Original Article

Expression of high mobility group protein B1 in the lungs of rats with sepsis

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BACKGROUND: *Vibrio vulnificus* inside the body could activate the NF- κ B signaling pathway and initiate the inflammatory cascade. The lung is one of the earliest organs affected by sepsis associated with acute lung injury. High mobility group protein B1 (HMGB1) is an important late-acting pro-inflammatory cytokine involving in the pathophysiology of sepsis. It is also involved in the injury process in the lung, liver and intestine. There has been no report on the involvement of HMGB1 in *Vibrio vulnificus* sepsis-induced lung injury.

METHODS: Sixty rats were randomly divided into a normal control group (group A, $n=10$) and a *Vibrio vulnificus* sepsis group (group B, $n=50$). Sepsis was induced in the rats by subcutaneous injection of *Vibrio vulnificus* (concentration 6×10^8 cfu/mL, volume 0.1 mL/100g) into the left lower limbs. The rats in group B were sacrificed separately 1, 6, 12, 24, and 48 hours after the infection. Their lungs were stored as specimens, lung water content was measured, and lung pathology was observed under a light microscope. The expressions of the HMGB1 gene and protein in the lungs were detected by RT-PCR and Western blot. Data were analyzed with one-way analysis of variance (ANOVA) and the LSD method for pair-wise comparison between the two groups. $P < 0.05$ was considered statistically significant.

RESULTS: Compared to group A (0.652 ± 0.177), HMGB1 mRNA expression in the lungs of group B was significantly higher at 0 hour (1.161 ± 0.358 , $P=0.013$), 24 hours (1.679 ± 0.235 , $P=0.000$), and 48 hours (1.258 ± 0.274 , $P=0.004$) ($P < 0.05$), and peaked at 24 hours. Compared to group A (0.594 ± 0.190), HMGB1 protein expression at 6 hours (1.408 ± 0.567 , $P=0.026$) after infection was significantly increased ($P < 0.05$), and peaked at 24 hours (2.415 ± 1.064 , $P=0.000$) after infection. Compared to group A (0.699 ± 0.054), lung water content was significantly increased at 6 hours (0.759 ± 0.030 , $P=0.001$), 12 hours (0.767 ± 0.023 , $P=0.000$), 24 hours (0.771 ± 0.043 , $P=0.000$) and 48 hours (0.789 ± 0.137 , $P=0.000$) after infection ($P < 0.05$). Compared to group A, pathological changes at 12 hours in group B indicate marked pulmonary vascular congestion, interstitial edema and inflammatory infiltration. Alveolar cavity collapse and boundaries of the alveolar septum could not be clearly identified.

CONCLUSION: *Vibrio vulnificus* sepsis can lead to injury in rat lungs, and increased HMGB1 expression in lung tissue may be one of the mechanisms for injury from *Vibrio vulnificus* sepsis.

KEY WORDS: *Vibrio vulnificus*; Sepsis; Lung injury; High mobility group protein B1; Reverse transcription polymerase chain reaction; Western blot; Lung water content; Histopathology

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INTRODUCTION

Vibrio vulnificus sepsis is acute in onset and progresses rapidly. Multiple organ dysfunction syndrome (MODS) usually occurs within 24-48 hours with a high mortality.^[1,2] *Vibrio vulnificus* inside the body could activate the NF- κ B signaling pathway and initiate the inflammatory cascade.^[3] The lung is one of the earliest organs affected by sepsis characterized by acute lung injury (ALI), which develops into acute respiratory distress syndrome (ARDS) and eventually leads to MODS and death.^[4] HMGB1 is an important late-acting pro-inflammatory cytokine involved in the pathophysiology of sepsis. The role of HMGB1 in tissue and organ injury has been reported that it is involved in the tissue and organ injury process in the lung, liver and intestine.^[5,6] In this study we established a *Vibrio vulnificus* sepsis model in rats and observed HMGB1 expression, lung water content and dynamic histopathological changes in the lung tissue. We also investigated the dynamic expression of HMGB1 in the lung tissue of rats with *Vibrio vulnificus*-induced sepsis so as to understand the pathogenesis of *Vibrio vulnificus* sepsis.

METHODS

Animals, bacteria and reagents

Sixty Sprague-Dawley rats, 30 males and 30 females, weighing 180-260 g, were provided by the Life Sciences Laboratory of Wenzhou Medical College, Zhejiang, China.

Fluid was extracted from hemorrhagic blisters of the lower limbs of patients with *Vibrio vulnificus* infection. It was inoculated into blood agar plates to culture and purify *Vibrio vulnificus* at 37 °C for 24 hours. Bacteria were identified as *Vibrio vulnificus* and the strain number was 705258. *Vibrio vulnificus* suspension was produced by the conventional method at a concentration of 6×10^8 cfu/mL for use.

