BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH

High mobility group box 1 as a mediator of endotoxin administration after hemorrhagic shock-primed lung injury

F. Guo, Y. Shi, H. Xu and J. Ding

High mobility group box 1 as a mediator of endotoxin administration after hemorrhagic shock-primed lung injury

F. Guo[1,2], Y. Shi[1], H. Xu[1] and J. Ding[1]

[1]Department of Respiratory Diseases, School of Medicine, Nanjing University,
Nanjing General Hospital of Nanjing Military Command, Nanjing, Jiagsu, China
[2]Department of Critical Care Medicine, Zhong-Da Hospital and School
of Clinical Medicine, South-east University, Nanjing, Jiagsu, China

Abstract

High mobility group box 1 (HMGB1) was discovered as a novel late-acting cytokine that contributes to acute lung injury (ALI). However, the contribution of HMGB1 to two-hit-induced ALI has not been investigated. To examine the participation of HMGB1 in the pathogenesis of ALI caused by the two-hit hypothesis, endotoxin was injected intratracheally in a hemorrhagic shock-primed ALI mouse model. Concentrations of HMGB1 in the lung of the shock group were markedly increased at 16 h (1.63 ± 0.05, compared to the control group: 1.02 ± 0.03; $P < 0.05$), with the highest concentration being observed at 24 h. In the sham/lipopolysaccharide group, lung HMGB1 concentrations were found to be markedly increased at 24 h (1.98 ± 0.08, compared to the control group: 1.07 ± 0.03; $P < 0.05$). Administration of lipopolysaccharide to the hemorrhagic shock group resulted in a notable HMGB1 increase by 4 h, with a further increase by 16 h. Intratracheal lipopolysaccharide injection after hemorrhagic shock resulted in the highest lung leak at 16 h (2.68 ± 0.08, compared to the control group: 1.05 ± 0.04; $P < 0.05$). Compared to the hemorrhagic shock/lipopolysaccharide mice, blockade of HMGB1 at the same time as lipopolysaccharide injection prevented significantly pulmonary tumor necrosis factor-alpha, interleukin-1beta and myeloperoxidase. Lung leak was also markedly reduced at 16 h; blockade of HMGB1 24 h after lipopolysaccharide injection failed to alter lung leak or myeloperoxidase at 48 h. Our observations suggest that HMGB1 plays a key role as a late mediator when lipopolysaccharide is injected after hemorrhagic shock-primed ALI and the kinetics of its release differs from that of one-hit ALI. The therapeutic window to suppress HMGB1 activity should not be delayed to 24 h after the disease onset.

Key words: Acute lung injury; High mobility group box 1; Two-hit-induced ALI; Hemorrhagic shock; Endotoxin

Introduction

Acute lung injury (ALI) is associated significantly with major insults such as multiple trauma, blood loss, sepsis, and aspiration of gastric content (1). Studies have demonstrated that ALI is characterized by an intense inflammatory response in the lung. The outcome of this process in the lung is migration and accumulation of activated leukocytes in the pulmonary capillaries, and diffuse alveolar-capillary membrane damage. Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) have been implicated in the mediation of the ini-

tiation and maintenance of ALI during the inflammatory process (2-4).

High mobility group box 1 (HMGB1), which was originally discovered as a nuclear DNA-binding protein about 30 years ago (5), also is a novel late-acting cytokine (6). Serum HMGB1 concentrations increased in mice within 8 to 32 h after the administration of lipopolysaccharide (LPS) and blockade of HMGB1 decreased the severity of LPS-induced lung injury and improved the survival of rats with sepsis (6-8). In endotoxin-induced murine sepsis, HMGB1

given intratracheally produced acute inflammatory injury to the lung, with neutrophil accumulation, the development of lung edema, and increased pulmonary production of TNF-α, IL-1β and macrophage inflammatory protein-2. Specific inhibition of HMGB1 activity with anti-HMGB1 antibodies decreased the migration of neutrophils to the lung as well as lung edema, and increased survival even when administered 24 h after endotoxemia (7,9). These observations suggest that HMGB1 is a key mediator of cell injury. Elevated concentrations of HMGB1 have been observed in the serum of septic patients and in patients presenting hemorrhagic shock, being highest in nonsurvivors and indicating that HMGB1 may play a role in reducing tissue perfusion and that its inhibition may play a key role in improving clinical outcomes (10,11).

Studies have suggested that the global ischemia-reperfusion related to resuscitation from hemorrhagic shock promotes the development of lung injury by priming the innate immunity for an exaggerated inflammatory response to a second, often trivial, inflammatory stimulus, the so-called two-hit hypothesis (12). Two-hit has a significant role in the development of ALI. However, the contribution of HMGB1 to two-hit-induced ALI has not been investigated. We conducted a study to examine the participation of HMGB1 in the pathogenesis of ALI caused by two-hit. To test the hypothesis that HMGB1 plays a key role as a late mediator and that the kinetics of its release is different from one-hit ALI we first measured the changes in its concentration at pre-established times. We then inhibited HMGB1 activity specifically at different times in order to determine the appropriate time window for treatment.

Material and Methods

Animals

Male BALB/c mice, 10-12 weeks of age, were purchased from the Compare Medical Center of Yangzhou University. The mice were maintained on a 12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Nanjing University.

