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# Early and Late Changes of MMP-2 and MMP-9 in Bleomycin-Induced Pulmonary Fibrosis

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**Purpose:** Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of pulmonary fibrosis. To understand the role of MMP-2 and MMP-9 in pulmonary fibrosis, we evaluated the sequential dynamic change and different cellular sources of the 2 MMPs along the time course and their differential expression in the bronchoalveolar lavage (BAL) fluid and in the lung parenchyma of the bleomycin-induced pulmonary fibrosis models in rats. **Materials and Methods:** The level of MMPs in BAL fluid of 54 bleomycin-treated rats was assessed by zymography from 1 to 28 days after intratracheal bleomycin instillation. The level of MMPs in lung parenchyma was evaluated by immunohistochemistry. **Results:** MMP-2 and MMP-9 were markedly increased in both the BAL fluid and in the lung parenchyma of the bleomycin-treated rats, especially in the early phase with the peak on the 4th day. The levels of both MMPs in the BAL fluid correlated generally well to those in lung parenchyma, although the level of MMP-9 in BAL fluid was higher than MMP-2. In the lung parenchyma, the 2 MMPs, in early stage, were predominantly expressed in the inflammatory cells. In late stage, type II pneumocytes and alveolar epithelial cells at the periphery of the fibrotic foci retained MMP expression, which was more prominent in the cells showing features of cellular injury and/or repair. **Conclusion:** In bleomycin-induced pulmonary fibrosis, MMP-2 and MMP-9 may play important roles, especially in the early phase. In the late stage, the MMP-2 and MMP-9 may play a role in the process of repair.

**Key Words:** Pulmonary fibrosis, bleomycin, matrix metalloproteinase, matrix-metalloproteinase-2, matrix metalloproteinase-9

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## INTRODUCTION

Idiopathic pulmonary fibrosis is a cryptic, chronic disease, resulting in a progressive and severe pulmonary insufficiency, however, the pathogenesis of idiopathic pulmonary fibrosis is not completely elucidated to date. Its main features include epithelial injury, fibroblastic proliferation, inflammation, and increased matrix production.[1] The ultimate feature is the progressive fibrosis, resulting from dysregulated extracellular matrix control, where matrix metalloproteinase(MMP)s are believed to play important roles. Matrix metalloproteinase (MMP) is a group of Zn-dependent proteinases activated by proteolytic removal of their amino terminal, and their actions are inhibited by specific inhibitors, tissue inhibitors of matrix metalloproteinases (TIMP).[2] MMP-2 and MMP-9 are designated as 72-kDa gelatinase A and 92-kDa gelatinase B, respectively, and well known by their ability of degrading collagen type IV, the major constituent of the basement membrane. Studies showed that the activity of MMP-2 and MMP-9 is upregulated in many pulmonary diseases including pulmonary fibrosis,[3-7] and they are secreted by many different kinds of cells in the lungs.[8-11] But, the studies dealing with human lung can provide only a snap shot at a certain time point in its long course of disease. Thus, many studies so far rely mostly upon *in vitro* experiments or animal models among which bleomycin-induced pulmonary fibrosis is considered to be the most common and acceptable model.[12] However, even in bleomycin-induced pulmonary fibrosis, most studies about MMPs have been dealing with only 1 or 2 cell types or focused only on targeting certain mediators at a

certain time point. Since the idiopathic pulmonary fibrosis is a long-term dynamic and progressive disease, the investigation of changing levels of the MMPs and their changing cellular sources along the time course may have significance in understanding the real pathophysiology of the condition. In this study, we tried to investigate the changes in the level of MMP-2 and MMP-9 and their various cellular sources in different stages of pulmonary fibrosis by estimating the changes of the activity of those MMPs at different time intervals in bleomycin-induced pulmonary fibrosis in rats.

## MATERIALS AND METHODS

### Animal model and experimental groups

Eighty one Sprague-Dawley male rats with body weight of around 200gm were divided into control group (n = 27) and bleomycin-administered experimental group (n = 54). Six rats from experimental group and 3 from the control group were sacrificed at 1, 2, 3, 4, 5, 7, 14, 21, and 28 days after bleomycin instillation.

### Bleomycin administration

After rats were positioned prone under ether anesthesia, 1.5 unit/kg bleomycin (Nippon Kayaku Co., Tokyo, Japan) dissolved in saline was instilled into the bronchi using appropriate laryngoscope.

