Lung Matrix Metalloproteinase-9 Correlates with Cigarette Smoking and Obstruction of Airflow

Cigarette smoking is the most important risk factor for obstruction of airflow in chronic obstructive pulmonary disease (COPD). Matrix metalloproteinases (MMPs) or an imbalance between MMPs and their inhibitors, the tissue inhibitors of MMP (TIMPs), is considered to play a role in the pathogenesis of COPD. We investigated whether the MMPs expression or the imbalance between MMPs and TIMP-1 is associated with the amount of cigarette smoking and the FEV_1 value, in the lung parenchyma of 26 subjects (6 non-smokers and 20 cigarette smokers). First, we performed zymographic analysis to identify the profile of the MMPs, which revealed gelatinolytic bands mainly equivalent to MMP-9 in the smokers. We then measured, using enzyme immunoassay, the concentrations of MMP-9 and its inhibitor, TIMP-1. Correlation analysis revealed that both the MMP-9 concentrations and the molar ratios of MMP-9 to TIMP-1 (MMP-9/TIMP-1) were correlated with the amount of cigarette smoking. Furthermore, MMP-9 concentrations were inversely correlated with FEV_1. In conclusion, this study shows that MMP-9 expression in human lung parenchyma is associated with cigarette smoking and also with the obstruction of airflow, suggesting that MMP-9 may play a role in the pathogenesis of the cigarette smoke-induced obstruction of airflow known as the characteristic of COPD.

Key Words : *Pulmonary Disease, Chronic Obstructive; Smoking; Matrix Metalloproteinases; Tissue Inhibitor of Metalloproteinases; Lung Diseases; Obstructive*

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is generally defined as a disease state characterized by the obstruction of airflow, which is not fully reversible (1). The major risk factor for the development of fixed obstruction of airflow in patients with COPD is cigarette smoking. The adverse effect of smoking on the rate of decline in FEV_1 have been well documented in many cross-sectional and longitudinal studies in normal populations. For example, Fletcher and colleagues found that the average rate of decline of FEV_1 was 0.03 L/yr among non-smokers, and that decline was twice as fast among smokers (2). Broadly comparative figures for rate of decline in FEV_1 have been reported from several other longitudinal studies (3-5).

Currently, the major hypothesis for the pathogenesis of emphysema, which is a major component of the morbidity and mortality of COPD, is the protease-antiprotease hypothesis (6, 7). This hypothesis states that an imbalance between the levels of degradative enzymes and their respective inhibitors damages the connective tissue matrix components of the lung. Among various proteases that have long been proposed to damage connective tissue components in lung parenchyma, there is now increasing evidence that matrix metalloproteinases (MMPs) play a role in the pathogenesis of COPD. In patients with emphysema, MMP-1 (collagenase) and MMP-9 (gelatinase B) was increased in bronchoalveolar lavage fluid (8). In addition, it increased the activities of MMP-2 and MMP-9 in the lung parenchyma of patients with emphysema (9). The interest in MMPs has been further heightened by the demonstration in mice that emphysema induced by chronic exposure to cigarettes is prevented by deletion of the gene encoding MMP-12 (macrophage metalloelastase) (10). The question remains how cigarette smoking brings about the obstruction of airflow in COPD. Increased expression of MMPs or an imbalance between MMPs and their specific inhibitors, the tissue inhibitors of metalloproteinase (TIMPs), may play a role in the generation

of cigarette smoke-induced obstruction of airflow. However, there is still no direct evidence of this results, although some recent reports have revealed that there is an increased expression of MMP-9 by alveolar macrophages in cigarette smokers compared to non-smokers (11, 12).

Therefore, we hypothesized that the smoke from cigarette would induce MMPs expression or an imbalance between MMPs and TIMPs in lung parenchyma, and that it would be associated with the degree of obstruction in airflow. To test the hypothesis, we evaluated whether the amount of cigarette smoking and FEV$_1$ (% predicted) value, an indicator of airflow obstruction, are correlated with the MMPs expression or the imbalance between the MMPs and TIMP-1 in human lung parenchyma.