Model for sepsis after hemorrhagic shock-primed lung injury

The model of mouse endotoxin injection after hemorrhagic shock-primed lung injury has been described previously (13,14). Mice were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine administered intraperitoneally. Cardiac puncture was used to remove 30% of the calculated total blood volume (0.27 mL/10 g body weight) over 60 s into a 1-mL heparinized syringe to prevent clotting. One hour after the induction of hemorrhage, mice were again anesthetized and resuscitated by transfusing the previously removed blood and Ringer's solution in a volume equal to that of the blood removed into a tail vein over a period of 30 min. Sham animals underwent the same procedure involving cardiac puncture under anesthesia without blood removal, followed by a second episode of anesthesia and tail vein puncture without resuscitation 1 h later. Animals then underwent tracheotomy using a 20-gauge catheter 1 h after the end of resuscitation (or sham procedure) and received LPS (*Escherichia coli* O111:B4; Sigma, USA), 50 μg in 50 μL saline (SAL), or SAL alone intratracheally. Animals were assigned to the following four groups: sham/SAL (control), hemorrhagic shock/SAL, sham/LPS, and hemorrhagic shock/LPS (N = 6 in each group).

Administration of anti-HMGB1 antibody

Neutralizing polyclonal mouse IgY anti-HMGB1 antibody (ab18256, Abcom Inc., USA; 200 μg/mouse) or control mouse IgY antibody (200 μg/mouse) was injected into the peritoneum at the same time as the induction of hemorrhagic shock/LPS and 24 h after the hemorrhagic shock/LPS. The therapeutic effects of anti-HMGB1 antibody injection at the same time as hemorrhagic shock/LPS were evaluated at 4 h for cytokines and lung levels of myeloperoxidase (MPO) activity, and at 16 h for lung leak by the Evans blue dye (EBD) assay. The effects of anti-HMGB1 antibody injection 24 h after hemorrhagic shock/LPS were evaluated at 48 h for MPO activity and lung leak was determined by the EBD assay.

Preparation of lung homogenate for ELISA and Western blot analysis

Lung tissue was homogenized in ice cold lysis buffer containing 1 mM protease inhibitor (Sigma). Homogenates were centrifuged at 14,000 *g* for 15 min, and supernatants were collected. The protein concentration of each sample was determined using the micro-BCA protein assay kit standardized with BSA, according to the manufacturer protocol (13).

Western blot analysis

Western blotting was used to determine HMGB1 levels in the lung. One hundred microgram lung homogenate protein was loaded onto a 10% Tris-HCL, SDS-polyacrylamide gel and run for 1 h at 120 V. Protein was electrotransferred to a nitrocellulose membrane and then blocked with 5% (v/v) nonfat dry milk and Tris-buffered saline with 0.1% Tween 20. After blocking, the membrane was incubated overnight at 4°C with a specific polyclonal rabbit primary antibody to HMGB1 (Abcam, UK) at 1:2000 dilution followed by an anti-rabbit horseradish peroxidase-coupled secondary antibody

(Abcam) at 1:5000 dilution. After three washings, bands were detected using enhanced chemiluminescence plus Western blotting detection reagents (Sant Cruz Biotechnology, Inc., USA). The membranes were then stripped using stripping buffer (63 mM Tris-HCL, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol; Bio-Rad, USA) and reprobed with β-actin-specific antibodies (Abcam) to ensure equal loading of the protein on the gel.

Cytokine ELISA

Immunoreactive TNF-α and IL-1β were quantified in duplicate using commercially available ELISA kits (R&D Systems, USA) according to manufacturer instructions.

Lung myeloperoxidase assay

The level of lung MPO, a marker of neutrophil infiltration, was also measured. Lung tissue was homogenized in 1 mL 50 mM potassium phosphate buffer, pH 6.0, containing the reducing agent N-ethylmaleimide (Sigma) for 30 s on ice and the homogenate was centrifuged at 12,000 g for 30 min at 4°C. The protein pellet was homogenized once more in ice-cold buffer and the homogenate was centrifuged. The pellet was resuspended and sonicated on ice for 90 s in a 10X volume of hexadecyltrimethylammonium bromide buffer (0.5% in 50 mM potassium phosphate, pH 6.0). Samples were incubated in a water bath (56°C) for 2 h and then centrifuged at 12,000 g for 10 min. The supernatant was collected for assay of MPO activity at 460 nm.

Assessment of lung leak

EBD (Sigma) solution (5 mg/mL, 200 μL) was injected through a tail vein (50 mg/kg) and 1 h later the chest was opened. The pulmonary vasculature was flushed free of blood by gentle infusion of 10 mL PBS into the beating right ventricle. The lung was then excised, weighed, and dried at 60°C for 24 h and placed in 3 mL formamide at 60°C for 36 h to extract EBD. Dye content was evaluated by spectrophotometry at 620 nm.

Statistical analysis

Data are reported as means ± SEM. Variance at baseline and during the study period was determined by one-way analysis of variance using the SPSS for Windows 11.0 software, and a difference was accepted as significant if the P value was <0.05, as verified by the Duncan and Tukey *post hoc* tests.