### Methods

*Bronchoalveolar lavage*

After rats were sacrificed by arterial exsanguinations, lungs were dissected, and the left bronchus was ligated at its proximal part. Bronchoalveolar lavage was done only from the right lung. In detail, 3 mL of PBS was administered through a three way tube at 25 cm $H_2O$ pressure by gravity, remained in lung for 3 minutes, and withdrawn again by gravity. This process was repeated 5 times. Collected lavage fluid was palced in 50 mL tube packed in ice. Two thirds of lavage fluid was prepared for cell count, and the remainder was kept in - 80°C to measure the activity of MMP-2 and MMP-9 by zymography.

*Cell count in BAL fluid*

After centrifugation at 4°C and 1,500 rpm for 10 minutes, the supernatant was discarded. After removing the supernatant, the sediment was rinsed twice with RPMI 1,640. Cells were counted using hemocytometer. Total cell count was expressed as the number of cells per mL of BAL fluid. After adjusting the total cell number to $1.0 \times 10^6$/mL, 200 μL of the sample was taken, centrifuged, smeared on a glass slide, air dried, and stained with Wright-Giemsa. Differential count was done by counting 500 cells on a high power view (× 400) light microscopic field using ocular lens with a grid.

### Microscopic examination

For microscopic examination, the left lungs from the rats were fixed in 10 % formalin for 8 hours, processed as usual, and embedded in paraffin. Three μm serial section was stained with hematoxylin-eosin. Masson's trichrome stain was done to evaluate the degree of collagenous fibrosis.

### Immunohistochemical examination

Three μm sections were prepared with formalin-fixed, paraffin-embedded tissue, deparaffinized, and rehydrated by serial alcohol treatment. Endogenous peroxidase quenching was done by 3 % $H_2O_2$ treatment for 15 minutes. After rinsing with distilled water (D.W.) for 3 minutes, the sections were microwaved for 10 minutes in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Then, the sections were incubated with mouse monoclonal MMP-2 (CA-4001), or mouse monoclonal MMP-9 (IIA5) (Neomarkers, Fremont, CA, USA) (dilution 1 : 50) for 1 hour at room temperature (RT), and then, overnight at 4°C. The sections were reacted with Biotin labeled secondary antibody and streptavidin HRP for 15 minutes each using LSAB II Rat kit (Dako Co., Carpinteria, CA, USA). After rinsing with PBS buffer, the sections were reacted with aminoethyl carbazole (AEC) and counterstained with Mayer's hematoxylin. The expression of the antibodies was semiquantita-

tively evaluated through Allred score,[13] modified as follows; Grade 0, 1, 2, and 3 were determined according to the numbers of the positive cells (0 = < 50 positive cells; 1 = 50 - 100 positive cells; 2 = 101 - 200 positive cells; 3 => 200 positive cells), and the intensity was evaluated as 1, 2, and 3 (1 = weakly positive; 2 = moderately positive; 3 = strongly positive). Immunohistochemical score was obtained by multiplying these two indices. All these examinations were done on 5 different high power fields (400 ×).

### Gelatin zymography

The zymography of MMP-2 and MMP-9 from the BAL fluid was performed according to Laemmli method.[14] In detail, 4X non-reducing sample buffer [0.5 M Tris-HCL, 10% (w/v) sodium dodecyl sulfate (SDS) 4 mL, 0.1% bromophenol blue 0.5 mL, glycerol 2.0 mL, and add distilled water to a total volume of 10 mL] was added to the same volume of sample, incubated for 10 minutes at RT, and then injected to the wells in a precast 10% (w/v) acrylamide gel (Novex, San Diego, CA, USA) containing 0.1% gelatin. Electrophoresis was performed at 4°C and 20 mA (125 V) for 3 hours. After electrophoresis, the gels were washed twice with 2.5% (v/v) triton X-100 renaturing buffer, rinsed with water, incubated overnight at 37°C in the developing buffer (50 mM Tris-based, 200mM NaCl, 5 mM $CaCl_2$, pH 7.6, 0.02% Brij). The gels were stained with 0.5% (w/v) Coomassie brilliant blue and destained in a solution of 25% ethanol 10% acetic acid, and then examined for the bright appearing bands where the gelatin was degraded and disappeared. To assess the gelatinolytic activity of the MMPs, the width and optical density of the bands were analyzed by the computer-assisted image analyzer (Bioprofile, Vilber Lourmat Biotechnology, Marne La Vallee, Cedex, France). The relative values of the bands were calculated using the HT-1080 cell lines as standards.

### Statistical analysis

Statistical analysis was done with Wilcoxon Rank Sum Test using SAS program.

