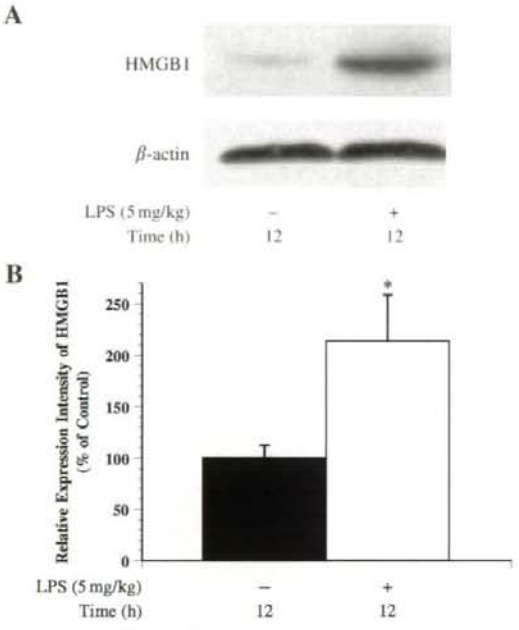

Fig 2. Effects of LPS treatment on high mobility group box 1 (HMGB1) expression in cardiac tissue. (A) Immunoblots showing HMGB1 and β-actin protein expression in the heart tissue from a representative control or LPS animal. (B) Signal intensities for the density of HMGB1 protein immunoblot bands (n=4 for each group). HMGB1 protein levels were quantified using an image analyzer and normalized to β-actin levels. Combined expression intensities of HMGB1 are shown as percentages of the control. Data are mean ± standard error of the mean (SEM). *p<0.05 relative to the control group. See Fig 1 for other abbreviation.

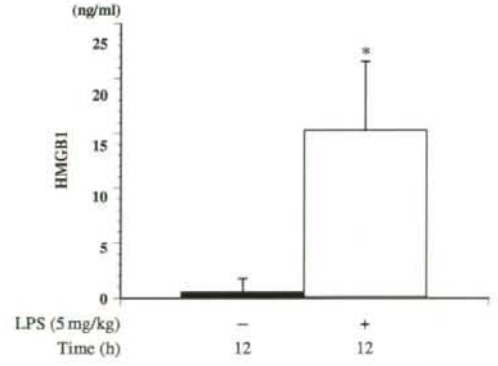

Fig 3. Differences in HMGB1 serum concentration between control (white bar; n=6) and LPS rats (black; n=6). All data are mean ± SEM. An asterisk denotes a difference vs administration of LPS (p<0.05). See Figs 1,2 for abbreviations.

A latex balloon was inserted through the left atrium into the left ventricle, and filled with water (0.18–0.28 ml). LVDP, LVEDP, +dP/dtmax, and –dP/dtmax were continuously recorded with a computerized pressure amplifier-digitizer (BIOPAC Systems, Inc, Goleta, CA, USA). After 20 min of perfusion (to reach equilibrium), the inotropic effects were measured in the presence of varying concentrations of recombinant HMGB1 added to the perfusion solution. Myocardial temperature was maintained at 37°C through the circulation of warm water.

Statistical Analysis

All descriptive data are presented as the means ± standard error of the mean. The data were analyzed by repeated measurement ANOVA for multiple comparisons, and by unpaired t-test for single comparisons. A p-value <0.05 was considered to be statistically significant.

Results

Cardiac Tissue Pathology

We investigated whether LPS-induced sepsis would affect cardiac histopathology. No histological alterations were observed, however, in either the control or the LPS-treated group (Figs 1A–D).

HMGB1 Expression in Cardiac Tissue and Serum

We next examined whether LPS treatment would affect the levels of HMGB1 in cardiac tissue and serum. At 12 h after LPS treatment, heart tissue was isolated and analyzed by immunoblotting. Levels of HMGB1 protein in tissue from LPS-treated animals were at least 2-fold higher than those in the control group (Figs 2A,B). Prior to LPS administration, HMGB1 was barely detectable in the sera of rats from either group. Following LPS injection, HMGB1 levels increased markedly, so that by 12 h post-injection the expression was higher than in the control group (Fig 3).