MATERIALS AND METHODS

Subjects and Specimens

In a total of 26 subjects (6 non-smokers and 20 cigarette smokers), specimens were obtained from lungs resected for the treatment of malignancy or for the evaluation of solitary pulmonary nodule. Immediately after resection, lung specimens were collected at locations as far as possible from the pathologic lesion. Subjects were excluded if the specimens examined had microscopic evidence of ongoing infection or malignant cellular infiltration. The study was approved by Human Ethics Committee of Hallym University Sacred Heart Hospital, and all subjects gave written informed consent.

Determination of the Amount of Cigarette Smoking

The definition of cigarette smoking was determined as follows. Current smokers were defined as those who had smoked at least 100 cigarettes in their lifetime and either still have smoked or had quit smoking within the preceding year. Among current cigarette smokers, the amount of cigarette smoking was calculated as pack-year basis (PYs), using a detailed questionnaire derived from a similar study (13). If subjects had smoked at least 100 cigarettes in their lifetime but had quit smoking more than one year earlier, then they were classified as former smokers in terms of smoking status and excluded from this study. Subjects who had smoked less than 100 cigarettes including those who had never smoked, were considered "never to have smoked" and their exposure to environmental tobacco smoke was estimated by determining the following the number of people living in the household who smoked at home, the number of cigarettes smoked at the home each day, and the number of hours the subjects spent daily outside the home in a place where people were smoking. If these subjects suffered less than 2 hr of passive smoking in a day, they were classified as non-smokers.

Protein Extraction from Lung Specimens

For analysis of gelatinolytic activities and enzyme immunoassay for MMP-9 and TIMP-1, pieces of lung samples weighing between 0.5 and 1.5 g were kept in air-tight plastic bottles at -80°C until tissue extraction. After removal of small vessels and bronchi under the microscope, the specimens, which weighed 0.5 g each, were minced into small pieces and homogenized with ULTRA-TURRAX T25 (Janke & Kunkel GmbH & Company, KG, IKA Laboratory Technology, Staufen, Germany) in an ice bath after the addition of neutral salt extraction buffer (150 mM NaCl, 50 mM NaF, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 1 mM Na$_3$Vo$_4$, 1 mM PMSF, 10 mM CaCl$_2$, 2M KCl, and 50 mM Tris-HCl, pH 7.5; 0.5 g tissue/2 mL buffer) (14). After incubation of 1 hr at 4°C, homogenates were centrifuged at 7.5×10^4 g for 1 hr at 4°C. The supernatants were dialyzed against matrix metalloproteinase buffer (5 mM CaCl$_2$, 10 mM ZnCl$_2$, 200 mM NaCl, and 50 mM Tris-HCl, pH 7.5) for 24 hr at 4°C. Protein concentration of the samples was analyzed by a spectrophotometer (DU 640; Beckman, Fullerton, CA, U.S.A.) according to the Bradford protein analysis method, and adjusted equally to levels of 1.0 mg/mL (total protein concentration) for each sample. The samples were stored at -80°C until analysis.

Gelatin Zymography

MMPs present in lung tissue protein extracts were detected by their capacity to degrade gelatin according to a method previously described (15). Briefly, protein extracts obtained from lung tissue specimens were subjected to electrophoresis on 12% polyacrylamide gels containing 1 mg/mL of gelatin, in the presence of sodium dodecyl sulfate (SDS-PAGE) under non-reducing conditions. After electrophoresis, gels were washed twice in 1% Triton X-100 (vol/vol) for 1 hr, rinsed briefly, and incubated at 37°C for 24 hr in buffer containing 10 mM CaCl$_2$, 5 μM ZnCl$_2$, pH 7.8 in Tris-HCl. After incubation, the gels were stained with Coomassie Brilliant Blue R250 and then destained in a solution of 10% acetic acid with 30% methanol. Zones of enzymatic activity were indicated by negative staining, with areas of proteolysis appearing as clear bands against a blue background. Molecular weight of the gelatinolytic bands was estimated relative to size markers.