## RESULTS

### Analysis of bronchoalveolar lavage fluid

*Total cell count of BAL fluid*

Total cell count of BAL fluid was significantly higher in the bleomycin-treated experimental group compared to those of the control group. Total cell count of the experimental group increased by 1.5 to 3 times from the first day. It reached its peak on the 4th day and decreased slowly thereafter. Total cell count on the 28th day was nearly the same in the two groups (Fig. 1).

*Differential count of BAL fluid*

In contrast to the control group, in which more than 95% of the total cells in BAL fluid were mononuclear cells, the neutrophil count in the experimental group increased from the first day, reaching its peak (52.1%) on the 4th day, and decreased dramatically until the 14th day, reaching to a level of 3.7% on the 28th day (Fig. 2). Eosinophils were temporarily increased from the 4th day to 7th day, and decreased thereafter. Lymphocyte count increased until the 3rd day and remained at a constant level.

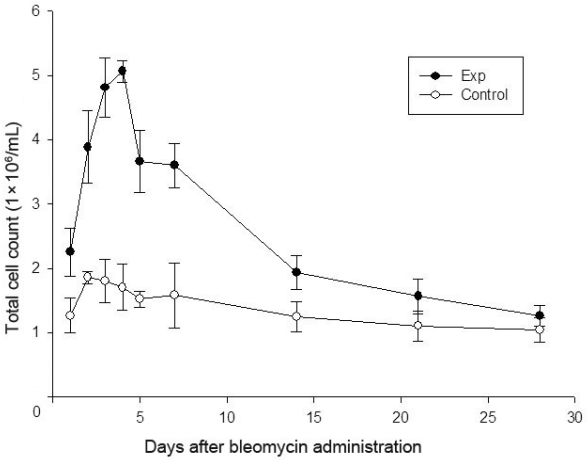


**Fig. 1.** Total cell count in the BAL fluid of bleomycin-treated group was significantly higher than in the control group ($p < 0.05$). Total cell count increased rapidly from the first day after bleomycin instillation, reaching the peak level on the 4th day. Then, it continuously decreased till the 28th day. Exp: bleomycin-treated experimental group: n = 54, Control: control group: n = 27, Average cell count from each group was used.

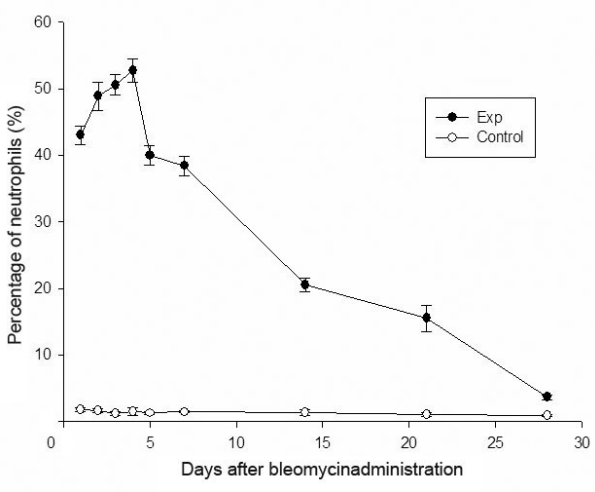


**Fig. 2.** The percentage of neutrophil in the BAL fluid in the bleomycin-treated group was significantly higher than in the control group ($p < 0.05$). The percentage of neutrophil was markedly increased from the first day after bleomycin instillation, reaching the peak level on the 4th day, and then decreased rapidly thereafter. Exp: bleomycin-treated experimental group: n = 54, Control: control group: n = 27. Average value from each group was used.

## Light microscopic features

*Hematoxylin-eosin stain findings*

In saline-treated control group, the inflammatory reaction was only mild throughout the whole experimental period, except focal mild lymphocytic infiltration around the small vessels. Fibrosis was scarce. On the other hand, in the bleomycin-treated experimental group, prominent inflammatory reaction was noted from the first day, showing some inflammatory infiltrate around the small vessels and interstitium along with mild interstitial edema. Inflammatory infiltration began around the small vessels and terminal bronchioles, and gradually spread to adjacent parenchyma, forming scattered foci of discrete inflammatory aggregate throughout the pulmonary parenchyma. A few neutrophils, lymphocytes, and macrophages were present in the edematous alveolar spaces, and these intraalveolar inflammatory foci were continuous to the inflammatory infiltrates around the small vessels and terminal bronchioles. On the

