Inhibition of matrix metalloproteinase-9 prevents neutrophilic inflammation in ventilator-induced lung injury

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Inhibition of matrix metalloproteinase-9 prevents neutrophilic inflammation in ventilator-induced lung injury. Male Sprague-Dawley rats were subjected to high tidal volume (HVT), low tidal volume (LVT), and aerosol high tidal volume (HVT/Aerosol) ventilation for 6 hours. Neutrophilic inflammation of ventilator-induced lung injury (VILI) is characterized by neutrophil accumulation within the alveoli, basement membrane disruption, and endothelial cell necrosis (1-3). Neutrophils are central to the pathogenesis of VILI (4-6). Neutrophils adhere to the pulmonary endothelial cell layer and migrate through the interendothelial cell junctions down to the underlying basement membrane; the neutrophils that reach the basement membrane traverse this barrier via an undetermined mechanism (7, 8). MMPs have been postulated to participate in neutrophil transmigration. The purpose of this study was to investigate the role of MMP-9 in the neutrophilic inflammation of VILI. Male Sprague-Dawley rats were divided into three groups: 1) low tidal volume (LVT), 7 ml/kg of tidal volume (Vt); 2) high tidal volume (HVT), 30 ml/kg of Vt; and 3) HVT with MMP inhibitor (HVT+MMPI). As a MMPI, CMT-3 was administered daily from 3 days before mechanical ventilation. Degree of VILI was assessed by wet-to-dry weight ratio and acute lung injury (ALI) scores. Neutrophilic inflammation was determined from the neutrophil count in the lung tissue and myeloperoxidase (MPO) activity in the bronchoalveolar lavage fluid (BALF). MMP-9 expression and activity were examined by immunohistochemical staining and gelatinase zymography, respectively. The wet-to-dry weight ratio, ALI score, neutrophil infiltration, and MPO activity were increased significantly in the HVT group. However, in the HVT+MMPI group, pretreatment with MMPI decreased significantly the degree of VILI, as well as neutrophil infiltration and MPO activity. These changes correlated significantly with MMP-9 immunoreactivity and MMP-9 activity. Most outcomes were significantly worse in the HVT+MMPI group compared with the LVT group. In conclusion, VILI mediated by neutrophilic inflammation is closely related to MMP-9 expression and activity. The inhibition of MMP-9 protects against the development of VILI through the downregulation of neutrophil-mediated inflammation.

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in lung tissues and of MMP-9 activity in the BALF with or without MMPI pretreatment.

MATERIALS AND METHODS

Animals and mechanical ventilation. Specific pathogen-free, male, Sprague-Dawley rats, each weighing 280–320 g, were housed in pathogen-free rooms and maintained on laboratory chow with free access to food and water. This study was performed in accordance with the guidelines of the Animal Research Committee of Korea University and with the approval of the Ethics Committee of Korea University Medical Center.

The animals were randomly divided into the following experimental groups: 1) low tidal volume (VT) group (LVT group, n = 12) in which the rats were ventilated with LVT with positive end-expiratory pressure (PEEP); 2) high tidal volume group (HVT group, n = 12) in which the rats were ventilated with HVT without PEEP; and 3) HVT with MMPI pretreatment group (HVT+MMPI group, n = 12) in which the rats were pretreated with MMPI and ventilated with the same settings as for the HVT group. Each group subdivided into the histological set (n = 6) for wet-to-dry weight ratio measurements, histology, immunohistochemical staining, and the BAL set (n = 6) for myeloperoxidase (MPO) activity assays and gelatinase zymography.