Measurement of MMP-9 and TIMP-1 by ELISA

The MMP-9 and TIMP-1 concentrations in protein extracts were measured by enzyme-linked-immunosorbent assay (ELISA) (MMP-9 & TIMP-1: R&D Systems Inc., MN, U.S.A.). MMP-9 ELISA measured total MMP-9 released (pro and active MMP protein together with that complexed to TIMP-1+2). TIMP-1 ELISA measured both bound and free TIMP-1.

Statistical Analysis

Results are expressed as medians and ranges. Statistical analysis was performed using χ^2 test and Mann-Whitney U test to assess differences between the two groups. Spearman's rank correlation was calculated to assess correlation.

RESULTS

Characteristics of Subjects

Pathologic diagnoses of 26 subjects were lung cancer (22 subjects), tuberculous granuloma (3 subjects), and chondroid hamartoma (1 subject). The study subjects were grouped as non-smokers and cigarette smokers as follows;

Non-smokers: The six subjects (4 lung cancer, 2 tuberculous granuloma) with no history of cigarette smoking ranged from 31 to 67 yr of age (median: 62.5 yr). All six subjects were females. The FEV$_1$/FVC ratios (%) in the subjects ranged from 73 to 87%, with a median value of 82.5%. The FEV$_1$ (% predicted) values ranged from 89 to 118%, with a median value of 105.5%.

Cigarette smokers: The 20 subjects (18 lung cancer, 1 tuberculous granuloma, 1 chondroid hamartoma) with current smoking history ranged from 27 to 78 yr of age (median: 58 yr), with 19 males and one female. Cigarette smoking history ranged from 3 to 104 pack-years (median: 40 pack-years). The FEV$_1$/FVC ratios (%) in the subjects ranged from 54 to 84%, with a median value of 73.5%. The FEV$_1$ (% predicted) values ranged from 45 to 118%, with a median value of 85.5%.

The comparison of the two groups is shown in Table 1. Age distribution was similar between two groups, but gender distribution was different ($p<0.001$). FVC (% predicted) values were similar between the two groups. However, FEV$_1$ (% predicted) value and FEV$_1$/FVC were significantly lower among the cigarette smokers than the non-smokers ($p<0.005$ and $p<0.03$, respectively), suggesting the presence of obstruction of airflow in cigarette smokers.

Table 1. Characteristics of the subjects*

Characteristics	Non-smokers (n=6)	Cigarette Smokers (n=20)	p value[†]
Age (yr)	62.5 (31-67)	58 (27-78)	NS
Sex, male : female	0:6	19:1	<0.001
Cigarette Smoking (PYs)	0	40 (3-104)	<0.001
Spirometry			
FEV$_1$ (% predicted)	105.5 (89-118)	85.5 (45-118)	<0.005
FVC (% predicted)	93.5 (80-106)	88.0 (54-110)	NS
FEV$_1$/FVC (%)	82.5 (73-87)	73.5 (54-84)	<0.03

*Results are expressed as median, with ranges in parentheses. [†]p value by χ^2 test for sex variable. For other variables, by Mann-Whitney U test. NS, not significant; PYs, pack-years; FEV$_1$, forced expiratory volume in one second; FVC, forced vital capacity.

Characterization of MMPs in Protein Extracts by Zymography

To identify the profile of MMPs in the lung tissue proteins of 26 subjects, we first performed zymographic analysis of the protein extracts. It revealed the presence of a major gelatinase species having molecular weights of 92-kDa and 82-kDa, which are equivalent to pro- and active- forms of MMP-9 molecules in the cigarette smokers (Fig. 1B). For the non-smokers, gelatinolytic bands were rarely observed (Fig. 1A).