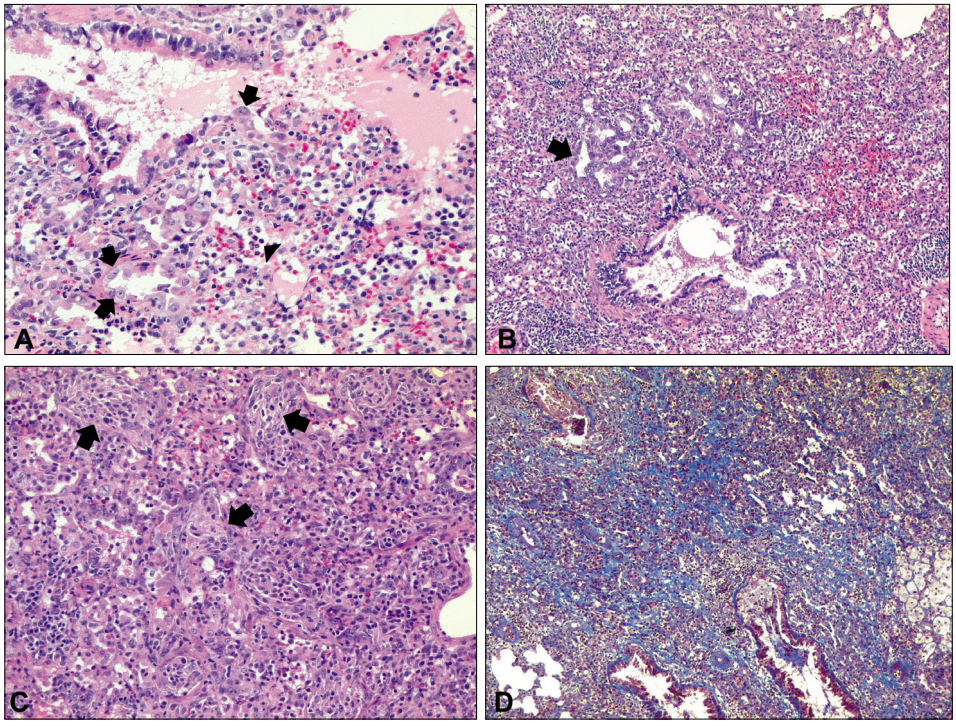


**Fig. 3.** Histological findings of bleomycin-treated group. (A) 3rd day. Swollen cuboidal epithelial cells of the terminal bronchiole and type II pneumocytes (arrows) and inflammatory cell infiltration in the peribronchiolar and perivascular area (arrowhead). (B) 5th day. A number of small tubular structures of bronchiolar epithelial cells, "bronchiolization" foci (arrow) are seen around the terminal bronchiole. (C) 14th day. Some foci of small intraalveolar fibrosis are seen (arrows). (D) 28th day. Dense mature collagen stained deep blue in Masson-trichrome stain is widening the alveolar septae and destroying the normal alveolar pattern (A, B, and C: H&E; D: Masson's trichrome, A and C: × 100; B, and D: × 40).

third and the 4th day, intraalveolar edema became prominent. More lymphocytes and macrophages were present in the alveolar spaces and, from the 7th day, they infiltrated in the alveolar septa and expanded them. This septal inflammatory infiltrate was specifically prominent on the 14th day. The bronchiolar epithelial cells were swollen showing vacuolar changes. Another characteristic feature was the so-called "bronchiolization",[15] the ingrowth of cuboidal cells from adjacent bronchioles to alveoli, forming a tubular structure, which was most apparent from the 4th to 7th days. Around the foci of bronchiolization, type II pneumocytes showed features such as swelling, cytoplasmic vacuolization, or nuclear enlargement, suggesting damage and/or activation. These changes of the type II pneumocytes were most prominent during the 3rd to 7th day and lasted thereafter, especially near the fibrotic foci. In addition, a layer of flat epithelial cells was often found to surround the fibrotic foci and appeared to be connected to the activated type II pneumocytes. In regard to fibrosis, immature-appearing collagen fibers, which were stained reddish purple on Masson trichrome stain, began to deposit focally on the 7th day at around the arterioles, bronchioles, and alveolar septae where inflammatory changes were present. On the 14th day, the area of collagen accumulation became widened, and intraalveolar fibrosis became apparent. On the 28th day, multiple fibrotic foci with mature collagen which was stained deep blue on Masson trichrome were noted at the alveolar septae, distorting the normal parenchymal architecture (Fig. 3).