Each rat was anesthetized with an intraperitoneal injection of 37 mg/kg thiopental, tracheostomized, and paralyzed with an intramuscular injection of 2 mg/kg vecuronium bromide. Mechanical ventilation was performed with a rodent volume ventilator (model 7025; Ugo Basile Biological Research Apparatus, Comerio-Varese, Italy). The rats in the LVT group were ventilated with the settings of 7 ml/kg VT, 3 cmH2O PEEP, and 40 breaths/min (36). Adequate modality for the formation of the VILI model was determined in preliminary studies using the following mechanical ventilator settings: 1) 20 ml/kg VT + 0 cmH2O PEEP + 25 breaths/min; 2) 30 ml/kg VT + 0 cmH2O PEEP + 40 breaths/min; and 3) 42 ml/kg VT + 0 cmH2O PEEP + 40 breaths/min. Examination of the lung tissues every 30 min allowed determination of the time and setting that gave pathological findings typical of VILI (12), i.e., severe diffuse alveolar damage, hyaline membranes, alveolar hemorrhage, and neutrophil infiltration. With the settings of 20 ml/kg VT + 0 cmH2O PEEP + 25 breaths/min, typical ALI was not apparent until after 4 h. With the settings of 42 ml/kg VT + 0 cmH2O PEEP + 40 breaths/min, severe pulmonary hemorrhage occurred 30 min after mechanical ventilation, and most of the rats expired after 30–60 min. Histological examination showed severe intra-alveolar hemorrhage with minimal evidence of other ALI indicators. With the settings of 30 ml/kg VT + 0 cmH2O PEEP + 40 breaths/min, typical ALI findings developed within the period of the experiment. These changes were most prominent at about 2 h after mechanical ventilation. Therefore, the optimal mechanical ventilator modality for the HVT and HVT+MMPI groups was the setting of 30 ml/kg VT + 0 cmH2O PEEP + 40 breaths/min for 2 h, and the rats in the LVT group were also ventilated for 2 h.

MMPI. The rats in the HVT+MMPI group were administered 6-demethyl-6-deoxy-4-dedimethylamino-tetracycline (CMT-3; donated by Collagenex Pharmaceuticals, Newtown, PA). This chemically modified, non-antibiotic tetracycline was administered by gavage daily from 3 days before mechanical ventilation at 20 mg/kg in 1 ml of vehicle, which consisted of N-methyl pyrrolidone, propylparaben, methylparaben, ethanol, and 2% carboxymethyl cellulose. The rats in the LVT and HVT groups were gavaged with 1 ml of vehicle during the same period.

Tissue preparation, wet-to-dry weight ratios, and BALF assays. After mechanical ventilation, the rats’ chests were opened by mid-sternal incision, and the rats were exsanguinated. The heart and lungs were removed en bloc. After ligation of the left main bronchus, the left lung was excised and weighed in a tared container. The lung was then dried in a drying oven until a constant weight was obtained, and the wet-to-dry weight ratio was calculated. After excision of the left lung, the lung tissues were prepared as follows for the histological and immunohistochemical examinations. The right main bronchus was instilled and fixed immediately with 4% paraformaldehyde at a hydrostatic pressure of 20 cmH2O, and the specimen was floated in fixative for 48 h. After excision of the lower lobe, paraffin blocks were prepared by dehydration of the lung tissues with ethanol and embedding in paraffin.

For the BAL group rats, after euthanasia by exsanguination, the thorax was opened, and three BAL procedures were performed, each with 2 ml of PBS. The retrieval fluid was centrifuged (2,000 g at 4°C) for 10 min, and the supernatants were divided into aliquots and stored at −70°C until analysis for MPO activity and gelatinase zymography.

Evaluation of degree of VILI and neutrophil infiltration. The posterior portions of the right lower lobe were sectioned at 4-μm thickness, placed on glass slides, and stained with hematoxylin-eosin. A pathologist, who was blinded to the protocol and experimental groups, examined the degree of lung injury and graded the specimens by ALI score. ALI was scored based on: 1) alveolar capillary congestion; 2) hemorrhage; 3) infiltration or aggregation of neutrophils in the alveolar wall or the vessel wall; and 4) thickness of the alveolar wall/hyaline membrane formation (19). Each item was graded according to the following five-point scale: 0 = minimal (little) damage; 1 = mild damage; 2 = moderate damage; 3 = severe damage; and 4 = maximal damage (19). The degree of VILI was assessed by sum of scores of items from 0 to 16 in five high-power fields (HPF, magnification ×400) randomly. The average sum of each field score was compared among groups.

To evaluate more accurately the recruitment of polymorphonuclear (PMN) leukocytes, we stained sections with 3,3’-diaminobenzidine and counterstained with toluidine blue. The PMN cells, which were observed as peroxidase-positive blue cytoplasmic cells, were counted in 10 consecutive HPFs (magnification ×400) (41).

