Research Communication

The effect of HMGB1 A box on lung injury in mice with acute pancreatitis

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Abstract.
The objective of this study is to observe the effect of high-mobility group protein B1 A Box (HMGB1 A) box on lung injury in mice with acute pancreatitis and its effect on the level of high-mobility group protein B1 (HMGB1) in lung, to explore the mechanism. A total of 60 male Institute of Cancer Research mice were randomly divided into control group (n = 30) and treatment group (n = 30). Severe acute pancreatitis mice model was induced by 20% L-Arg intraperitoneal injection. The recombination HMGB1 A box was used in treatment after modeling. All the mice were killed under anesthesia at 24 and 48 h after the modeling injection. The level of HMGB1 and activity of myeloperoxidase (MPO) in lung were measured. The pathological changes of lung were observed. The level of HMGB1 in lung of A box treatment group decreased more significantly 24 h and 48 h after modeling compared with control group. The activity of MPO in lung of A box treatment group decreased more significantly 24 h after modeling compared with control group. The lung tissue pathologic score of A box treatment group decreased more significantly 48 h after modeling compared with control group. HMGB1 expression levels in the lungs were positively related to histological score of injured lung in acute pancreatitis. It indicates that HMGB1 A box is remarkably protective to lung injury induced by acute pancreatitis.

Keywords: acute pancreatitis, injury of lungs, high mobility group box protein 1 A box, mediators of inflammation

1. Introduction

Acute pancreatitis is an acute necrotic and inflammatory patho-genetic condition that suddenly occurs in peripheral and internal areas of pancreas. In recent years, its incidence is on the rise. Acute pancreatitis associated lung injury (APALI) is a major factor contributed to the high mortality rate of severe acute pancreatitis (SAP), which mostly due to difficult-to-treat complications, such as acute respiratory distress syndrome (ARDS), which involves disorders of inflammation/anti-inflammation and the increased production of chemokines [1,2]. Despite many attempts, the mechanisms of APALI have not yet fully clear, and there is no breakthrough in treatment. Studies have shown that high-motility group box protein1 (HMGB1), a DNA-binding intranuclear protein, is involved in systemic inflammatory response of SAP as a downstream cytokine of early inflammatory factors TNF-α, IL-1β when released into the extracellular space [3]. HMGB1 contains two structure domains, A box and B box, which are functional structure fields for combining DNA: B box is a major domain for proinflammatory response [4], whereas A box can inhibit HMGB1 function of proinflammatory response competitively [5]. Although researches have shown that antioxidant inhibits HMGB1 expression and reduces pancreas injury in rats with SAP [6], moreover, HMGB1 A box has a protective effect on the lipopolysaccharide-induced acute lung injury in mice [7], the effect of HMGB1 A box on lung injury in mice with acute pancreatitis is not directly stated. The aim of this study is to investigate the impact of A box administration on levels of HMGB1 in lung tissue and lung histopathology changes in mice with APALI.

2. Materials and methods

2.2. Animal model and grouping

All animal care and this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the
US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and was approved by the Ethical Review Board of Shandong University. Male Institute of Cancer Research (ICR) mice, each weighing 20–25 g (Shandong University Experimental Animal Center) were used in this study. A total of 60 male ICR mice were randomly divided into control group (n = 30) and treatment group (n = 30). The groups were then randomly allocated to 24- and 48-h groups, with 15 mice in each group. All the 60 mice were injected a solution of 20% L-arginine (200 mg/100 g body weight) [8] intraperitoneally twice, and the interval time was 1 h. The treatment group was administrated intraperitoneally with recombinant HMGB1 A box protein at a dose of 600 μg in 12 and 24 h after the modeling injection, and the other group was given an abdominal cavity injection of Phosphate Buffered Saline (PBS) with equivalent volume. All the male ICR mice were killed under anesthesia at 24 and 48 h after the modeling injection. Tissues from the right inferior pulmonary lobe were accessed, one part was frozen in liquid nitrogen, and stored in a refrigerator at -80°C, whereas the other part was fixed by a solution of 10% formaldehyde and embedded in paraffin wax for cutting sections. The dose and time interval of L-arginine and recombinant A box administration was discussed in our previous experiment [9].