**Immunohistochemical stain findings**

In saline-instilled control groups, MMP-2 and MMP-9 were expressed in moderate degrees in the bronchiolar epithelial cells and smooth muscle cells in the arteriolar wall, and weakly expressed in a few alveolar macrophages and type II pneumocytes as well as in some lymphocytes around the arterioles and bronchioles. In the experimental groups, however, the bronchiolar epithelial cells showed intense immunoreactivity throughout the entire course. The cells showing features of cellular injury, activation and/or repair particularly showed more prominent expression of both MMPs. The inflammatory cells were also strongly positive. MMP-9 was almost exclusively stained in the neutrophils, whereas MMP-2 was expressed more strongly in the macrophages. Small numbers of mast cells were also positive to the MMPs. The MMPs were also expressed in the intraalveolar edema fluid which was most prominent on the 3rd and the 4th days. Atypical type II pneumocytes started to stain strongly for MMP-2 and MMP-9 from the 3rd day, most intensely on the 4th day. From the 7th day, there were fewer inflammatory cells, and the intensity of the MMP-2 and MMP-9 staining was weakened. On the 14th day, positive reaction of the macrophages and atypical type II pneumocytes was retained at the periphery of the fibrotic foci, and the single-layered attenuated alveolar epithelial cells surrounding the fibrotic foci were also positive to both MMPs (Fig. 4). The overall expression of MMP-2 and MMP-9 along the time sequence dynamically changed according to the immunohistochemical score. The expression was dramatically increased from the 1st day, reaching the peak on the 4th day. From the 5th day, they were slightly diminished, but still remained at significantly higher levels till the 14th day, compared to the control groups ($p < 0.05$). Although there was no statistically significant difference in the expression between the MMP-2 and MMP-9, MMP-2 appeared to be expressed at a higher level on the 4th day, compared to MMP-9 and also showed a tendency to decline less rapidly (Fig. 5).

**Gelatin zymographic analysis**

Zymographic analysis of the BAL fluid showed distinct bands of gelatinolytic activity at 72-kDa and 92-kDa, which correspond to MMP-2 and MMP-9, respectively. The gelatinolytic activity increased from the first day, reaching the maximum level on the 4th day (Fig. 6). The change of MMP-9 was more profound, reaching 6 times the level on the 4th day compared to that of the 1st day, and then rapidly decreased and remained unchanged at 2 times the level from the 5th to 14th day. Finally, it started to decrease again on the 21st day (Fig. 7B). The change of MMP-2 was not as profound as MMP-9, nevertheless, still showed a similar pattern, reaching the maximum on the 4th