Immunohistochemical staining for MMP-9. Immunohistochemical staining for MMP-9 was performed as follows. PBS that contained 0.05% Tween 20 and 2% normal goat serum was used as the antibody diluent after blocking endogenous peroxidase with 0.05% H2O2 in methanol. The sections were incubated overnight at 4°C with mouse monoclonal antibodies against MMP-9 (diluted 1:500; Oncogene Science, Cambridge, MA) and then washed with PBS to remove excess primary antibody. The sections were then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 for 1 h at room temperature. Bound antibody was visualized according to the standard avidin-biotin-peroxidase complex protocol. The immunoreactivities of the lung tissue specimens were scored independently by two people using the following scheme: 0 = none; 1 = mild; 2 = moderate; 3 = strongly abundant immunoreactivity. MMP-9 expression was examined randomly in five HPFs (magnification ×400), and the sums of the scores for each field score were compared.

Determination of MPO activity. Pulmonary hemorrhage is one of the characteristic findings in the VILI model. Because neutrophil-mediated inflammation may be overestimated in examinations of lung tissues and BALF cell counts, MPO activity was determined in cell-free BALF according to a previously described method (16), with minor modifications. Aliquots of 50 μl of cell-free BALF were mixed in microtiter plates with 200 μl of O-dianisidine dihydrochloride (1.25 mg/ml in PBS) plus BSA (0.1% wt/vol) containing H2O2 (0.05% = 0.4 mM). The MPO activities are expressed as changes in absorbance at 450 nm.

Gelatinase zymography. Gelatinase zymography was performed with modifications to a previously published protocol (15, 23). The protein concentration of each sample was measured with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). The volume of BALF that contained 100 μg of protein was mixed with an equal volume of sample buffer [80 mmol/l Tris-·HCl (pH 6.8), 4% SDS, 10% glycerol, 0.01% bromphenol blue]. Recombinant human MMP-9 (Sigma Chemical, St. Louis, MO) was diluted in collagenase buffer,

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mixed with an equal volume of sample buffer, and loaded onto the gel as a standard. As a negative control, sample buffer was loaded onto each gel. For electrophoresis, 8% SDS-polyacrylamide resolving gels that contained 1 mg/ml gelatin were overlaid with 5% stacking gels, and the samples were loaded and run at 4°C (25 mA/gel). After electrophoresis, the gels were rinsed briefly with distilled water and washed three times (15 min each) with 150 ml of 2.5% Triton X-100 solution on a rotary shaker. The gels were then incubated at 37°C for 66 h in 250 ml of 50 mmol/l Tris-HCl (pH 7.5) that contained 10 mmol/l CaCl2 and 0.02% NaN3. After incubation, the gels were stained with 50% methanol, 10% acetic acid, and 0.1% Coomassie blue R-250 and then destained with 10% methanol and 10% acetic acid. After being destained, the gels were immersed in distilled water for 20 min and scanned immediately. MMP activity was determined by densitometry and Quantity One 1-D Analysis Software (Bio-Rad Laboratories). Normalization of integrated densities was performed by dividing values obtained from each sample by the fraction of recombinant human MMP-9 standards run in parallel in the same gel.

Statistical analyses. All the data are expressed as means ± SD. Statistical analysis was performed using the nonparametric Mann-Whitney’s U-test and Kruskal-Wallis methods to determine intergroup differences. A P value of < 0.05 was taken to be statistically significant. Spearman’s rank correlation coefficient was used to correlate the MMP-9 activity in BALF and immunoreactivity in tissue with neutrophil infiltration and degree of VILI.

RESULTS

Wet-to-dry weight ratios. The wet-to-dry weight ratios of the LVT, HVT, and HVT+MMPI groups were 4.70 ± 0.14, 6.82 ± 1.28, and 4.92 ± 0.28, respectively, with significant differences between the groups (P = 0.001 by the Kruskal-Wallis test). The HVT group had a significantly higher wet-to-dry weight ratio than either the LVT or HVT+MMPI group (P = 0.002). In addition, the HVT+MMPI group showed a higher wet-to-dry weight ratio than the LVT group (P = 0.041; Fig. 1A).