Results

HMGB1 concentration in the lung

As shown in Figure 1A, in the hemorrhage shock/SAL

group, HMGB1 concentrations in the lung were slightly increased at 4 h of hemorrhage and more markedly increased at 16 h, with the highest concentration being observed at 24 h. Compared with the control group, difference was statistically significant (P < 0.05). HMGB1 concentration started to decrease at 48 h but did not return to basal levels. In the sham/LPS group, HMGB1 concentrations in the lung were found to be increased at 16 h, and the highest concentration was found at 24 h, and compared with the control group, difference was statistically significant (P < 0.05), as shown
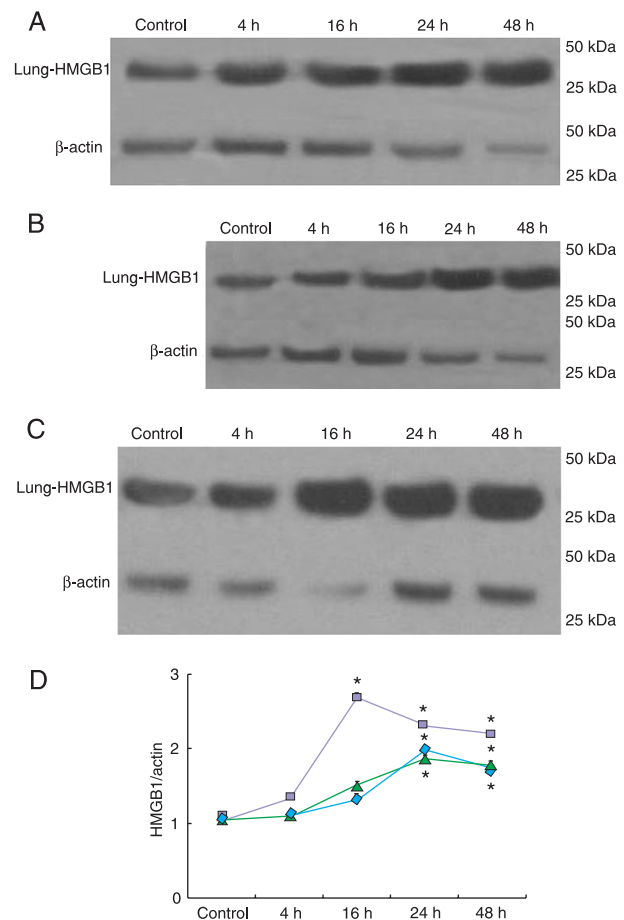
Figure 1. Western blot analysis of high mobility group box 1 (HMGB1) in the lungs of the hemorrhagic shock/saline (SAL) (A), sham/lipopolysaccharide (LPS) (B) and hemorrhagic shock/LPS (C) groups. *D*, HMGB1/actin ratio (mean ± SEM) calculated from Western blots performed on the hemorrhagic shock/SAL (triangles), sham/LPS (lozenges) and hemorrhagic shock/LPS (squares) groups at each time. Bands of 46 kDa represent signals of β-actin and bands of 28 kDa represent signals of HMGB1 protein. Compared to control, hemorrhage shock and LPS separately induced a significant increase at 24 h and hemorrhagic shock/LPS induced a significant increase at 16 h after LPS injection. Each experimental group consisted of 6 mice and data shown are representative. *P < 0.05 compared to control.

in Figure 1B. The changes in HMGB1 concentrations in the lung in the hemorrhage shock/LPS group are shown in Figure 1C. Administration of LPS to hemorrhagic shock animals resulted in a notable increase by 4 h, with a further increase by 16 h. Compared with the control group, this difference was statistically significant (P < 0.05). The levels remained elevated for more than 48 h.

Cytokine expression in the lung

TNF-α and IL-1β were determined at the designated times in the lung homogenates. As shown in Figure 2A and B, compared to control, both TNF-α and IL-1β increased significantly at 4 h (P < 0.05) in the hemorrhage shock/SAL group, sham/LPS group, and hemorrhage shock/LPS group, with a return to baseline values at an early time and no elevation for prolonged periods of time as did HMGB1. In the hemorrhage shock/LPS group, the changes in the expression of two cytokines in the lung were more significant

than in the hemorrhage shock/SAL group and sham/LPS group at 4 h (P < 0.05), although the change trends were similar for all groups.

MPO in the lung

Neutrophil accumulation in the lung was evaluated with the MPO assay. Compared to control, MPO peaked at 4 h in the hemorrhage shock/SAL, sham/LPS and hemorrhage shock/LPS groups (P < 0.05), with the change being more significant in the hemorrhage shock/LPS group than that in the hemorrhage shock/SAL group and sham/LPS group at all times (P < 0.05), as shown in Figure 2C.