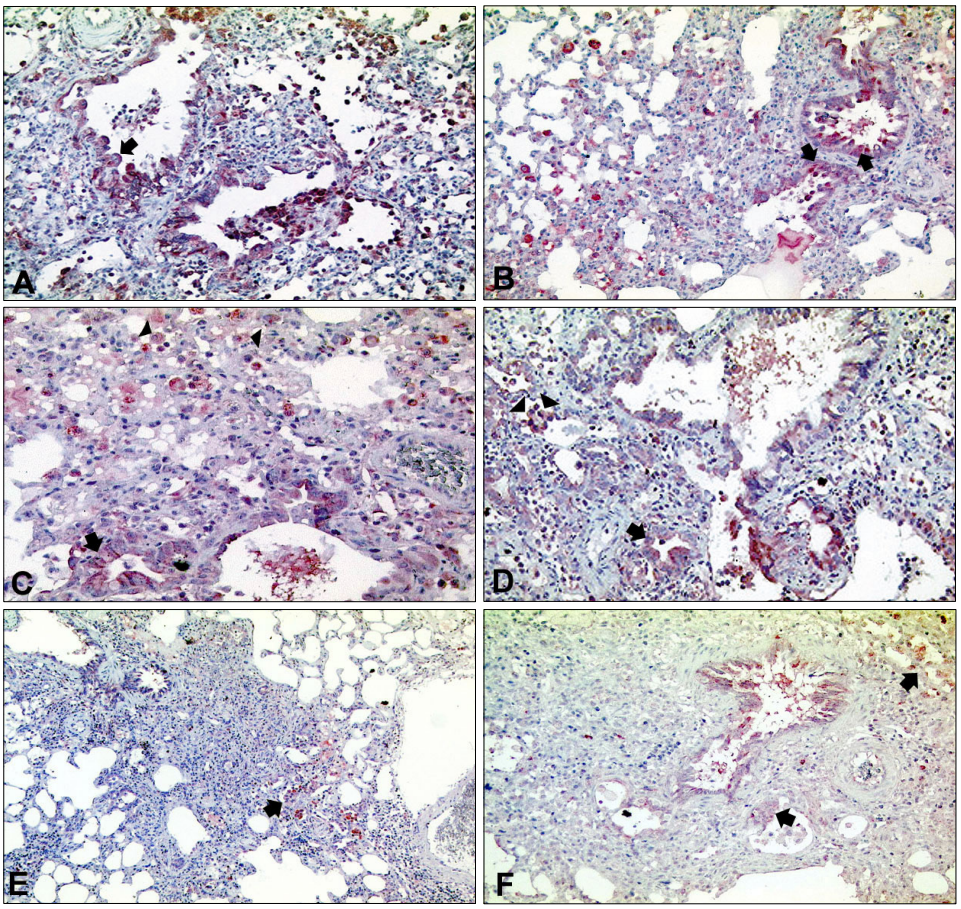


**Fig. 4.** The immunohistochemical expression of the MMP-2 and MMP-9 in bleomycin-treated group (A, C, and E: MMP-2; B, D, and F: MMP-9). (A and B) On the 4th day, the bronchiolar epithelial cells that are swollen and activated show prominent MMP positivity (arrows). MMPs are also strongly expressed in the inflammatory cells in the surrounding parenchyma. (C and D) On the 7th day, the MMP-2 and MMP-9 are expressed in the foci of bronchiolization (arrows). Some inflammatory cells, such as neutrophils and macrophages, still show expression (arrowheads). (E and F) On 14th day, the expression of MMP-2 (E) and MMP-9 (F) is decreased in most part, but still retained at the periphery of the fibrotic foci (arrows).

day and decreasing thereafter. Interestingly, the MMP-2 stayed in a relatively higher level than MMP-9 on the 14th and 21st day (Fig. 7A). On the 14th day, a small amount of active form of MMP-2 (62 kDa) was also observed (Fig. 6).

## DISCUSSION

In normal condition, the homeostasis of the extracellular matrix of the lung is maintained and tightly controlled. In idiopathic pulmonary fibrosis, however, this homeostasis is severely disrupted and accumulation of ECM continues, resulting in progressive fibrosis. The pathogenesis of IPF is not yet completely understood. Inflammation after epithelial injury is believed to be the integral part of the pathophysiology of pulmonary fibrosis,[16] especially in early phase, although the links between fibrosis and inflammation have been debated.[17-19] Dysregulated wound repair is also believed to be important pathogenetic mechanisms.[18,19] Key players, such as TGF-beta 1, TNF, and IL-1 beta have been reported to be crucial in sustaining inflammation and promoting progressive fibrosis.[10,20,21] MMPs and their inhibitors, TIMPs, have been known to play important roles in pulmonary fibrosis through their function in matrix protein degradation and its control.[3-5] Among many types of MMPs, MMP-2 and MMP-9 have been known

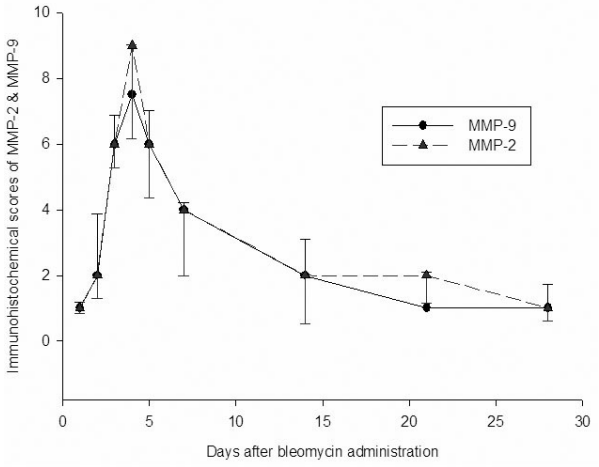


**Fig. 5.** The change in the levels of MMP-2 and MMP-9 in the lung parenchyma of bleomycin-treated group, shown by immunohistochemistry. The immunohistochemical score was evaluated semiquantitatively as described in methods. Both MMP-2 and MMP-9 rapidly increased from the first day to the 4th day, and decreased thereafter till the 28th day.

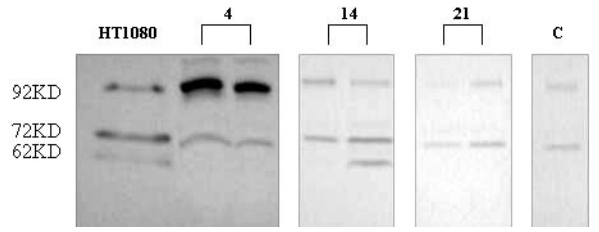


**Fig. 6.** Gelatin zymography of MMP-2 (72 kDa) and MMP-9 (92 kDa) in the BAL fluid of the bleomycin-treated group. The representative gelatin zymography sample shows that the band of MMP-9 is strong, particularly on the 4th days. It gets weaker from the 14th to 21st days. On the 14th day, weak band of active MMP-2 (62 kDa) is noted. 4, 14, and 21: experimental group; C: control group.