Isolated Heart Perfusion and General Effects of LPS Treatment

Heart rate (HR) did not differ between the control and LPS groups (Fig 4A), but LPS group animals demonstrated a higher LVEDP at 12 h after exposure to LPS compared with the control groups (Fig 4B). As shown in Fig 4C, hearts subjected to LPS exhibited a significant impairment of LVDP. LV+dP/dtmax, the rate of pressure rise during contraction (Fig 4D), was lower in the LPS group than in the control group ($p<0.05$). LV–dP/dtmin, the maximum rate of relaxation (Fig 4E), was also lower in the LPS group ($p<0.05$). Twelve hours after exposure to LPS, the isolated hearts demonstrated significantly lower LVDPs.

Effect of HMGB1 on Myocardial Function in Control Animals

Perfusion with 0.1 μg/ml HMGB1 had no apparent effect on HR, LVEDP, LVDP, LV+dP/dtmax, or LV–dP/dtmin (data not shown). We then investigated the effect of 1 and 10 μg/ml HMGB1 on cardiac performance in the control animals. Perfusion with 10 μg/ml HMGB1 had no effect on HR (Fig 4A), LVEDP (Fig 4B) or LV–dP/dtmin (Fig 4E). It did, however lead to small decreases in LVDP (Fig 4C) and LV+dP/dtmax (Fig 4D), although these changes were not significantly different.

Effect of Combined LPS Treatment With HMGB1 on Myocardial Function

After LPS treatment, hearts were removed and perfused with HMGB1 for a period of 10 min and cardiac function was measured. Perfusion with 1 or 10 μg/ml HMGB1 resulted in no changes in HR (Fig 4A). Perfusion of 0.1 μg/ml HMGB1 had no effect on HR, LVDP, LVEDP, LV+dP/