Comparison of MMP-9 and TIMP-1 between Non-smokers and Cigarette Smokers

According to the zymographic analysis, we selected MMP-9 as one of the most probable candidate enzyme, and then measured the concentrations of MMP-9 and one of its inhibitor, TIMP-1. The concentrations of MMP-9 were higher in protein extracts of the cigarette smokers (median: 51.45,
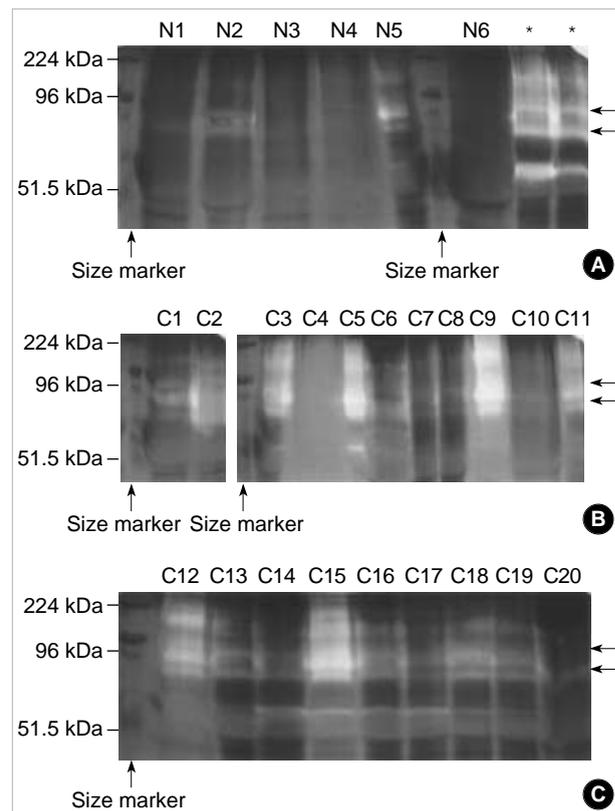