EBD in the lung

EBD reflected lung leak. In the hemorrhage shock/SAL group and sham/LPS group, the highest lung leak occurred at 24 h. Compared to control, EBD increased significantly at 16 and 24 h (P < 0.05) in both groups. Administration of LPS to
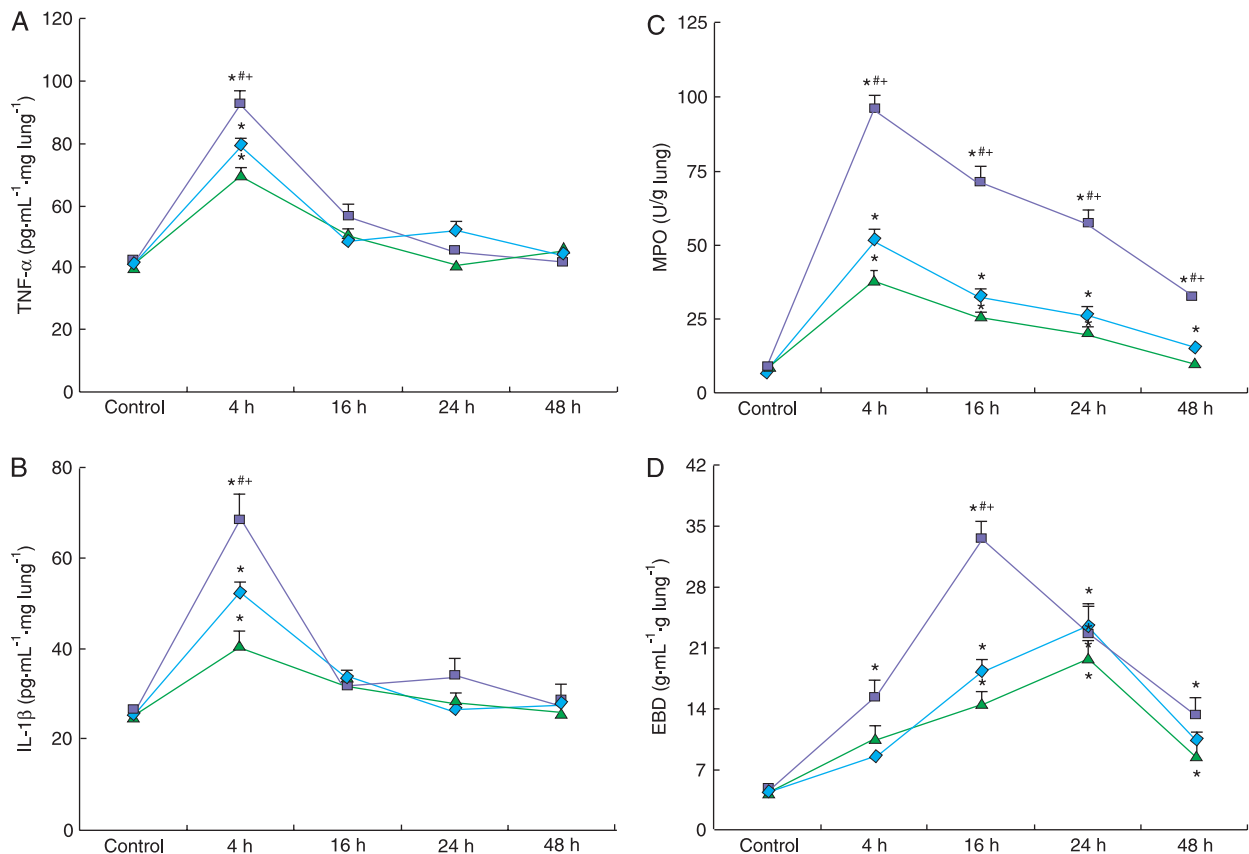

Figure 2. Hemorrhage shock/saline (SAL), sham/lipopolysaccharide (LPS) and hemorrhage shock/LPS induced an increase in the expression of tumor necrosis factor-alpha (TNF-α) (Panel A) and interleukin-1beta (IL-1β) (Panel B) in the lungs and also an increase in the levels of myeloperoxidase (MPO) activity (Panel C) and Evans blue dye (EBD) (Panel D) in the lungs. Triangles: hemorrhage shock/SAL group; lozenges: sham/LPS group; squares: hemorrhage shock/LPS group. *P < 0.05 compared to control; #P < 0.05 compared to hemorrhage shock/SAL at the same time; +P < 0.05 compared to sham/LPS at the same time (Bonferroni modification of the t-test).

hemorrhagic shock animals resulted in a notable increase in EBD by 4 h, and the highest value was observed at 16 h (Figure 2D), in agreement with the change of HMGB1 in the hemorrhagic shock/LPS group. Compared to control, EBD was increased significantly at each time (P < 0.05) in the hemorrhagic shock/LPS group. The highest EBD was more notable in the hemorrhage shock/LPS group than in the hemorrhage shock/SAL group or the sham/LPS group (P < 0.05).