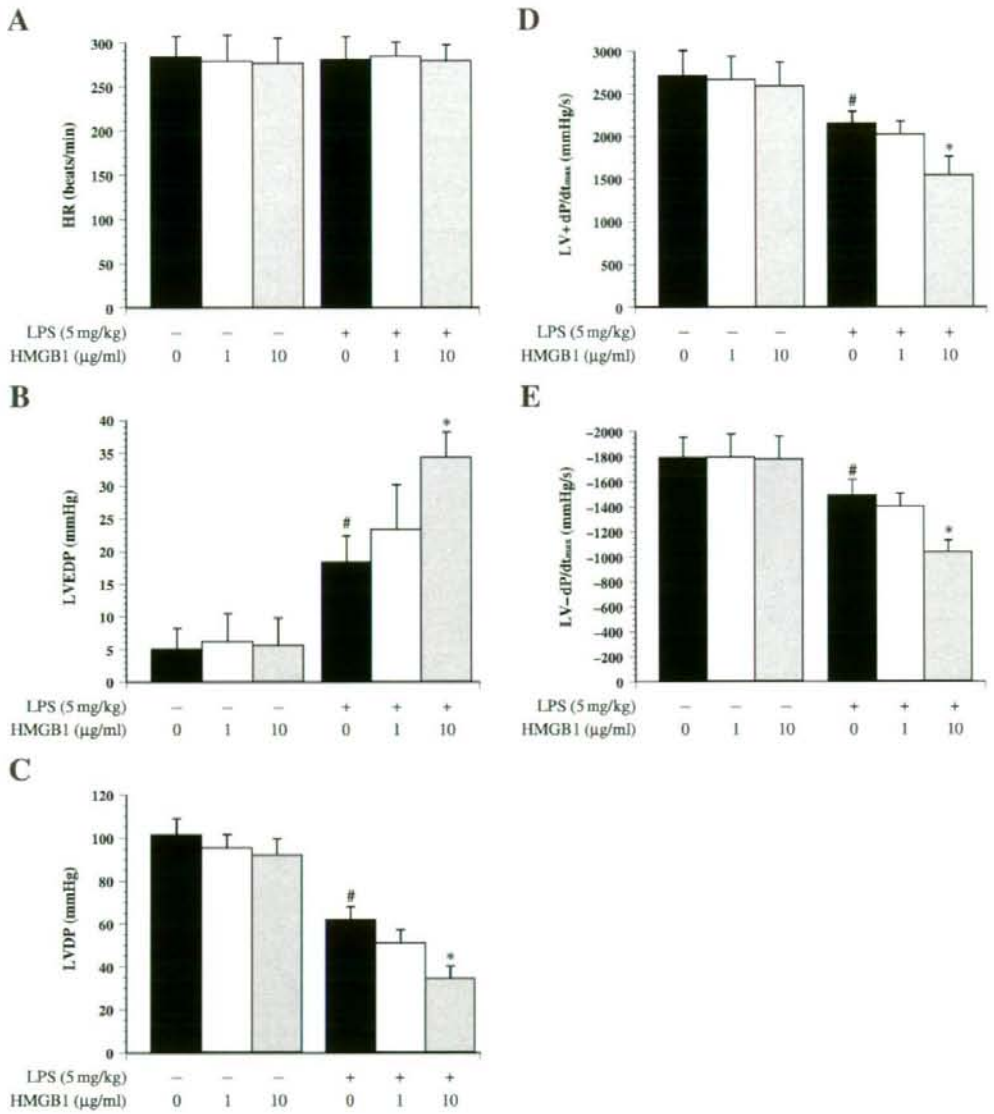

Fig 4. Effects of LPS and HMGB1 treatment on heart function in rats from a LPS-induced sepsis model. (A) Heart rate, (B) LVEDP, (C) LVDP, (D) +dP/dtmax, (E) −dP/dtmin. Data are mean ± SEM from 6 animals in the control and LPS-treated groups. *p<0.05 vs LPS (+) HMGB1 (0). #p<0.05 vs LPS (−) HMGB1 (0). LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure. See Figs 1,2 for other abbreviations.

dtmax or LV−dP/dtmin (data not shown). Hearts perfused with HMGB1 at concentrations of 1 or 10μg/ml, however, showed significantly increased LVEDP in the LPS group, compared with the control group (Fig 4B). In contrast, hearts perfused with HMGB1 at concentrations of 1 or 10μg/ml showed markedly decreased LVDP (Fig 4C), LV+dP/dtmax (Fig 4D), and LV−dP/dtmin (Fig 4E), relative to the control group. These observed effects on cardiac function occurred in an HMGB1 dose-dependent manner. We next examined the effect of 10μg/ml HMGB1 on hemodynamic parameters over time. All of the previously described effects were apparent within 1 min after exposure and remained relatively stable throughout the 10-min period of exposure (Figs 5A–D). After 10 min, HMGB1 was washed from the perfused hearts and all parameters immediately returned to baseline.

Discussion

Our results suggest that HMGB1 played an important role in causing decreased myocardial activity in a rat model of septic shock. Within 12 h of administration, LPS caused a significant increase in the level of HMGB1 in serum and cardiac tissue. In addition, recombinant HMGB1 repro-