Fig. 1. The profile of matrix metalloproteinases (MMPs) in lung tissues by gelatin zymography. The figure shows multiple clear bands in the cigarette smokers (C1-C20) (**B**), as compared with the non-smokers (N1-N6), in which gelatinolytic bands are rarely seen (**A**). Arrows indicate the positions of pro- and active- forms of MMP-9 (92 and 82 kDa, respectively). Zones of enzymatic activity are indicated by negative staining, with areas of proteolysis appearing as clear bands against a blue background. *Positive control.
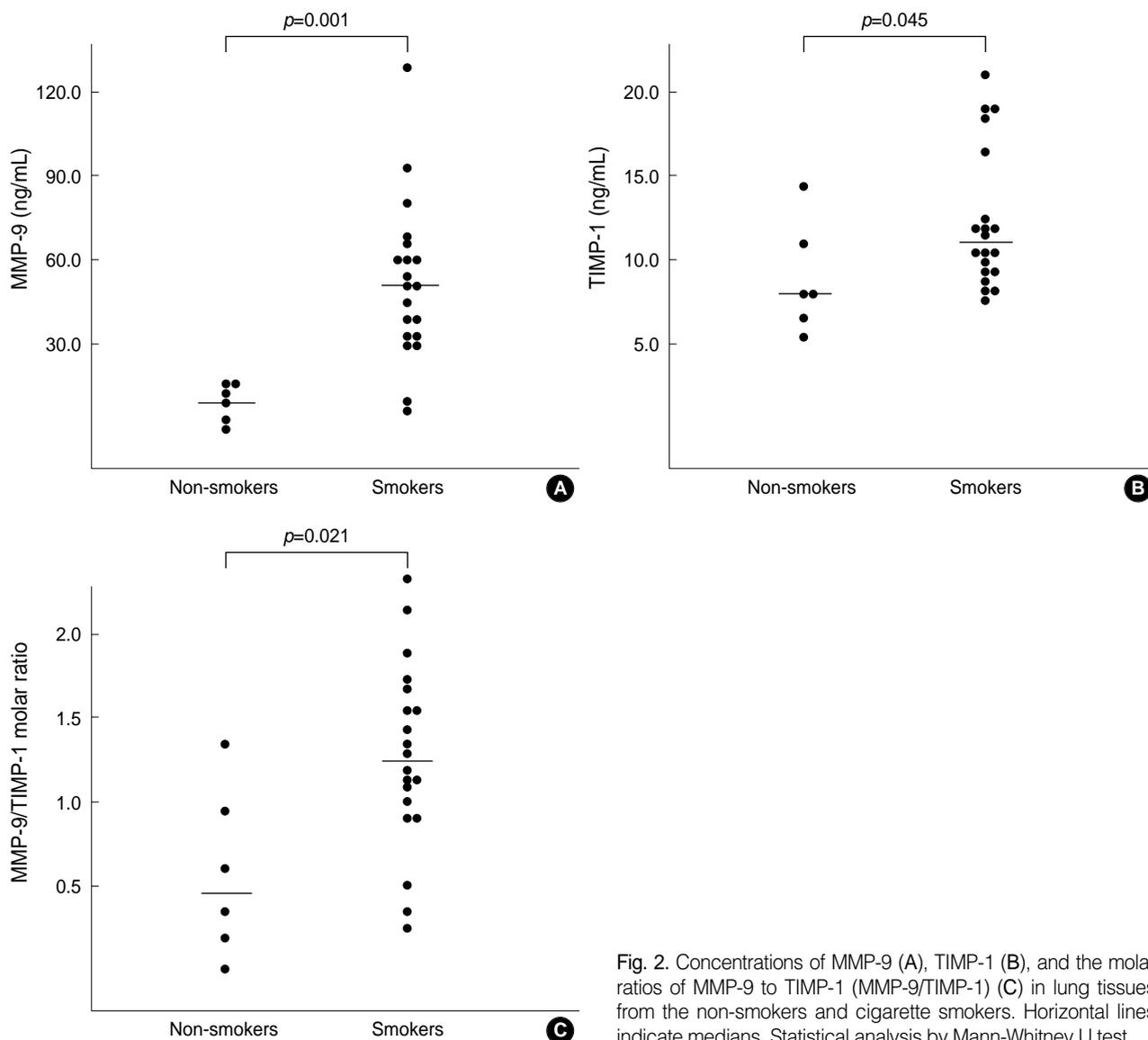
Fig. 2. Concentrations of MMP-9 (A), TIMP-1 (B), and the molar ratios of MMP-9 to TIMP-1 (MMP-9/TIMP-1) (C) in lung tissues from the non-smokers and cigarette smokers. Horizontal lines indicate medians. Statistical analysis by Mann-Whitney U test.

range: 7.20 to 129.90 ng/mL) than the non-smokers (median: 14.70, range: 1.40 to 16.10 ng/mL) ($p<0.001$, Mann-Whitney U test) (Fig. 2A).

The concentrations of TIMP-1 were higher in the cigarette smokers (median: 11.15, range: 7.90 to 20.80 ng/mL) than the non-smokers (media: 8.10, range: 5.40 to 14.40 ng/mL) ($p<0.045$, Mann-Whitney U test) (Fig. 2B).

The molar ratio of MMP-9 to TIMP-1 (MMP-9/TIMP-1) was higher in the cigarette smokers (median: 1.26, range: 0.24 to 2.36) than the non-smokers (median: 0.49, range: 0.01 to 1.35) ($p<0.021$, Mann-Whitney U test) (Fig. 2C).