ALI scores. After mechanical ventilation, the HVT group showed high levels of intra-alveolar exudates, hyaline membrane formation, inflammatory cell infiltration, intra-alveolar hemorrhage, and interstitial edema (Fig. 2B). However, significant ALI findings were absent and only mild inflammatory cellular infiltration was present in the LVT group (Fig. 2A). The HVT+MMPI group displayed moderately increased inflammatory cellular infiltration and intra-alveolar hemorrhage, compared with the LVT group, but the overall degree of lung injury in the HVT+MMPI group was significantly lower than in the HVT group (Fig. 2C).

The degrees of VILI, as expressed by ALI scores, in the LVT, HVT, and HVT+MMPI groups were 3.25 ± 0.001, 12.83 ± 1.17, and 4.67 ± 0.52, respectively, with significant differences between the three groups (P = 0.001 by the Kruskal-Wallis test). The HVT group showed a significantly higher ALI score than the LVT and HVT+MMPI groups (P = 0.001 compared with the LVT group and P = 0.002 compared with the HVT+MMPI group). In comparisons of the LVT and HVT+MMPI groups, the HVT+MMPI group showed the higher ALI score (P = 0.029; Fig. 1B).

Neutrophil infiltration and BALF MPO activity. The levels of neutrophil infiltration in 10 HPFs of the LVT, HVT, and HVT+MMPI groups were 24.33 ± 3.33, 202.17 ± 17.61, and 86.50 ± 13.55, respectively, with significant differences between the three groups (P = 0.001 by the Kruskal-Wallis test). The HVT group showed significantly higher neutrophil infiltration than the LVT and HVT+MMPI groups (P = 0.002 compared with LVT and HVT+MMPI groups). In comparisons of the LVT and HVT+MMPI groups, the HVT+MMPI group showed the higher level of neutrophil infiltration (P = 0.002; Fig. 3A).

The optical densities of the MPO activities in the BALFs of the LVT, HVT, and HVT+MMPI groups were 0.0296 ± 0.0138, respectively, with significant differences between the groups (P = 0.001 by the Kruskal-Wallis test). The HVT group showed significantly higher MPO activity than the LVT and HVT+MMPI groups (P = 0.002 compared with LVT and HVT+MMPI groups). In comparisons of the LVT and HVT+MMPI groups, the HVT+MMPI group showed the higher level of MPO activity (P = 0.015; Fig. 3B).
Immunohistochemical staining for MMP-9. The expression of MMP-9 was examined by immunohistochemical staining. In the LVT group, the immunoreactivity of MMP-9 was minimal in the low (×100) and high (×400) power fields (Fig. 4A). However, the HVT group showed strong heterogeneous expression in low power fields, and immunoreactivity was observed at or around the inflammatory cells in HPF (Fig. 4B). The HVT+MMPI group expressed significantly less MMP-9 than the HVT group (Fig. 4C).

According to the semiquantification system using the four-point scale, the levels of MMP-9 expression in the LVT, HVT, and HVT+MMPI groups were 3.33 ± 2.07, 12.17 ± 2.79, and 3.60 ± 1.95, respectively, with significant differences between the three groups (P = 0.004 by the Kruskal-Wallis test). The HVT group showed significantly higher immunoreactivity than the LVT and HVT+MMPI groups (P = 0.004 compared with the HVT+MMPI group and P = 0.002 compared with the LVT group). In comparisons of the LVT and the HVT+MMPI groups, the HVT+MMPI group showed the higher level of MMP-9 expression, although this difference was not statistically significant (P = 0.931; Fig. 4A).

Using Spearman’s correlation, MMP-9 immunoreactivity in tissues was found to have a significant correlation with neutrophil infiltration (r = 0.713, P = 0.001), MPO activity (r = 0.713, P = 0.001), and HPFs, high-power fields.
0.674, \( P = 0.002 \)), wet-to-dry weight ratio (\( r = 0.750, P = 0.000 \)), and ALI score (\( r = 0.704, P = 0.001 \)).

