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ORIGINAL ARTICLE

Contrary Regulation of TIMP-1 and MMP-9 by Hepatocyte Growth Factor Antibody after Lung Injury

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Key words: hepatocyte growth factor; matrix metalloproteinases-9; tissue inhibitor of metalloproteinases-1

Objective To study the influence of hepatocyte growth factor (HGF) antibody on the lung expression level of matrix metalloproteinases-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1).

Methods Thirty male Wistar rats were randomly divided into 3 groups: control group, model group, and intervention group. Endotoxin was intratracheally infused in the model and intervention groups. HGF antibody was injected in the rats of the intervention group from day 1 to day 14, while the same volume of saline was injected in the control group. The rats were sacrificed on day 28 after endotoxin treatment. The amounts of MMP-9 mRNA and TIMP-1 mRNA were measured by reverse transcription-polymerase chain reaction, and protein expression levels of MMP-9 and TIMP-1 were measured by immunohistochemistry.

Results In the model group, both mRNA and protein expression levels of TIMP-1 were significantly increased, the same as MMP-9. In the intervention group, the increase of TIMP-1 was remarkably reduced compared with the model group, while the mRNA and protein expression levels of MMP-9 were still increased.

Conclusion HGF activity may accelerate the repair of lung injury through contrary regulating the expression levels of TIMP-1 and MMP-9.

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WITH regard to the mechanism of lung damage and disordered remodeling, therapeutic strategies fall into 3 categories: (1) to counteract active inflammatory reactions caused by pro-inflammatory agents; (2) to avoid apoptotic loss of epithelial cells and stimulate ordered tissue repair; (3) to eliminate chronic stimuli for fibrosis.¹

Current therapeutic approaches are mostly confined to the early anti-inflammatory phase. However, strong therapeutic support to repair cells and attenuate collagen accumulation is essential.

Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent neutral endopeptidases capable of degrading all extracellular matrix (ECM) components. They play an important role in ECM remodeling in embryonic development, tissue regeneration, and wound repair.² MMP-9 is a critical extracellular remodeling enzyme in wound healing, especially in degradation of basement

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membrane and antifibrosis.³ Tissue inhibitor of metalloproteinase-1 (TIMP-1), a representative natural MMP inhibitor, inhibits almost all MMPs by binding to the catalytic zinc atom at the MMP active site.⁴ A recent study revealed that the disruption of the balance between MMPs and TIMPs played a critical role in the pathogenesis of several well-recognized pulmonary disorders, such as lung injury, chronic obstructive pulmonary disease, and asthma.⁵ However, how the balance is regulated remains completely unknown. Hepatocyte growth factor (HGF), also known as scatter factor, is a reparative factor for the regeneration of injured lung. HGF exerts multiple biological functions such as stimulating epithelial cell proliferation and mobility, inducing branching tubule formation, and inhibiting tissue fibrosis.⁶ As diminishing the deposits of both endogenous and exogenous basement membranes constituents, HGF is considered to have the potential to induce proteolytic activity or regulate MMPs expression. HGF-treated SV40-T2 cells partly present HGF-associated induction of MMP-9.⁷ The present study investigates the effect of HGF in the endotoxin-induced acute lung injury, focusing on the expression levels of MMP-9 and TIMP-1.

MATERIALS AND METHODS

Materials

The endotoxin O55B5 of *Escherichia coli* and rabbit anti-mouse & HGF antibody were obtained from Sigma Company (St. Louis, MO, USA). The reverse transcription-polymerase chain reaction (RT-PCR) kits of MMP-9 and TIMP-1 were purchased from Shanghai Sangon Institute of Biology (China). MMP-9 and TIMP-1 immunohistochemistry kits were from Beijing Zhongshan Biotechnology Company (China). Hydroxyproline and Coomassie brilliant blue determination kits were from Nanjing Jiancheng Bioengineering Institute (China).