Trizol was purchased from Invitrogen in the USA. The reverse transcriptase kit was purchased from Takara Biotechnology (Dalian) Co. Ltd, China. The PCR kit was purchased from Fermentas in the USA. RIPA lysis buffer (strong), the BCA protein concentration assay kit, color prestained protein molecular weight markers, horseradish peroxidase labeled goat anti-mouse IgG (H+L), horseradish peroxidase labeled goat anti-rabbit IgG (H+L), ECL reagents and the developer fuser kit were purchased from the Beyotime Institute of Biotechnology, China. Rabbit anti-rat HMGB1 antibodies and mouse anti-rat HMGB1 antibodies were purchased from Abcam in the USA.

Groups

The rats were fed for 1 week, then fasted for 12 hours

before the experiment, but they were allowed free access to water. They were randomly divided into control group and model group. The rats of the control group (group A, $n=10$) were sacrificed after anesthesia. The rats of the *Vibrio vulnificus* sepsis model group (group B, $n=50$) were injected subcutaneously with *Vibrio vulnificus* suspension in the left lower limbs. The concentration of the suspension was 6×10^8 cfu/mL and the dosage was 0.1 mL/100g. The rat *Vibrio vulnificus* sepsis model was described previously.^[7,8] The rats in group B were sacrificed after anesthesia at 1, 6, 12, 24 and 48 hours after infection. Ten rats were used at each time point. Parts of the lung tissues were collected sterily, rinsed with normal saline at 4 °C, and then stored immediately in liquid nitrogen for later use.

Measurement of lung tissues water content

About 60 mg of lung tissue was taken for determination of wet weight. The tissue specimens were transferred to the oven to dry at 60 °C for 48 hours to a constant weight and dry weight. Then dry weight was measured according to the formula: lung water content = (wet weight of the lung - dry weight of the lung)/ wet weight of the lung.

Pathological manifestations of lung tissue

A light microscope was used to observe the pathological changes of lung tissue in group A. These changes were used as a reference to compare the changes in group B at each time point.

Determination of HMGB1 mRNA

According to the instructions of the RT-PCR kit, the reaction conditions were as follows: denaturation at 94 °C for 30 seconds, annealing at 59 °C for 30 seconds, and extension at 72 °C for 60 seconds. The first cycle pre-denaturation at 94 °C for 5 minutes was followed by 27 cycles with a final extension at 72 °C for 10 minutes (primer sequences and PCR conditions, Table 1). After electrophoresis, images of PCR products were generated by a gel imaging system and were analyzed by Gelpro32

Table 1. RT-PCR primer sequences and conditions

Gene	Primer sequences	Tm	PCR products (°C) (bp)
β-actin	Forward CATTGAACACGGCATTG	48	590
	Reverse GAAGGAAGGCTGGAAGAG		
HMGB1	Forward TGATTAATGAATGAGTTCGGGC	59	214
	Reverse TGCTCAGGAACTTGACTGTTT		

Tm: temperature

analysis software. They were compared with the relative values of gene expression using target gene/ β -actin.

HMGB1 levels

Protein was extracted according to the instructions on the RIPA lysis buffer. Ten μ g of protein was loaded into each well. After polyacrylamide gel electrophoresis in an ice-water bath, the membrane was transferred at 320 mA in an ice-water bath for 35 minutes and sealed at room temperature for 90 minutes. Rabbit anti-HMGB1 antibody (1:1 000) and mouse anti- β -actin antibody (1:10 000) were incubated at 4 °C overnight. Horseradish peroxidase labeled goat anti-rabbit antibody (1: 500) and horseradish peroxidase labeled goat anti-mouse antibody (1:1 000) were incubated at room temperature for 2 hours. The film was developed and images were generated after exposure. They were analyzed by Quantity One analysis software and then compared with the relative values of protein expression using target protein/ β -actin.

Statistical analysis

Experimental data were expressed as mean $\pm$ standard deviation. SPSS16.0 (SPSS Chicago, IL USA) was used for statistical analysis. One-way analysis of variance and LSD method were used for a pair-wise comparison between the two groups and $P < 0.05$ was considered statistically significant.

RESULTS

HMGB1 mRNA expression

HMGB1 mRNA expression in the lung tissue of rats in group A was (0.652 ± 0.177). After *Vibrio vulnificus* infection in group B compared with group A, HMGB1 mRNA expression in the lung tissue was significantly higher at 12 hours (1.161 ± 0.358 , $P = 0.013$), 24 hours (1.679 ± 0.235 , $P = 0.000$) and 48 hours (1.258 ± 0.274 , $P = 0.004$) ($P < 0.05$) (Figure 1).

Figure 1. HMGB1 mRNA expression in the lung tissue of rats with *Vibrio vulnificus* sepsis shown by RT-PCR.