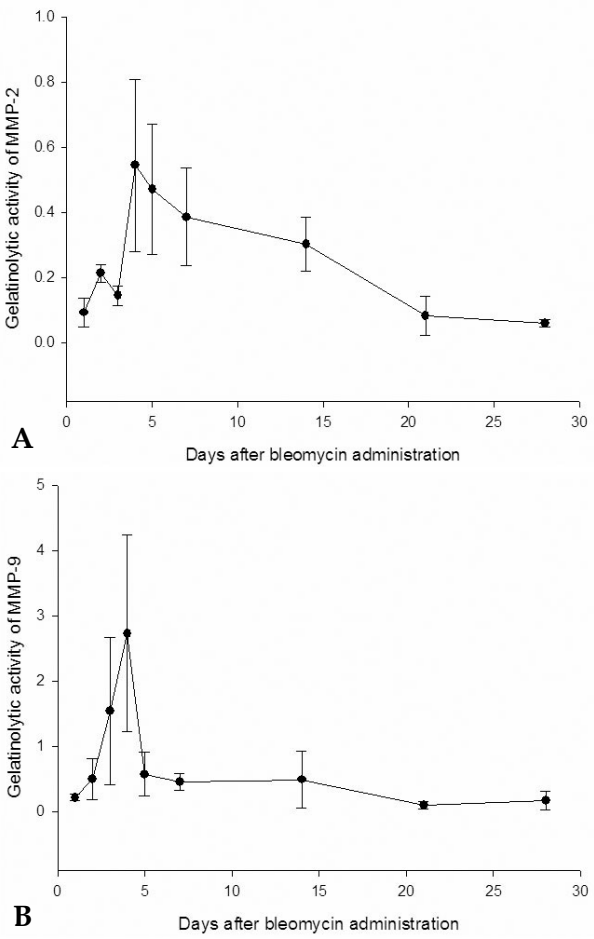


**Fig. 7.** The change of the gelatinolytic activity of MMP-2 and MMP-9 in the BAL fluid of the bleomycin-treated group. The activity of MMP-2 (A) and MMP-9 (B) is rapidly increased and reaches the peak level on the 4th day and then decreases thereafter. The change of the MMP-9 is more pronounced than that of the MMP-2. The MMP-2 declined less rapidly.

to be important and to show increased activity in many pulmonary diseases including pulmonary fibrosis.[4,5,22,23] Herein, we demonstrated that the activity of MMP-2 and MMP-9 in the bronchoalveolar lavage fluid and lung parenchyma increased rapidly in the early phase, reaching the peak levels on the 4th day, and then consistently decreased thereafter. This is in accordance with the previous studies,[24,25] which showed that the activity of the MMP-2 and MMP-9 increases in the early stage and decreases thereafter. However, the timing of the peak level in previous studies is somewhat different, and this may be due to the difference of experimental animals used or to the different time schedules when animals were sacrificed. The changes of the MMPs in the BAL fluid correlated to the total/differential cell counts in the BAL fluid. The level of the 2 MMPs in the lung parenchyma was not significantly different from each other, however, the change of the gelatinolytic activity of the MMP-9 in the BAL fluid was more prominent than that of MMP-2. This is maybe due to that in early phase, the MMP-9 was expressed strongly in neutrophils which more frequently existed in the intraalveolar spaces rather than in the lung parenchyma per se.

There was a temporal change in the major sources of the MMPs. In early phase until the 4th

day, both two MMPs were mainly expressed in the inflammatory cells. However, more specifically, MMP-2 was noted more frequently in alveolar macrophages, whereas MMP-9 in neutrophils. Since previous studies showed that these MMPs were co-localized with the type IV collagen in the basement membrane,[3] the MMP-2 and MMP-9 in this early phase secreted by macrophages and neutrophils may play an important role in degrading the basement membrane, thereby facilitating the inflammatory cell migration. In mid-phase (the 5th to 7th day), parenchymal cells, such as bronchiolar epithelial cells and type II pneumocytes, in addition to the inflammatory cells, also comprise important cellular sources expressing these MMPs. Especially, those with the features of cellular injury, such as cellular swelling, vacuolization, and nuclear atypia, expressed MMP-2 and MMP-9 more prominently. The bronchiolization foci[15] observed frequently from the 4th to 7th day also showed prominent MMP-2 and MMP-9 reactivity. In later phase (from 14th to 28th days), the main cellular sources of the MMPs were again the type II pneumocytes, however, mainly the ones surrounding the fibrotic foci. After the 14th day when fibrosis progressed, the overall expression of the MMP-2 and MMP-9 was very low. The fibroblasts, immature or mature collagen fibers at the center of the fibrotic foci, didn't show much MMP expression, while a few alveolar macrophages and type 2 pneumocytes retained the expression of the MMPs at the periphery of the fibrotic foci, presumably the advancing front of the fibrosis.

