposed that the activity of TIMP-1 was higher in patients with COP than those with IPF [14]. Our study showed that the expression of both TIMP-1 and -2 was higher in patients with IPF than in patients with NSIP/COP and control subjects. Therefore, based on our results, we can assume that the expression of TIMPs in IPF patients has different characteristics than those of control subjects and patients with NSIP/COP, and that this difference is associated with the progression of pulmonary fibrosis. TIMP-2 was primarily located in the fibroblsts and myofibroblasts of the fibroblast foci, and TIMP-1 was found to have diffuse distribution

upon immunohistochemical analysis. We also observed that MMP-2 and TMP-2 were primarily located in the fibroblast foci, which is the most characteristic manifestation of ongoing lung injury in IPF patients. King *et al.* reported that the degree of fibroblast foci is associated with survival of patients with IPF [20].

In our study, we observed that the levels of pMMP-9 and TIMP-1 in patients with NSIP/COP were decreased compared to those of control subjects, and that MMP-2 and TIMP-2 expression in patients with NSIP/COP was similar to that of the control subjects. It is known that the expression of MMPs and TIMPs is greater in lungs afflicted with diseases such as pulmonary fibrosis, chronic airway diseases, and lung cancer than in healthy control lungs. However, it should be noted that our study was limited because the normal lung tissue used for the controls was obtained from patients who had surgery for lung cancer. In lung cancer patients, MMP-2 and -9 expression may be associated with tumor angiogenesis and the invasion of malignant cells through the basement membrane [8] [21]. Therefore, the activities of MMP-9 and TIMP-1 in the control subjects may have been high because the control subjects in our study were patients with lung cancer.

6. Conclusion

Patients with IPF showed higher expressions of MMPs and TIMPs than NSIP/COP patients and control subjects, and this expression was particularly high in IPF patients with fibroblastic foci in which fibrosis was actively progressing by fibroblasts and myofibroblasts. This difference in the expression of MMPs and TIMPs suggests that the degradation of the basement membrane by gelatinases and the accumulation of collagen into the ECM as a result of TIMP-1 and -2 activity play an important role in the pathogenesis of irreversible pulmonary fibrosis. Further studies should be conducted to identify the pathogenesis that induces the different expressions of MMPs and TIMPs in patients with IPF and NSIP/COP.

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Abbreviations

BALF = Bronchoalveolar Lavage Fluid
COP = Cryptogenic Organizing Pneumonia
DLCO = Diffusion Capacity of the Lung for Carbon Monoxide
ECM = Extracellular Matrix
FVC = Forced Vital Capacity
IIP = Idiopathic Interstitial Pneumonia
IPF = Idiopathic Pulmonary Fibrosis
MMPs = Matrix Metalloproteinases
NSIP = Nonspecific Interstitial Pneumonia
TIMPs = Tissue Inhibitor of Metalloproteinases

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