Model construction

Thirty male Wistar rats (Center of Medical Experimental Animal, Tongji Medical College, Huazhong University of Science of Technology) weighing 250 ± 50 g were divided into 3 groups ($n=10$) using random number table sampling: control group, model group, and intervention group. The rats were anesthetized with intraperitoneal injection of 3% pentobarbital 30 mg/kg. The rats in the control group were injected with saline, while the other rats were intratracheally injected with endotoxin 3 mg/kg on day 1. HGF antibody [1.25 ug/(kg·d)] was injected from day 1 to day 14 after intratracheal injection of endotoxin in the intervention group. The rats in all the groups were sacrificed

under ether anesthesia on day 28.

Histopathological analysis

The lung tissue was excised and inflated with 10% formalin. Fixed samples were embedded in paraffin, sectioned into 6-um pieces, and stained with hematoxylin and eosin. A pathologist unaware of the group division analyzed the samples according to the histopathological scoring system of lung injury by Mrozek et al.⁸ Lung injury was scored using a 5-point scale according to combined assessments in 4 aspects, namely alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation: 0, minimum damage; 1, mild damage; 2, moderate damage; 3, severe damage; 4, maximum damage. The minimum and maximum possible scores were 0 and 16, respectively.

Measurement of lung wet-to-dry weight ratio

To quantify the magnitude of pulmonary edema, we calculated the wet/dry weight (W/D) ratio of the lung. Briefly, portions of the harvested wet lungs were weighed and then placed in an oven at 80°C for 24 hours, and weighed again when the lung portions were dried.

Hydroxyproline measurement

The total collagen content of the lung was determined by hydroxyproline assay and the hydroxyproline content in the same protein amount was measured. The hydroxyproline content was tested according to the protocol of the hydroxyproline detection kit.

RT-PCR analysis of MMP-9 mRNA and TIMP-1 mRNA

Total RNA was extracted from the lung parenchyma. mRNA was converted into the first stand cDNA using M-MLV reverse transcriptase, PCR was carried out using specific homo MMP-9 primers (upper primer, 5'-CTGAGG GTC ACA GAC TTC G-3'; lower primer, 5'-AGC TCA GAA CCG ACC GGC TT-3'; product length, 529 bp). The cycling parameters were as follows: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute, 35 cycles. TIMP-1 primers were 5'-ACGACC GAC ATA CTC TGC G-3' and 5'-TGC TCA GAA CCG AGG TTC AA-3'. The product length of TIMP-1 was 712 bp. The cycling parameters were as follows: denaturation at 96°C for 40 seconds, annealing at 60°C for 45 seconds, extension at 70°C for 1 minute, 36 cycles. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The intensity of MMP-9 mRNA and TIMP-1 mRNA band were quantified by densitometry using a gel documentation and analysis system (GDS 8000, Ultra-Violet

Products, UK) and normalized to the values of GAPDH.

Immunohistochemistry

Immunohistochemical detection for MMP-9 and TIMP-1 was performed according to the Envision two-step method. The primary antibodies were rabbit anti-mouse antibody against MMP-9 (1 : 100 working solution) and TIMP-1 (1 : 80 working solution). A mouse anti-rabbit IgG was used as the second antibody. The expressions of MMP-9 and TIMP-1 were evaluated semiquantitatively according to the percentage of positive cells: 0, negative; +1, focal, 1%-10% of the cells in the lesion were positive; +2, moderate, 11%-50% of the cells in the lesion were positive; and +3, marked, >50% of the cells in the lesion were positive. The percentage of positively stained area was measured and scored in 10 fields of each sample in a blind manner.

Statistical analysis

Data were presented as means±SD. The statistical analysis was conducted with SPSS 10.0 software. $P<0.05$ was considered statistically significant.

RESULTS

Histological evaluation

The rats in the control group, which were injected with physiological saline, showed normal lung histology. Model group rats showed extensive morphological lung damage: edema, thickening of the alveolar wall, and infiltration of inflammatory cells into interstitium and alveolar spaces. In the intervention group, injection of HGF antibody significantly improved the lung injury (Fig. 1). The extent of these histological abnormalities was assessed with lung injury score (Table 1).

Wet-to-dry weight ratio

The lung W/D ratio significantly increased after the endotoxin injection ($P<0.05$). The W/D ratio in the intervention group was significantly lower than that in the model group ($P<0.05$, Table 1).