Figure 2. HMGB1 protein expression in the lung tissue of rats with *Vibrio vulnificus* sepsis shown by Western blot.

Figure 3. Lung tissue of rats in groups A and B (HE, magnification $\times 200$); A: group A; B: group B at 1 hour; C: group B at 6 hours; D: group B at 12 hours; E: group B at 24 hours; F: group B at 48 hours.

HMGB1 protein expression

The lung tissue of rats in group A showed a lower level of HMGB1 (0.594 ± 0.190). Compared with group A, HMGB1 expression in the lung tissue 6 hours after *Vibrio vulnificus* infection increased significantly in group B (1.408 ± 0.567 , $P=0.026$) ($P<0.005$). HMGB1 expression peaked at 24 hours in the lung tissue (2.415 ± 1.064 , $P=0.005$) (Figure 2).

Lung water content

The lung water content of rats in group A was lower (0.699 ± 0.054). It was significantly increased at 6 hours (0.759 ± 0.030 , $P=0.001$), 12 hours (0.767 ± 0.023 , $P=0.000$), 24 hours (0.771 ± 0.043 , $P=0.000$) and 48 hours (0.789 ± 0.137 , $P=0.000$) after *Vibrio vulnificus* infection in group B compared with group A ($P<0.05$).

Pathological changes in lung tissue

Microscopic study of group A showed marked alveoli, thin alveolar septum and no congestion or edema (Figure 3). Pathological changes in the lung tissues of group B 1 hour and 6 hours after *Vibrio vulnificus* infection were not significant as compared with group A (Figure 3). However, 12, 24 and 48 hours after the infection, group B showed significant pulmonary vascular congestion, local bleeding, and pulmonary interstitial edema associated with inflammatory infiltration. The boundaries of the alveolar septum could not be clearly identified, and significant alveolar collapse and more severe edema were observed (Figure 3).

DISCUSSION

Studies have shown that *Vibrio vulnificus* is a halophilic gram-negative bacillus. Sepsis induced by this bacterium is severe with rapid progression and a high mortality. But the specific pathogenesis of *Vibrio vulnificus* sepsis is not very clear.^[9,10]

Sepsis is a systemic inflammatory response syndrome caused by infection. It causes the release of a large number of inflammatory mediators and other injury factors. It is a common cause of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS).^[11,12] ALI and ARDS are the major causes of death caused by sepsis induced by gram-negative bacterial infections. A large number of pro-inflammatory cytokines can lead to pulmonary vascular endothelial injury and increased pulmonary vascular permeability, which are important causes of lung injury.^[13] The results of this experiment showed that the water content of the lung

tissue of rats with *Vibrio vulnificus* sepsis increased gradually after *Vibrio vulnificus* infection. Similarly, the histopathological changes of the lung increased progressively, but they were not significant during the early phase. In the mid to late phase, pulmonary vascular congestion, local bleeding, severe pulmonary edema, and alveolar collapse could be clearly observed. Lung tissue water content and pathological changes indicated that lung injury was more severe as the disease progressed, particularly in the mid and late phases.

According to other reports, HMGB1 as a DNA binding protein is found extensively in eukaryotic cells. Its functions include stable nucleic acid structure, transcriptional regulation and gene expression. HMGB1 can be secreted into the extracellular space and acts as an important late-acting pro-inflammatory cytokine involving in the pathogenesis of sepsis. LPS, TNF- α and IL-1 can induce the production of HMGB1 directly or indirectly. HMGB1 can further stimulate monocytes to secrete other pro-inflammatory cytokines TNF- α , IL-1 and IL-6. The inflammatory cascade continues and eventually leads to an uncontrolled inflammatory response and the occurrence of MODS. HMGB1 expression in the lung tissue of rats with CLP sepsis increased significantly.^[14] In addition, HMGB1 given within the trachea caused acute lung injury with manifestations of pulmonary edema, neutrophil accumulation and an increase in early-acting pro-inflammatory cytokines TNF- α and IL-1 in the lung tissue.^[15] HMGB1 is closely related to the occurrence of sepsis-induced acute lung injury. The results of this experiment showed that HMGB1 mRNA expression in the lung tissue of rats with *Vibrio vulnificus* sepsis peaked in the mid and late phases and maintained a relatively high level in the late phase. It also confirmed that HMGB1 acted as a late-acting pro-inflammatory cytokine involving in the pathophysiology of sepsis.^[16] HMGB1 expression and injury to the lung tissues of rats with *Vibrio vulnificus* sepsis showed significant pathological changes in the mid and late phases, suggesting a relationship between HMGB1 expression and lung tissue injury.

In conclusion, *Vibrio vulnificus* sepsis can cause lung injury in rats. There is a relationship between HMGB1 expression and lung injury. The increase in HMGB1 expression could be one of the mechanisms for lung injury in rats with *Vibrio vulnificus* sepsis.

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