Effects of anti-HMGB1

To determine the effects of HMGB1 in the hemorrhage shock/LPS group and to determine the appropriate treatment time window, we inhibited HMGB1 activity with a specific anti-HMGB1 antibody at different times, i.e., 4, 16, and 48 h after intratracheal LPS injection. Compared with the hemorrhage shock/LPS mice that were injected with control mouse IgY antibody, blockade of HMGB1 at the same time as intratracheal LPS injection significantly reduced pulmonary TNF-α, IL-1β (Figure 3) and MPO at 4 h (Figure 4A), with EBD also being markedly reduced at 16 h (Figure 4B). Blockade of HMGB1 24 h after intratracheal LPS injection failed to alter EBD and MPO levels at 48 h (Figure 4A and B).
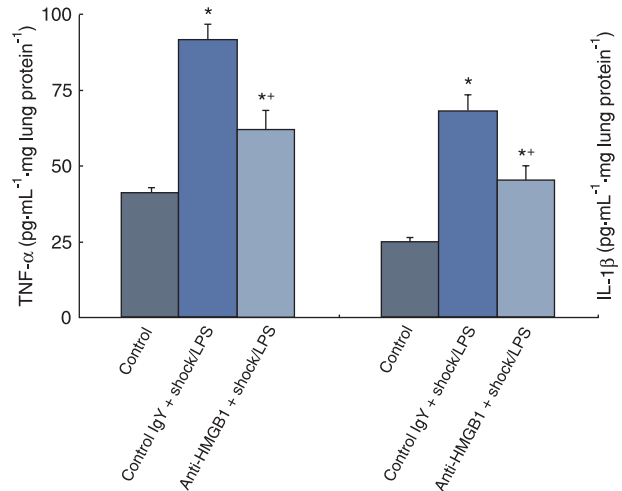
Figure 3. Blockade of high mobility group box 1 (HMGB1) by intraperitoneal injection of anti-HMGB1 antibody at the same time as hemorrhagic shock/lipopolysaccharide (LPS). The therapeutic effect of anti-HMGB1 antibody was evaluated at 4 h after hemorrhagic shock/LPS regarding tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) levels in lung homogenates. Each experimental group consisted of 6 mice. *P < 0.05 compared to control, +P < 0.05 anti-HMGB1 + shock/LPS compared to control IgY + shock/LPS (Bonferroni modification of the *t*-test).
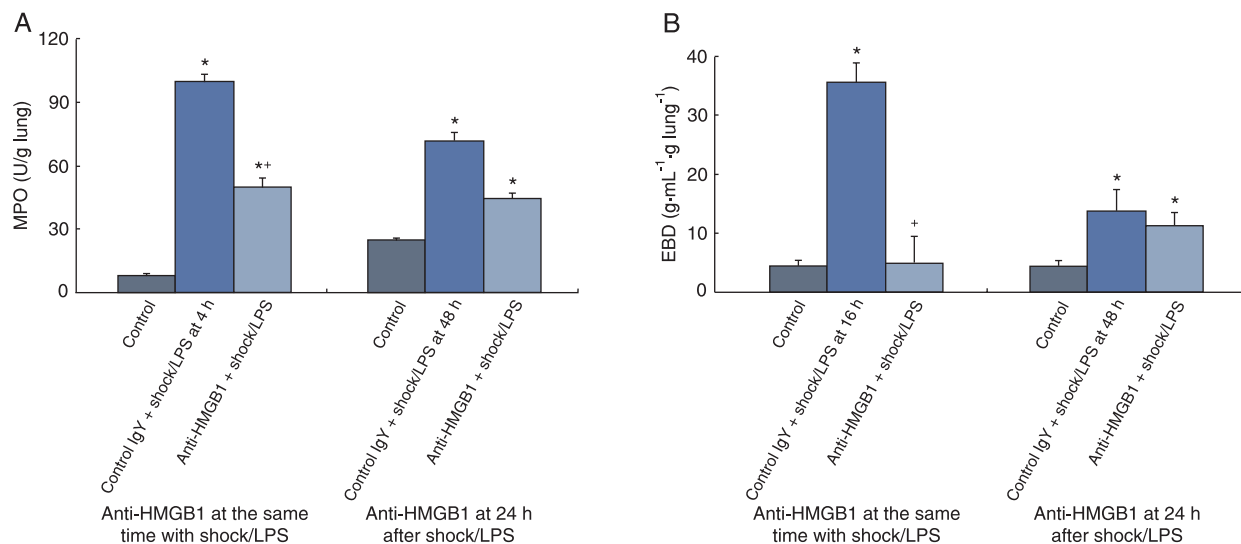
Figure 4. Blockade of high mobility group box 1 (HMGB1) by intraperitoneal injection of anti-HMGB1 antibody at the same time as hemorrhagic shock/lipopolysaccharide (LPS) and 24 h after hemorrhagic shock/LPS. The therapeutic effects of anti-HMGB1 antibody injection at the same time as hemorrhagic shock/LPS were evaluated at 4 h for myeloperoxidase (MPO) activity (A) and at 16 h for the Evans blue dye (EBD) assay (B). The effects of anti-HMGB1 antibody injection 24 h after hemorrhagic shock/LPS were evaluated at 48 h for MPO activity and lung leak was assessed by the EBD assay. Each experimental group consisted of 6 mice. *P < 0.05 compared to control, +P < 0.05 anti-HMGB1 + shock/LPS compared to control IgY + shock/LPS (Bonferroni modification of the *t*-test).
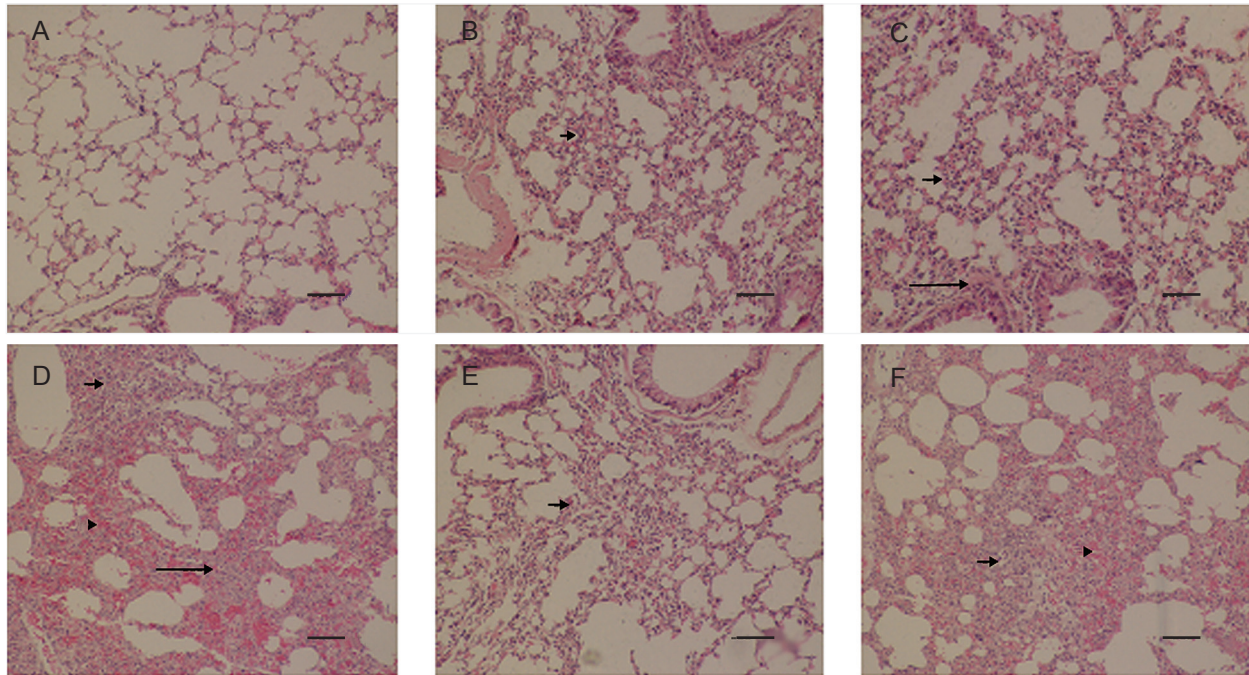
Figure 5. Histopathological samples from sham/saline (SAL) (A), hemorrhagic shock/SAL (B), sham/lipopolysaccharide (LPS) (C), and hemorrhagic shock/LPS mice (D). Abundant neutrophil infiltration (short arrow) was observed in the hemorrhagic shock/SAL mice. In addition to neutrophil accumulation, interstitial edema (long arrow), and pulmonary hemorrhages (arrowhead) were observed in the hemorrhagic shock/LPS mice. Blockade of high mobility group box 1 (HMGB1) at the same time as intratracheal LPS injection (E), accumulation of neutrophil, interstitial edema, and pulmonary hemorrhages were all attenuated. However, histopathological changes were not notable after blockade of HMGB1 at 24 h after intratracheal LPS injection (F). Magnification bars = 100 μm.