Correlation of MMP-9 and MMP-9/TIMP-1 with Cigarette Smoking

Among the 26 subjects, there was a positive correlation of

MMP-9 concentrations with the amount of cigarette smoking ($r_s=0.577$, $p<0.002$) (Fig. 3A). In addition, there was also a positive correlation of the molar ratio of MMP-9 to TIMP-1 with the amount of cigarette smoking ($r_s=0.430$, $p<0.028$) (Fig. 3B).

Correlation of MMP-9 and MMP-9/TIMP-1 with FEV₁

Among the 26 subjects, there was an inverse correlation of MMP-9 concentrations with the FEV₁ (% predicted) values ($r_s=-0.553$, $p<0.003$) (Fig. 4A). However, correlation analysis of the molar ratio of MMP-9 to TIMP-1 with the FEV₁ (% predicted) values failed to show statistical significance, although it tended to increase according to the severity of obstruction of airflow ($r_s=-0.356$, $p<0.074$) (Fig. 4B).
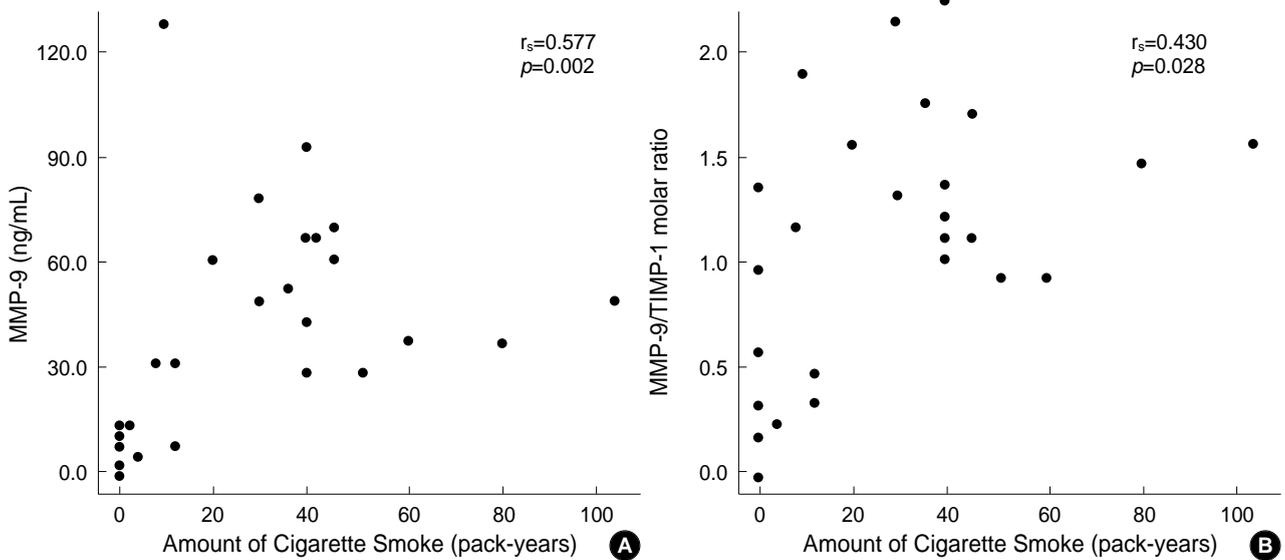
Fig. 3. Correlation of MMP-9 (A), and the molar ratio of MMP-9 to TIMP-1 (MMP-9/TIMP-1) (B) with the amount of cigarette smoking in lung tissues from the non-smokers and cigarette smokers. PYs, pack-years. Statistical analysis by Spearman's rank correlation analysis.
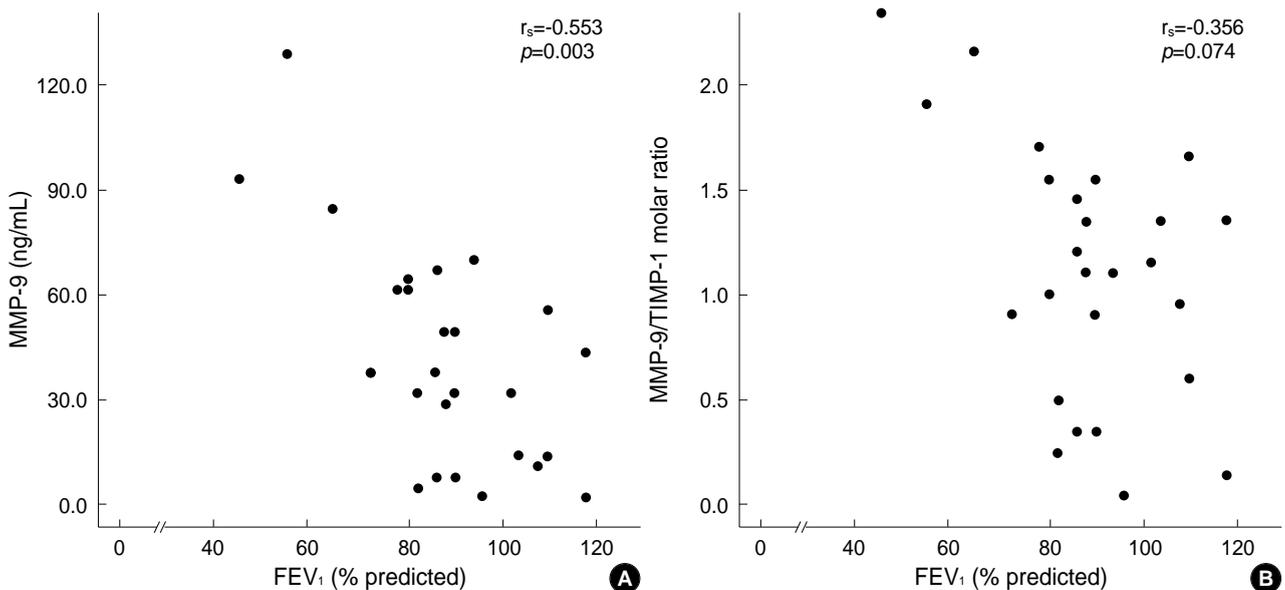