Gelatinase zymography. Gelatinase activity was examined in the BALFs using the zymography techniques described previously. We found bands of degradation around 92 kDa that were consistent with MMP-9 (Fig. 5B). In addition, incubation with EDTA completely eliminated this band of gelatinase activity, which is consistent with the known properties of MMP-9. The relative densitometry units of MMP-9 activity for the LVT, HVT, and HVT/MMPI groups were 84.12 ± 4.37, 158.44 ± 23.45, and 95.77 ± 5.95, respectively, which were significantly different between the groups (\( P = 0.001 \) by the Kruskal-Wallis test). The HVT group showed significantly higher MMP-9 activity than the LVT and HVT/MMPI groups (\( P = 0.002 \) compared with the LVT and HVT/MMPI groups). In comparisons of the LVT and HVT/MMPI groups, the HVT/MMPI group showed the higher level of MMP-9 activity (\( P = 0.002 \); Fig. 5C).

In Spearman’s correlation analysis, MMP-9 activity in BALF correlated significantly with neutrophil infiltration (\( r = 0.909, P = 0.000 \)), MPO activity (\( r = 0.785, P = 0.000 \)), wet-to-dry weight ratio (\( r = 0.775, P = 0.000 \)), and ALI score (\( r = 0.846, P = 0.000 \)).

Fig. 4. A: in the LVT group, MMP-9 immunoreactivity is minimal in the low- (×100) and high- (×400) power fields. B: the HVT group shows strong heterogeneous expression in the low-power fields, and immunoreactivity is observed at or around the inflammatory cells in the HPFs. C: the HVT/MMPI group expresses significantly less MMP-9 than the HVT group.

Fig. 5. A: semiquantification of MMP-9 immunoreactivity. The levels of MMP-9 immunoreactivity are significantly different among the 3 groups (\( P = 0.004 \) by the Kruskal-Wallis test). The HVT group shows significantly higher immunoreactivity than the LVT and HVT/MMPI groups. Comparisons of the LVT and HVT/MMPI groups show that the HVT/MMPI group shows higher expression of MMP-9, although this difference is not statistically significant. Gelatinase zymography (B) and the relative densitometry units (C) of MMP-9 activity show significant differences among the groups (\( P = 0.001 \) by the Kruskal-Wallis test). The HVT group shows significantly higher MMP-9 activity than the LVT and HVT/MMPI groups. Comparing the LVT and HVT/MMPI groups, the HVT/MMPI group shows the higher MMP-9 activity (*\( P < 0.05 \)).
In the present study, injurious mechanical ventilation strategy with HVT without PEEP increased the degree of VILI and neutrophil infiltration, which were significantly correlated with the level of MMP-9 expression in lung tissue and MMP-9 activity in the BALF. In the case of MMP-9 inhibition by CMT-3 pretreatment, VILI was significantly prevented, and this was associated with decreases in neutrophil infiltration and MMP-9 expression and activity. These results indicate that MMP-9 plays an important role in promoting neutrophil transmigration and in the development of VILI in rats.

MMPs are zinc- and calcium-dependent endopeptidases that have the ability to cleave one or several extracellular matrix constituents. MMPs can be classified into four groups on the basis of sequence homology and substrate specificity: interstitial collagenase, neutrophil collagenase, collagenase-3, and membrane-type metalloproteinases. We investigated the unusual activity of neutrophil collagenase in the pathogenesis of VILI, which is associated with neutrophil-mediated inflammation. Neutrophil collagenase consists of the 72- and 92-kDa gelatinases, MMP-2 and MMP-9, respectively. MMP-2 is synthesized by a wide variety of cells, including fibroblasts, endothelial cells, and alveolar epithelial cells. MMP-9 is produced mainly by inflammatory cells, such as polymorphonuclear neutrophils, monocytes, macrophages, eosinophils, and lymphocytes. In particular, activated neutrophils secrete significant amounts of MMP-9, which is a major elastolytic MMP (40) but not of MMP-2 (34).