Histopathological findings

No destructive change was observed in the sham/SAL group (Figure 5A). Abundant neutrophil infiltration was present in the hemorrhage shock/SAL group and sham/LPS group (Figure 5B and C). In addition to the above changes, interstitial edema and pulmonary hemorrhage occurred in the hemorrhage shock/LPS group (Figure 5D). After blockade of HMGB1 at the same time as intratracheal LPS injection, neutrophil infiltration, interstitial edema, and pulmonary hemorrhage were all attenuated in the hemorrhage shock/LPS group (Figure 5E). However, histopathological changes were not marked after blockade of HMGB1 24 h after intratracheal LPS injection (Figure 5F).

Discussion

Abundant evidence supports the view that HMGB1 is a late inflammatory mediator. Investigators have reported that HMGB1 is released systemically in animals and humans during endotoxemia, severe sepsis and hemorrhage, including ALI (15). In addition, administration of recombinant HMGB1 to normal animals is lethal. Blocking HMGB1 with either HMGB1 antibodies or specific antagonists from dis-

eased animals improves survival from established sepsis or endotoxemia (16).

Not only risk factors are associated with the development of ALI but two-hit plays a significant role in the development of the condition. Studies have supported a two-hit hypothesis in the pathogenesis of lung injury in trauma patients, namely that an initial stimulus may prime for subsequent organ damage in response to a second, often minor, insult (17). However, the contribution of HMGB1 to two-hit-induced ALI has not been previously investigated. Since endotoxemia is often observed in severely injured trauma patients suffering from severe blood loss, we conducted the present study using a rodent model of hemorrhagic shock/LPS.

The kinetics of HMGB1 release is delayed in animal models of endotoxemia and hemorrhagic shock, a feature that is relatively unique compared with other cytokines, most of which are released within a few hours after inflammation or injury (6,16). Hemorrhagic shock is characterized by activation of a cytokine cascade that can stimulate the systemic release of HMGB1 (18,19). In the present study, lung HMGB1 levels increased significantly at 16 h after the induction of hemorrhagic shock, and the highest concentration was observed at 24 h after the onset of

hemorrhagic shock. At the same time, lung HMGB1 levels increased significantly at 24 h after intratracheal LPS injection, a delayed event compared to hemorrhagic shock in mice. This result is consistent with a previously conducted study (20). However, lung HMGB1 levels increased notably within 4 h of LPS administration to hemorrhagic shock animals, an earlier event compared to ALI induced by hemorrhagic shock and intratracheal LPS injection (6,20).