Fig. 4. Correlation of MMP-9 (A), and the molar ratio of MMP-9 to TIMP-1 (MMP-9/TIMP-1) (B) with the FEV$_1$ (% predicted) value in lung tissues from the non-smokers and cigarette smokers. Statistical analysis by Spearman's rank correlation analysis.

DISCUSSION

This study showed that in human lung parenchyma, MMP-9 expression and the molar ratio of MMP-9 to TIMP-1 were increased in cigarette smokers when compared with non-smokers, and that both the MMP-9 expression and the molar ratio of MMP-9 to TIMP-1 were correlated with the amount of cigarette smoking. Furthermore, this study also revealed inverse correlation of the concentration of MMP-9 in lung parenchyma with the FEV$_1$ (% predicted) value, an index of

the obstruction of airflow of cigarette smokers.

Obviously, cigarette smoking is the most important cause of obstruction of airflow, which is the characteristic of COPD. In humans, however, there has been no direct evidence confirming that MMPs expression is increased according to the amount of cigarette smoking or that their expression is associated with the degree of obstruction of airflow observed among cigarette smokers. To our knowledge, this is the first study showing that MMP-9 expression in human lung parenchyma is associated with the amount of cigarette smok-

ing, and also with the obstruction of airflow in cigarette smokers.