Hydroxyproline content in rat lung tissue

The hydroxyproline concentration of the model group was significantly higher compared with the control group ($P<0.05$). Hydroxyproline concentration in the intervention group was significantly decreased compared with that of the model group ($P<0.05$, Table 1).

Expression levels of MMP-9 mRNA and TIMP-1 mRNA

RT-PCR analysis showed a very weak staining of MMP-9 mRNA and TIMP-1 mRNA in normal lung tissue. The mRNA expression level of TIMP-1 was obviously increased after acute lung injury, and MMP-9 mRNA was also increased in the model group compared with the control group (both $P<0.05$). Interestingly, after the HGF antibody treatment, the expression of MMP-9 mRNA was significantly increased to a higher level ($P<0.05$). The TIMP-1 mRNA, on the contrary, was decreased in the intervention group compared with the model group ($P<0.05$, Table 2, Fig. 2).

Table 1. Histological evaluation, wet-to-dry weight (W/D) ratio, and hydroxyproline (HYP) content of the control, model, and intervention groups ($n=10$)[§]

Group	Histological evaluation	W/D ratio	HYP content
Control	1.02±0.01	1.02±0.01	1.12±0.12
Model	9.67±0.34*	3.67±0.21*	7.92±0.31*
Intervention	5.23±0.11	2.32±0.13 [#]	3.21±0.20 [#]

§: Plus-minus values are means±SD.

* $P<0.05$ compared with the control group; [#] $P<0.05$ compared with the model group.

Table 2. mRNA levels of MMP-9 and TIMP-1 in lung tissue[§]

Group	MMP-9 mRNA	TIMP-1 mRNA
Control	0.26±0.13	0.19±0.30
Model	0.42±0.23*	5.90±0.33*
Intervention	2.41±0.11 [#]	3.42±0.32 [#]

§: Plus-minus values are means±SD.

* $P<0.05$ compared with the control group; [#] $P<0.05$ compared with the model group.

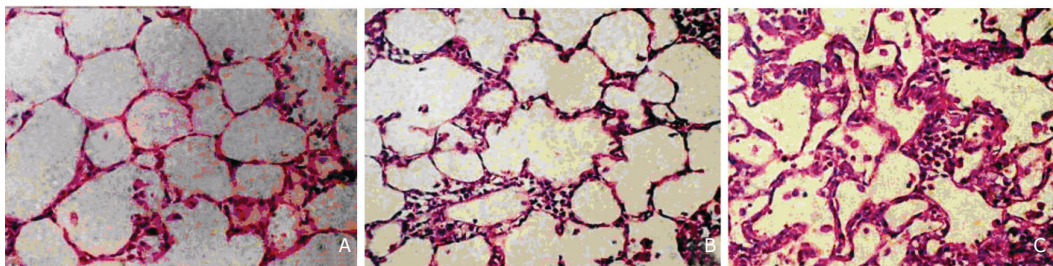


Figure 1. Pathology of lung tissue in the control group (A), model group (B), and intervention group (C). HE ×40

Expression levels of MMP-9 and TIMP-1 proteins

Immunohistochemistry revealed a very weak staining for MMP-9, TIMP-1 in the normal lung tissue. The protein level of MMP-9 was increased in the model group and increased more in the intervention group (both $P<0.05$, Table 3, Fig. 3). Consistent with the changes of TIMP-1 mRNA, the protein expression level of TIMP-1 was also obviously decreased in the intervention group when compared with the model group, but increased when compared with the control group (both $P<0.05$, Table 3, Fig. 4).

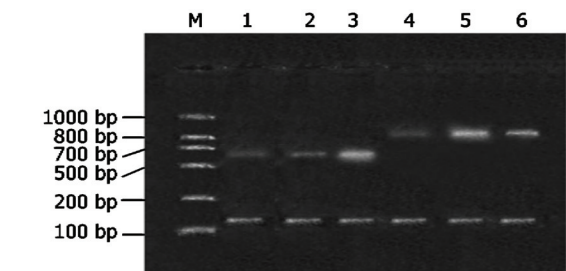


Figure 2. mRNA levels of MMP-9 and TIMP-1 in lung tissue. M: marker; 1: MMP-9 mRNA of the control group; 2: MMP-9 mRNA of the model group; 3: MMP-9 mRNA of the intervention group; 4: TIMP-1 mRNA of the control group; 5: TIMP-1 mRNA of the model group; 6: TIMP-1 mRNA of the intervention group.