ALI is characterized by an intense inflammatory response in the lung. Tissue ischemia and resuscitation lead to release of reactive oxygen intermediates, which has been demonstrated to contribute to the activation of proinflammatory cytokines and to the development of ALI (21). Endotoxin is responsible for initiating the inflammatory response that leads to ALI and other organ dysfunction in sepsis. In the shock/SAL, sham/LPS and shock/LPS mice, TNF-α and IL-1β were all released within the first few hours of the inflammatory response and returned to basal levels within hours. However, lung leak, as measured by EBD dye extravasations, began to increase at 4 h in the hemorrhagic shock/LPS group and at 16 h in the hemorrhagic shock/SAL and sham/LPS group, and increased for a prolonged period time, when TNF-α and IL-1β returned to basal levels and pulmonary HMGB1 levels were elevated.

These results show that HMGB1 elevation persisted after early proinflammatory cytokines returned to basal levels, indicating a role for HMGB1 as a late-acting mediator of inflammation in two-hit-induced ALI. Compared to one-hit-induced ALI, lung HMGB1 increased earlier in two-hit- than in one-hit-induced ALI (9).

The mechanisms involved in the kinetics of HMGB1 release in hemorrhagic shock/LPS have not been fully elucidated. The present study showed that lung neutrophils and toll-like receptor 4 (TLR4) contributed to the post-hemorrhage increase in pulmonary HMGB1 levels, which promoted the development of ALI. Using a rodent model of hemorrhage/resuscitation, Fan et al. (14) demonstrated that shock led to increased lung neutrophil sequestration and lung injury, in response to a small dose of intratracheal LPS, shock-activated neutrophil mediated TLR4-TLR2 cross-talk, and then promoted AMΦ and AMΦ priming, which acted in a positive feedback manner to amplify pulmonary neutropenia and inflammation.

In the present study, lung MPO increased significantly in the shock/SAL and sham/LPS groups and lung neutrophil sequestration further increased in the shock/LPS group, reflecting the increased pulmonary inflammation. Recent studies have shown that pulmonary neutrophil and TLR4 play a major role in the development of hemorrhage-induced lung injury, and HMGB1 is released from macrophages late after exposure to inflammatory stimuli (22,23). Thus, the mechanism cited above may participate in the kinetics of HMGB1 release in

animal models of hemorrhagic shock/LPS. Further study would be valuable to elucidate the mechanism involved in the kinetics of HMGB1 release in sepsis after hemorrhagic shock-primed ALI.

Specific inhibition of HMGB1 activity with anti-HMGB1 antibodies reduced neutrophil sequestration, pulmonary levels of inflammatory cytokines, and lung leak. These results suggest that HMGB1 contributed to ALI after hemorrhage plus endotoxin injection. The inflammatory cytokines TNF-α and IL-1β, although reduced in anti-HMGB1-treated mice, still remained significantly above baseline levels, indicating that HMGB1 stimulated proinflammatory cytokine synthesis and that other mediators were involved in this pathophysiological process (24,25). Therefore, MPO levels also increased significantly above baseline levels as a result of IL-1β, which is a cytokine with potent neutrophil chemoattractant properties (13).

Targeting the early and rapid kinetics of TNF-α and IL-1β is particularly problematic in the clinic, because even a minimal delay reduces the efficacy of this treatment approach (26). The delayed kinetics of HMGB1 release suggests that it may be targeted in a wider therapeutic window than is provided by inhibitors that target the early cytokine response (27-30). To investigate the appropriate time window for treatment, we specifically inhibited HMGB1 activity at different times. The results suggested that anti-HMGB1 antibodies failed to confer significant protection against shock/LPS-induced ALI when antibody administration was delayed by 24 h, in contrast to what occurs in sepsis and endotoxemia (15,31). Further investigation is needed to define the precise therapeutic window for the inhibition of HMGB1 activity.

In the present study, control mice were subjected to cardiac puncture, tracheotomy and intratracheal injection in order to eliminate the influence of the operation in both control and other animals. Zetterström et al. (32) have reported that HMGB1 produced and stored intracellularly in the adenoid gland contributes to the local antibacterial barrier system of the respiratory tract and may function in a host defense mechanism (32). This may explain why lung HMGB1 was above the detection limit in the control group in the present study. Lung HMGB1 concentrations were all significantly increased in the hemorrhagic shock/SAL, sham/LPS and hemorrhagic shock/LPS groups, suggesting that extracellular HMGB1 released from neutrophils and alveolar macrophages may play a pivotal pathogenetic role in ALI.

The present study demonstrated that HMGB1 plays a key role as a late mediator in intratracheal endotoxin injection after hemorrhagic shock-primed ALI, and the kinetics of its release is different from that of one-hit ALI. The therapeutic window for the suppression of HMGB1 activity should not be delayed by more than 24 h after the onset of the disease, and anti-HMGB1 antibodies should be administered as early as possible.

References

1. Hudson LD, Milberg JA, Anardi D, Maunder RJ. Clinical risks for development of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1995; 151: 293-301.