Previous studies have shown that MMP-9 is consistently increased in emphysema patients or cigarette smokers. Finlay and coworkers demonstrated that the secretion of MMP-1 and MMP-9 and the expression of MMP-1 and MMP-9 mRNA were increased in the alveolar macrophages from emphysema patients (8). Ohnishi and coworkers demonstrated that the elastolytic activities of MMP-2 and MMP-9 were increased from emphysematous lung tissues when compared with nonemphysematous lung tissues (9). Betsuyaku and coworkers demonstrated that in bronchoalveolar lavage fluid from 65 community-based elderly volunteers, the levels of MMP-9 and MMP-8 were significantly higher in current smokers with emphysema than in those without emphysema (16). However, none of the above human studies analyzed data in terms of the amount of cigarette smoking; instead they merely considered the presence or the absence of emphysematous change. Recently, Lim and coworkers demonstrated that alveolar macrophages obtained from bronchoalveolar lavage fluid in current healthy smokers released greater amount of MMP-9 and TIMP-1 compared with non-smokers (11). In addition, Russell and coworkers studied the production and the activities of macrophage-derived MMP-9 and TIMP-1 from alveolar macrophages from smokers with COPD, healthy smokers and non-smokers (12). Their study revealed that alveolar macrophages from smokers with COPD released the greatest amounts of MMP-9, followed by healthy smokers, and with MMP-9 expression from non-smokers being the smallest. There is also a report that MMP-9 was increased in serums from cigarette smokers when compared with those from non-smokers (17). Therefore, we consider that our results are well in agreement to the previously published experimental data.

There exists a study that failed to verify increased expression of MMP-9 of lung parenchyma from patients with emphysema (18). They examined the lung parenchyma of 23 patients with emphysema and of 8 normal control samples for the expression of MMP-1, MMP-9, and MMP-12 and reported that MMP-1 mRNA, protein, and activities were present in the lung parenchyma of patients with emphysema but not in the lungs of the controls. However, they did not investigate further for the expression of MMP-9 protein and its activities because they observed that MMP-9 mRNA was expressed throughout the study subjects, irrespective of the presence or absence of emphysema. In addition, they did not analyze the data from the aspect of cigarette smoking status or its amount.

Our study showed that MMP-9 expression and the molar ratio of MMP-9 to TIMP-1 were increased among cigarette smokers when compared with non-smokers, and that both the MMP-9 expression and the molar ratio of MMP-9 to TIMP-1 were correlated with the amount of cigarette smoking. There exists a study showing similar results, in which

alveolar macrophages from cigarette smokers released greater amounts of both MMP-9 and TIMP-1 than do alveolar macrophages from non-smokers, with a trend toward a higher molar ratio of MMP-9 to TIMP-1 (11). However, in another study that evaluated an imbalance between MMP-9 and TIMP-1 in induced sputum of chronic bronchitic subjects compared with controls, MMP-9 and TIMP-1 concentrations were greater in sputum of patients with chronic bronchitis than in control subjects but the molar ratio of MMP-9 to TIMP-1 was lower in chronic bronchitics than in control subjects (19). The difference might be explained from that induced sputum is mainly obtained from large airways and are unlikely to reflect pathophysiologic changes of lung parenchyma. The major site of the chronic airflow limitation in COPD are the small airways and lung parenchyma. Therefore, we suggest that our study presents a more close explanation for cigarette smoke-induced obstruction in airflow, which is characteristic of COPD.

Several studies have shown that cigarette smoking causes an inflammatory process in central airways (20, 21), peripheral airways (22-24), and lung parenchyma (25). Cigarette smoking leads to the accumulation of various inflammatory cells including macrophages and neutrophils that are well known to produce MMP-9. MMP-9 expression of these inflammatory cells may increase in response to various inflammatory cytokines and oxidative stress caused by cigarette smoking. It has previously been shown that an increase in oxidant stress may increase MMP-9 activation (26, 27). MMP-9 release from alveolar macrophages can be stimulated by lipopolysaccharide, interleukin-1β, and tumor necrosis factor-α (11, 12, 28). These findings suggest that proinflammatory stimuli induced by cigarette smoking might regulate MMP-9 activity in the COPD airways.

In conclusion, this study shows that MMP-9 expression in human lung parenchyma is associated with cigarette smoking and also with the obstruction of airflow. Further study should follow to investigate the role of MMP-9 in the pathogenesis of cigarette smoke induced airflow obstruction, which is the characteristic of COPD.

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