Table 3. Protein levels of MMP-9 and TIMP-1 in lung tissue[§]

Group	MMP-9 protein	TIMP-1 protein
Control	32.26±4.13	12.39±5.30
Model	71.37±6.23*	93.90±7.33*
Intervention	89.01±9.12 [#]	58.92±4.32 [#]

§: Plus-minus values are means±SD.
* $P<0.05$ compared with the control group; [#] $P<0.05$ compared with the model group.

DISCUSSION

The acute lung injury (ALI) is a complex and volatile condition with severe respiratory dysfunction. The balance between fibrosis and antifibrosis is thought to play a key role in ALI. An increase in collagen deposition in the bronchial wall is also observed in lung injury, whereas histopathologic evidence of ECM degradation is one of the hallmarks of long course ALI.⁹ The alveolar macrophages distribute in the alveolar space, and make up of an important line of defense of the lung. A recent research confirms that alveolar macrophages excrete the enzyme that induces the degradation of the albumen, especially the MMPs, which directly decompose and reconstruct ECM during the inflammation and damage of lungs.¹⁰ MMP-9 mainly degrades collagen type IV, which constitutes membranes of alveolar epithelium and pulmonary capillary. The release of MMP-9 is under the influence of several inflammatory mediators, cytokines, and surface molecules. Macrophages also produce TIMPs, which bind to the active forms of MMPs. MMP-9 is essentially inhibited by TIMP-1 *via* this binding.¹¹ In this study, we used the endotoxin to establish ALI rat model, which is effective according to the pathological results, hydroxyproline content, and the W/D ratio of the lung tissue. Both mRNA and protein of MMP-9 and TIMP-1 were higher in the model group compared with the control group, proving that the expressions of MMP-9 and TIMP-1 are influenced by the inflammatory reaction *in vivo*. Little is known about the promoting elements involved in MMP-9 and TIMP-1 regulation by endotoxin, but we assume that some inflammatory factors participate in this course.

HGF is a pleiotropic factor mainly produced by mesenchymal cells and acts on epithelial cells, which express

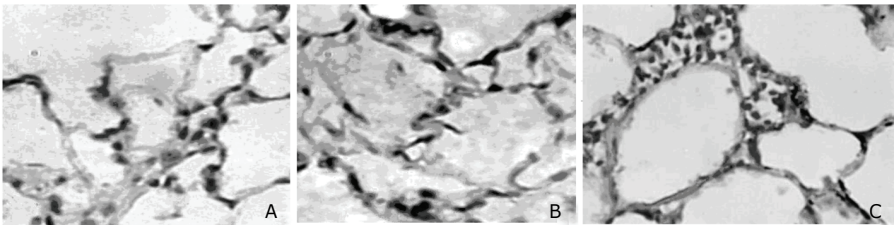


Figure 3. Expression of MMP-9 protein in lung tissue in the control group (A), the model group (B), and the intervention group (C). SP×200

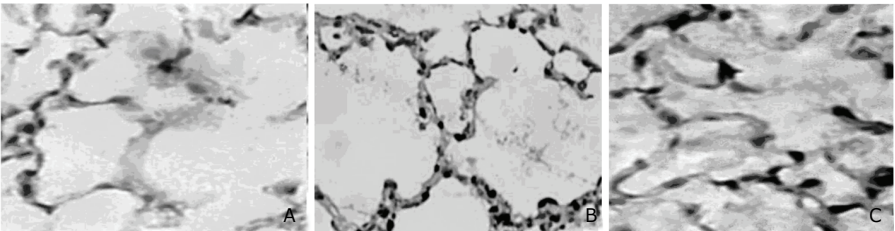


Figure 4. Expression of TIMP-1 protein in lung tissue in the control group (A), the model group (B), and the intervention group (C). SP×200