2.3. Preparation for recombinant HMGB1 A box protein

Recombinant HMGB1 A box was prepared as described by Zhang et al. [10]. First, recombinant prokaryon expression vector pQE-80L/DHFR/HMGB1 A box (gift from Prof. He Fengtian, Third Military Medical University, Chongqing, China) was transformed into competence coli bacillus M15 (Dingguo Biotech, Beijing, China) and incubated in Luria-Bertani culture media, which contained a solution of 100 μg/mL of aminobenzylpenicillin for 5–7 h at 37°C with vigorous shaking until OD600 between 0.6 and 0.8. Isopropylthio-β-D-galactoside (1 mmol/L) was administrated to induce the expression of target protein and further incubated for 3–4 h at 37°C with vigorous shaking. Second, bacteria were collected by centrifuging at 4,000 g, 4°C for 15 min and subjected to lysozyme treatment (1 mg/mL) for 30 min and subsequent sonication on ice to get supernatant fluid with 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Then 50% Ni-nitrilotriacetic acid slurry (Invitrogen, Carlsbad, CA) 1 mL and phenyl methyl sulfonyl fluoride were added into 4 mL of the supernatant fluid to achieve the final concentration of 1 mmol/L. Finally, this mixture which was intensively mixed at 200 round per min in the centrifuge at 4°C for 1 h was purified using the His-tagged affinity columns (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. Protein elute was dialyzed extensively against PBS before passing over polyminyxin B columns (Pierce, Rockford, IL) to prevent the possible effect of contaminating LPS. The purity and integrity of proteins was verified by Coomassie blue staining after SDS-PAGE.

2.4. Western blotting analysis

Lung tissue samples were homogenized, and protein was extracted by cell lysis buffer for western according to the manufacture’s instructions (Beyotime Institute of Biotechnology, Shanghai, China). After determining protein concentration, 200 μg of lung homogenate protein were resolved on a 10% Tris HCl-SDS-polyacrylamide gel. Protein was electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After being blocked, the membrane was incubated overnight at 4°C with a specific polyclonal rabbit primary antibody to HMGB1 (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA) followed, respectively, by secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:5000 for 1 h at room temperature. Bands were subsequently detected using Beyo ECL Plus detection reagents (Beyotime Institute of Biotechnology, Shanghai, China). The membrane was then exposed further to film; the band concentration was calculated by the quantification of the optical density of the appropriate band.

2.5. Measurements of MPO activity

Tissue myeloperoxidase (MPO) activity was measured to assay the sequestration of neutrophils in lung tissue samples [11,12]. Briefly, the frozen tissue samples were thawed, homogenized in 0.2-mmol/L potassium phosphate buffer (PPB, pH 7.4) with 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO). Homogenate was sonicated for 10 s, freeze-thawed three cycles, and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was evaluated for MPO activity with Enzyme-linked immunosorbent assay (ELISA) kits of MPO (R&D Systems, Minneapolis, MN) referring to the manufacturer’s instructions.

2.6. Histological score of injured lung

Lung tissue embedded in paraffin wax was cut into sections (4-μm thick), then were stained with H&E and examined under light microscope. Scores were evaluated based on the lung injury level judged by pulmonary edema, leukocyte infiltration, the area percentage of pulmonary parenchyma tissue, and empty alveolus according to the method of Hofbauer [13].

2.7. Statistical analyses

Data were expressed as mean ± standard deviation (SD). Student’s t-test was adopted for comparison among groups of measurement data; P < 0.05 was considered statistically significant. The correlations of HMGB1 levels in the lungs with histological score of injured lung were assessed with linear regression analysis.

3. Results

3.1. Effect of recombinant A box on HMGB expression level of lung tissue

Western blot analysis revealed that compared with the control group, recombinant HMGB1 A Box markedly weakened the signal of HMGB1 protein expression. Densitometric scan results showed that treatment group at 24-h time-point and 48-h time-point were respectively lower than the control.
group in the density of HMGB1 protein expression, and there was statistical difference between the above two groups \((P < 0.05)\) (Fig. 1).

3.2. MPO activity in lung tissue
Treatment of the animals with Recombinant HMGB1 A Box significantly reduced the MPO activity in lung at 24-h time-point \((P < 0.01)\). MPO activity in lung decreased in both control group and treatment group, and the treatment effect of A Box is not significant at 48-h time-point (Fig. 2).

3.3. Histological changes in lung tissues
Lung tissues of mice in control group swelled with exudates in the thorax at 24-h time-point after modeling. Examined under a microscope, the phenomena such as widespread alveolar wall thickness due to edema, obvious inflammatory cells infiltration, and hemorrhage in the alveolus, part of which collapsed were observed. Forty-eight hours after modeling, pathological changes in lung tissues of mice were aggravated, and hemorrhage was observed in alveolus (Fig. 3A). The histological changes in treatment group were obvious compared with those in control group (Fig. 3B), edema, infiltration of inflammatory cells and hemorrhage were attenuated greatly. The histopathological scores of injured lung were shown in Table 1. The histopathological score of injured lung indicated that there was no significant difference between treatment group and control group 24 h after modeling \((P > 0.05)\), whereas 48 h after modeling, there was significant difference between the two groups in the above: the score of injured lung in treatment group was lower than the score of control group \((P < 0.05)\).

3.4. Correlation analysis
Linear regression analysis showed that HMGB1 expression levels in the lungs was positively related to histological score of injured lung in acute pancreatitis \((r = 0.721, P < 0.01)\).