Bronchiolization, frequently observed from 4th to 7th day, is regarded as a reparative reaction to cellular injury to the alveolar epithelial cells.[15,26] Also, cells surrounding the fibrotic foci are known to be derived from regenerating pneumocytes.[27] The fact that the MMP-2 and MMP-9 were expressed in the cells with features of cellular injury/repair may suggest the roles of MMP-2 and MMP-9 in cellular regeneration. The roles of MMPs in relation with cellular regeneration in pulmonary fibrosis has also been suggested by several previous studies,[28-30] implicating their role in facilitating the migration of epithelial cells to the injured site.

Proinflammatory cytokines such as TNF-alpha and IL-6, which were increased in the early phase of pulmonary fibrosis and are essential in progression of early pulmonary inflammation to pulmonary fibrosis[31,32] have been associated with increased MMP levels[33] or induction of MMP production from various cell types including alveolar epithelial cells, alveolar fibroblasts, and alveolar macrophages.[34-36] They were also shown to induce other cytokines such as TGF-beta 1.[32] These proinflammatory cytokines may play roles in pulmonary fibrosis by recruiting inflammatory cells and inducing MMPs production from recruited cells, thus facilitating the migration of those inflammatory cells and further promoting fibrosis through a synergistic action with profibrotic cytokine TGF-beta 1. TGF-beta 1, the most potent fibrogenic cytokine, has been reported to increase in pulmonary fibrosis and to be expressed in alveolar epithelial cells in higher levels even in later phase of pulmonary fibrosis.[37,38] It was also reported that TGF-beta 1 induces transition of alveolar epithelial cells to mesenchymal/fibroblasts-like cells and MMP-2 expression in those cells,[39] possibly suggesting that MMP-2 production in alveolar epithelial cells by TGF-beta 1 may promote pulmonary fibrosis. However, the mechanisms of cytokines and MMPs need to be further elucidated, since many other cytokines such as IL-10[40] and IL-8[41] are also involved, and there are conflicting results, too.[42]

In our study, the levels of the MMP-2 and MMP-9 in the lung parenchyma corresponded well to those of BAL fluid. The antibodies used for immunohistochemistry could equally stain both latent and active forms without any preference, therefore, active forms of both MMPs were to be evaluated by the zymography. It was expected that the active forms of MMP-2 and MMP-9 might have been increased in the early phase of pulmonary fibrosis when the total MMP-2 and MMP-9 activity reached the maximum. However, the zymography of the BAL revealed no active forms of the MMPs from the 4th to 7th day when the MMP levels were the highest. Only a small amount of active MMP-2 was detected on the 14th day, and the active form of MMP-9 was not noted throughout the entire time course. It is quite possible that the active forms of MMPs were more likely present in the lung parenchyma where active protein degradation occurs and that the

level of the active MMPs in the BAL fluid might be too low to be detected, or might have been degraded too quickly after their normal function. There are some studies reporting that the levels of active MMP did not necessarily correlate to total MMP levels.[10,11,43] The fact that active form of only MMP-2, but not MMP-9, was detected in later phase of fibrosis is in accordance with an another study which showed similar result, suggesting different roles of MMP-2 and MMP-9 along in the time course.[25] Presumably, MMP-9 is more important in early phase when active degradation of the basement membrane and inflammatory cell migration occur, whereas MMP-2 may play roles in later phase in regard to the formation of the fibrotic foci and cellular repair.

In this study, we didn't investigate the activity of TIMPs. Considering the fact that MMP activity is regulated by TIMPs, the role of MMPs in pulmonary fibrosis has to be understood in association with that of TIMPs. The activities of TIMP-1 or TIMP-2[3,44-46] were reported to increase in pulmonary fibrosis, especially in the acute phase of pulmonary fibrosis or acute lung injury model. It has also been suggested that TIMP is related to the process of bronchial epithelial cell regeneration.[30] More detailed information on the functions of MMP in relation with TIMPs in pulmonary fibrosis needs to be clarified.

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