the HGF receptor c-Met. HGF has mitogenic, morphogenic, antiapoptotic, and fibrinolytic effects in injured tissue. Therefore, it has been widely considered as a potential protective agent for a variety of organ disorders, such as liver cirrhosis, myocardial ischemia, and renal fibrosis.¹² However, the molecular signaling pathway is still unknown. This study proved that the HGF antibody ameliorated the pathological changes of ALI induced by endotoxin. The mRNA and protein expression of MMP-9 were obviously increased in the intervention group injected with HGF antibody, while the expression of TIMP-1 declined. According to the previous researches, the balance between MMPs and TIMPs levels plays a very important role in the pathogenesis of ALI; in other words, the imbalance between MMP-9 and TIMP-1 would participate in airway remodeling leading to either short- or long-course ALI.^{13,14} The MMP-9/TIMP-1 ratio could be a predictive factor of the ALI evolution.¹⁵ The finding of this study suggests that HGF may promote the renovation of the lung injury by changing the MMP-9/TIMP-1 ratio.

REFERENCES

1. Ware LB, Matthay MA. Keratinocyte and hepatocyte growth factor in the lung: roles in lung development, inflammation, and repair. *Am J Physiol Lung Cell Mol Physiol* 2002; 282:L924-40.
2. Droppelmann CA, Gutiérrez J, Vial C, et al. Matrix metalloproteinase-2-deficient fibroblasts exhibit an alteration in the fibrotic response to connective tissue growth factor/CCN2 because of an increase in the levels of endogenous fibronectin. *J Biol Chem* 2009; 284:13551-61.
3. Carney DE, Lutz CJ, Picone AL, et al. Matrix metalloproteinase inhibitor prevents acute lung injury after cardiopulmonary bypass. *Circulation* 1999; 100:400-6.
4. Gibbs DF, Warner RL, Weiss SJ, et al. Characterization of matrix metalloproteinases produced by rat alveolar macrophages. *Am J Respir Cell Mol Biol* 1999; 20:1136-44.
5. Hagiwara S, Iwasaka H, Matsumoto S, et al. Antisense oligonucleotide inhibition of heat shock protein (HSP) 47 improves bleomycin-induced pulmonary fibrosis in rats. *Respir Res* 2007; 8:37.
6. Lianxu C, Hongti J, Changlong Y. NF- κ Bp65-specific siRNA inhibits expression of genes of COX-2, NOS-2 and MMP-9 in rat IL-1 β -induced and TNF- α -induced chondrocytes. *Osteoarthritis Cartilage* 2006; 14:367-76.
7. Morariu AM, Maathuis MH, Asgeirsdottir SA, et al. Acute isovolemic hemodilution triggers proinflammatory and procoagulatory endothelial microcirculation activation in vital organs: role of erythrocyte aggregation. *Microcirculation* 2006; 13:397-409.
8. Mrozek JD, Smith KM, Bing DR, et al. Exogenous surfactant and partial liquid ventilation: physiologic and pathologic effects. *Am J Respir Crit Care Med* 1997; 156:1058-65.
9. Coimbra R, Melbostad H, Loomis W, et al. LPS-induced acute lung injury is attenuated by phosphodiesterase inhibition: effects on proinflammatory mediators, metalloproteinases, NF- κ B, and ICAM-1 expression. *J Trauma* 2006; 60:115-25.
10. Stuelten CH, DaCosta Byfield S, Arany PR, et al. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF- α and TGF- β . *J Cell Sci* 2005; 118:2143-53.
11. Melchior B, Frangos JA. Shear-induced endothelial cell-cell junction inclination. *Am J Physiol Cell Physiol* 2010; 299:C621-9.
12. Verghese GM, McCormick-Shannon K, Mason RJ, et al. Hepatocyte growth factor and keratinocyte growth factor in the pulmonary edema fluid of patients with acute lung injury. biologic and clinical significance. *Am J Respir Crit Care Med* 1998; 158:386-94.
13. Russell RE, Culpitt SV, DeMatos C, et al. Release and activity of MMP-9 and TIMP-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2002; 26:602-9.
14. Mercer PF, Shute JK, Bhowmik A, et al. MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res* 2005; 6:151.
15. Michele YF, Amit G, Yao L, et al. Matrix metalloproteinase activity in pediatric acute lung injury. *Int J Med Sci* 2009; 6:9-17.