2. Luster AD. Chemokines - chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; 338: 436-445.

3. Friedman Z, Berkenstadt H, Preisman S, Perel A. A comparison of lactated ringer's solution to hydroxyethyl starch 6% in a model of severe hemorrhagic shock and continuous bleeding in dogs. *Anesth Analg* 2003; 96: 39-45.

4. Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, et al. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996; 154: 602-611.

5. Bustin M. Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends Biochem Sci* 2001; 26: 152-153.

6. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285: 248-251.

7. Gaini S, Pedersen SS, Koldkjaer OG, Pedersen C, Moller HJ. High mobility group box-1 protein in patients with suspected community-acquired infections and sepsis: a prospective study. *Crit Care* 2007; 11: R32.

8. Suda K, Kitagawa Y, Ozawa S, Saikawa Y, Ueda M, Ebina M, et al. Anti-high-mobility group box chromosomal protein 1 antibodies improve survival of rats with sepsis. *World J Surg* 2006; 30: 1755-1762.

9. Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. HMG-1 as a mediator of acute lung inflammation. *J Immunol* 2000; 165: 2950-2954.

10. Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. HMGB1 as a DNA-binding cytokine. *J Leukoc Biol* 2002; 72: 1084-1091.

11. Ombrellino M, Wang H, Ajemian MS, Talhouk A, Scher LA, Friedman SG, et al. Increased serum concentrations of high-mobility-group protein 1 in haemorrhagic shock. *Lancet* 1999; 354: 1446-1447.

12. Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. *Surg Clin North Am* 1995; 75: 257-277.

13. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L1137-L1145.

14. Fan J, Li Y, Vodovotz Y, Billiar TR, Wilson MA. Hemorrhagic shock-activated neutrophils augment TLR4 signaling-induced TLR2 upregulation in alveolar macrophages: role in hemorrhage-primed lung inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L738-L746.

15. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; 5: 331-342.

16. Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A* 2004; 101: 296-301.

17. Fan J, Marshall JC, Jimenez M, Shek PN, Zagorski J, Rotstein OD. Hemorrhagic shock primes for increased expression of cytokine-induced neutrophil chemoattractant in the lung: role in pulmonary inflammation following lipopolysac-

charide. *J Immunol* 1998; 161: 440-447.

18. Tamion F, Richard V, Bonmarchand G, Leroy J, Hiron M, Daveau M, et al. Reduced synthesis of inflammatory cytokines by a free radical scavenger after hemorrhagic shock in rats. *Crit Care Med* 2000; 28: 2522-2527.

19. Schwartz MD, Repine JE, Abraham E. Xanthine oxidase-derived oxygen radicals increase lung cytokine expression in mice subjected to hemorrhagic shock. *Am J Respir Cell Mol Biol* 1995; 12: 434-440.

20. Kim JY, Park JS, Strassheim D, Douglas I, Diaz del Valle F, Asehnoune K, et al. HMGB1 contributes to the development of acute lung injury after hemorrhage. *Am J Physiol Lung Cell Mol Physiol* 2005; 288: L958-L965.

21. Jarrar D, Wang P, Cioffi WG, Bland KI, Chaudry IH. Critical role of oxygen radicals in the initiation of hepatic depression after trauma hemorrhage. *J Trauma* 2000; 49: 879-885.

22. Barsness KA, Arcaroli J, Harken AH, Abraham E, Banerjee A, Reznikov L, et al. Hemorrhage-induced acute lung injury is TLR-4 dependent. *Am J Physiol Regul Integr Comp Physiol* 2004; 287: R592-R599.

23. Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep* 2002; 3: 995-1001.

24. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 2000; 192: 565-570.

25. Agnello D, Wang H, Yang H, Tracey KJ, Ghezzi P. HMGB-1, a DNA-binding protein with cytokine activity, induces brain TNF and IL-6 production, and mediates anorexia and taste aversion. *Cytokine* 2002; 18: 231-236.

26. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* 1987; 330: 662-664.

27. Yang H, Wang H, Czura CJ, Tracey KJ. HMGB1 as a cytokine and therapeutic target. *J Endotoxin Res* 2002; 8: 469-472.

28. Sims CA, Wattanasirichaigoon S, Menconi MJ, Ajami AM, Fink MP. Ringer's ethyl pyruvate solution ameliorates ischemia/reperfusion-induced intestinal mucosal injury in rats. *Crit Care Med* 2001; 29: 1513-1518.

29. Tawadrous ZS, Delude RL, Fink MP. Resuscitation from hemorrhagic shock with Ringer's ethyl pyruvate solution improves survival and ameliorates intestinal mucosal hyperpermeability in rats. *Shock* 2002; 17: 473-477.

30. Lin X, Yang H, Sakuragi T, Hu M, Mantell LL, Hayashi S, et al. Alpha-chemokine receptor blockade reduces high mobility group box 1 protein-induced lung inflammation and injury and improves survival in sepsis. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L583-L590.

31. Wang H, Yang H, Tracey KJ. Extracellular role of HMGB1 in inflammation and sepsis. *J Intern Med* 2004; 255: 320-331.

32. Zetterström CK, Bergman T, Rynnel-Dagöö B, Erlandsson HH, Soder O, Andersson U, et al. High mobility group box chromosomal protein 1 (HMGB1) is an antibacterial factor produced by the human adenoid. *Pediatr Res* 2002; 52: 148-154.