4. Discussions
SAP features serious pathogenetic condition with high mortality due to multiple organ dysfunction syndrome in lung, kidney, liver, and other organs at the early stage. Currently, clinical study found that 30–50% of SAP patients suffer from acute lung injury, its pathogenesis is still unclear, which leads to the lack of proper treatment in acute pancreatitis and its associated lung injury therapeutic strategy. Although studies have shown that proinflammatory cytokines plays a key role in the mechanism of SAP, acute lung injury and associated systemic inflammatory response syndrome and ARDS [14], the treatments by inhibiting “early” cytokines such as TNF-\( \alpha \), IL-1\( \beta \) have failed because the level of these cytokines quickly decreased toward normal level after increased to a peak, however, the injury still continued.

High-mobility group box protein-1 (HMGB1) is an intranuclear protein which is important in the regulation of genetic information [15]. Compared with other known inflammatory factors TNF-\( \alpha \), IL-1\( \beta \), recently, HMGB1 has been discovered to play a pivotal role as a late-phase mediator in the pathogenesis of proinflammatory disease such as sepsis, acute lung injury [5,16,17]. HMGB1 can be released from necrotic cells, or secreted by activated monocytes or macrophages to mediate cell-to-cell signaling by binding to the
receptor for advanced glycosylation end (RAGE) product, toll-like receptor 2 (TLR2), and Toll-like receptor 4 (TLR4) [18–20], then enhance the inflammatory response. HMGB1 inhibitors treatment has indicated beneficial effects in experimental model of sepsis, hepatic ischemia/reperfusion injury, SAP [5,21]. HMGB1 A box is to be part of HMGB1 as a domain, can replace 125I-labeled HMGB1 and bind to cultured macrophages significantly and A box dose-dependently inhibited HMGB1-induced TNF-α, IL-1β release. Its mechanism may be related to competitive inhibition. Animal models of sepsis 24 h after perforation of the colon and repeated to give HMGB1 A box proteins, can significantly improve survival [5]. So purified recombinant A box may be a most ideal HMGB1 antagonist of HMGB1 to be used in the clinical settings of inflammatory diseases.

Pathological examination has confirmed that HMGB1 given intratracheally can lead to acute inflammation injury in lung [17]. And studies [7] have shown that HMGB1 antagonist can reduce the lipopolysaccharide-induced acute lung injury, meanwhile in our experiments, the results show that HMGB1 stayed at elevated plateau levels for a relatively long time. Moreover, lung tissue damage became worse as HMGB1 expression levels increased and maintained(\(r = 0.721, P < 0.01\)), suggesting that HMGB1 also acted as a late cytokine mediator in the pathogenesis of acute lung injury with SAP.

In our study, for the first time, we have shown that the effect of recombinant A box on acute lung injury induced by SAP in mice. Data presented here demonstrated that A box exerted anti-inflammatory effects in the lungs of the APALI mice. It significantly reduced the expression level of HMGB1 at 24-h time-point and 48-h time-point, which may decreased HMGB1 binding with downstream receptors such as RAGE, TLR2, and TLR4, thereby reducing the inflammatory response.

SAP is associated with a rise in both pancreatic and lung MPO activity, indicating the presence of sequestered neutrophils [22]. MPO activity of the lung tissue in treatment group at 24-h time-point was also significantly reduced, which indicates that the activation and function of inflammatory cells were inhibited. The histopathologic findings also indicated that edema, inflammatory cell infiltration, and bleeding were less severe in A box treatment mice than in control group.

The mechanism above may be that, as reported in studies, activated monocytes and macrophages can secrete the nuclear HMGB1 into the extracellular initiatively [23], meanwhile, HMGB1 stimulates macrophage secretion of TNF-α, which, in turn, enhances the HMGB1 expression by macrophages, amplifying the inflammatory response [24]. The function of HMGB1 including the release of inflammatory factors was inhibited when A box administration. These perhaps are one explanation why the decreased activation of inflammatory cells and inhibition of HMGB1 positive feedback may reduce the expression level of HMGB1.

**Table 1**

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Control group ((n = 30))</th>
<th>Treatment group ((n = 30))</th>
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<tbody>
<tr>
<td>24</td>
<td>2.12 ± 0.39</td>
<td>1.82 ± 0.26</td>
</tr>
<tr>
<td>48</td>
<td>2.55 ± 0.40</td>
<td>1.87 ± 0.34</td>
</tr>
</tbody>
</table>

Result are expressed as mean ± SD.

*\(P < 0.05\) compared with control group.*
In addition, from the histopathologic results, we can find that recombinant A box did not completely improve the development of lung injury. Perhaps, it is because the network of cytokines is complex, such as TNF-α, IL-1β, and other proinflammatory mediators involve in the inflammatory response in ALI and the ARDS [25] and whereas HMGB1 participates in it, other mediators are also involved, which recombinant A box cannot or do not fully able to inhibit.

In conclusion, HMGB1 as a late cytokine mediator seems to play an important role in the pathogenesis of acute lung injury in mice with acute pancreatitis and A box is a specific blockade for endogenous HMGB1 to suppress the inflammatory response, thereby having a protective effect on lung injury in SAP mice. These findings suggest that HMGB1 A box might be a potential treatment for APALI. However, the molecular mechanism by which A box modulates HMGB1 expression remains to be further elucidated.

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**References**