The exuberant or aberrant expression of MMPs can cause tissue damage and has been associated with a variety of lung diseases. The higher concentration of MMP-9 may be induced by the increased number of neutrophils in the BALF of ARDS patients, as MMP-9 is the type IV collagenase that is found in neutrophils (27). Thus the MMPs have the ability to degrade the type IV collagen-rich basement membrane (29, 30). Although macrophages are known to produce MMP-2 and MMP-9, increases in the numbers of macrophages are not correlated with increases in MMP-9 levels (7). Because MMP-9 is expressed by inflammatory cells, the role of MMP-9 in neutrophil migration has been studied.

Physical stretching and shearing forces caused by mechanical ventilation induce the recruitment of inflammatory cells and the production of inflammatory mediators (18, 20, 46, 48, 50). Of these factors, neutrophils and their mediators have been implicated in the development of both VILI and ALI (4, 12, 19, 20, 28, 33, 35, 49, 50). However, VILI can be caused by mechanisms independent of neutrophils such as alveolar instability (44) or decreased lung edema clearance (24). Neutrophil involvement in lung injury can be conceptualized in terms of sequential stages, beginning with sequestration in the pulmonary microvasculature, followed by adhesion and activation, and culminating in the production of an "effector" response, i.e., the generation of reactive oxygen species or the release of proteolytic enzymes (25). Sequestered neutrophils adhere to the pulmonary endothelial cell layer and migrate through the interendothelial cell junctions to the underlying basement membrane. These processes involve several cellular adhesion molecules. Physiological studies have shown that the neutrophil that reaches the basement membrane pauses before traversing the basement membrane, using an undefined mechanism to penetrate this barrier (17, 52). Although MMPs have been postulated to be active in this process, studies clarifying their role have been controversial. According to some studies, MMP-9 is a major factor in neutrophil migration across the basement membrane in vitro (10) and promotes neutrophil migration in both pancreatitis-associated lung injury (21) and lipopolysaccharide-induced goblet cell metaplasia (22) in the rat. However, MMP-9 is ineffective at stopping neutrophil migration through intact endothelial cell monolayers and basement membrane matrices in vitro (26), and neutrophils from MMP-9-deficient mice showed no defect in transendothelial migration in vitro (1). Furthermore, MMP-9-deficient mice have normal neutrophil emigration into the lungs, peritoneum, and skin (5). Thus the role of MMP-9 release and digestion of extravascular matrix components during migration remains uncertain (51). Therefore, we investigated the role of MMP-9 and MMPI in the pathogenesis of VILI.

In the present study, the degree of MMP-9 expression observed by immunohistochemical staining and the levels of MMP-9 activity in the BALF detected by gelatinase zymography showed significant correlations with the levels of neutrophil infiltration, MPO activity in BALF, and the degree of VILI observed by ALI score and wet-to-dry weight ratio in rat model. This suggests that the migration of activated neutrophils through the extravascular milieu is aided by digestion with MMP-9. MMP-9 expression, MMP-9 activity, and neutrophil infiltration were decreased significantly by CMT-3 treatment, which suggests that MMPI prevents neutrophil migration. The attenuation of neutrophil infiltration by MMPI was related to a decrease in the degree of VILI. CMT-3, which was used as the MMPI in the present study, is a nonantimicrobial tetracycline, chemically modified to enhance its collagenase-inhibitory property. CMT-3 is the most potent inhibitor of both MMP-2 and MMP-9 (39), but since MMP-2 is not produced by activated neutrophils (34), we believe that CMT-3 treatment primarily blocked MMP-9 in the present study. However, in the results, all outcomes with an exception of MMP-9 immunoreactivity were significantly worse in the HVT+MMPI group compared with the LVT group. Although the exact mechanisms are unclear, the plausible explanation is that VILI is caused by complex of multiple pathogenesis other than neutrophils and MMP-9.

In conclusion, inhibition of MMP-9 significantly, but not completely, reduces the degree of lung injury induced by injurious mechanical ventilation by downregulating neutrophil-mediated inflammation as well as MMP-9 expression and activity. These findings suggest that MMP-9 plays a pivotal role in the pathogenesis of VILI and provide further evidence that MMPI has the potential to prevent to a substantial degree VILI that cannot be completely protected against by lung-protective ventilation strategies.

REFERENCES


ROLE OF MMP-9 IN NEUTROPHILIC INFLAMMATION DURING VILI