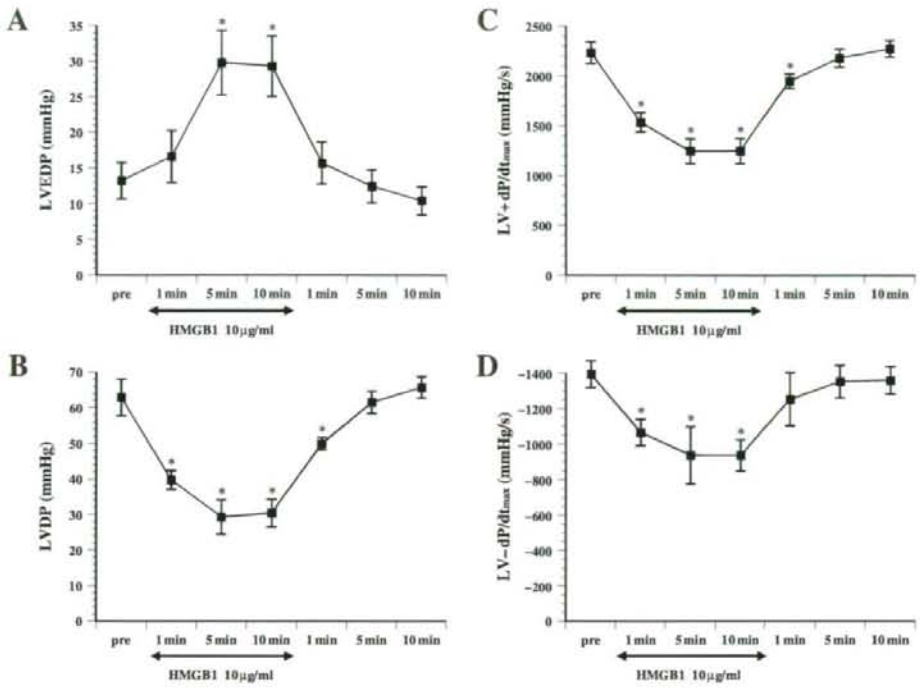

Fig 5. Time course analysis of hemodynamic parameters after exposure to 10μg/ml of HMGB1. (A) LVEDP; (B) LVDP; (C) +dP/dtmax; (D) −dP/dtmin. Data are mean±SEM from 6 animals in the LPS group. *p<0.05 vs pretreatment with HMGB1. See Figs 1,2,4 for abbreviations.

duced the effects of LPS-induced septic shock on rat cardiac function. This study is the first to demonstrate that HMGB1 serves as a late mediator of endotoxin-induced cardiac dysfunction in vivo. Taken together, our findings indicate that HMGB1 must be locally present in cardiac tissue within 12h after LPS challenge to cause cardiac dysfunction.

This result is critical because cardiac dysfunction during sepsis is associated with poor outcome in both humans and animals[3–5] Several previous studies have shown that sepsis-associated cardiac dysfunction is primarily related to circulating myocardial depressant factors, including tumor necrosis factor (TNF)-α[15] However, because TNF-α is a sentinel, rapid-response cytokine and is removed from the circulation several days before the resolution of myocardial dysfunction, it is clear that there are other mediators involved. HMGB1, widely known as a nuclear structural protein, has been identified as a late mediator of delayed endotoxin lethality[16] and is an excellent candidate protein to exert these effects. In humans, patients with sepsis or systemic inflammatory response syndrome have significantly elevated plasma levels of HMGB1[16]

HMGB1 is a nuclear protein that acts as a DNA chaperone in normal cells and promotes DNA–protein interactions[17] It has been shown to play an important role in various types of inflammation and is thought to contribute to the pathology and mortality of sepsis, presumably as a late phase inflammatory mediator[18] In addition, HMGB1 is an important mediator of cell death, and its expression occurs at a relatively late phase following injury[16]

In this study, we found that HMGB1 expression was elevated in cardiac tissue and serum 12h after LPS administration in a rat septic shock model. In this model, the hearts from LPS-treated animals showed increased LVEDP and decreased LVDP, LV+dP/dtmax, and absolute value of −dP/dtmin, compared with the hearts from untreated rats.

We also examined the direct effect of HMGB1 on cardiac function following endotoxin exposure by perfusing recombinant HMGB1 into the hearts from LPS-treated animals. This treatment did not affect HR, but it did augment the increase in LVEDP normally seen in LPS-treated animals, as well as further augment the decrease in LVDP, LV+dP/dtmax, and the absolute value of −dP/dtmin in a dose-dependent manner. Recent studies reported that exogenous HMGB1 improved cardiac function and myocyte regeneration after infarction;[19,20] however, in our model of septic shock, HMGB1 had a negative inotropic effect. This is the first report of exogenous HMGB1 having an inhibitory effect on cardiac function in a septic shock model, which is different from other reports. The mechanism by which HMGB1 inhibits cardiac function is unknown, and thus requires further study.

In summary, our results suggest that HMGB1 plays a critical role in the myocardial dysfunction that follows endotoxin-induced septic shock. Our study examined only the effects of recombinant HMGB1 in the endotoxin-treated rat heart. Further extension of these studies may confirm that HMGB1 could be a new therapeutic target for ameliorating myocardial dysfunction in septic shock.

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ORIGINAL

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High-dose intravenous immunoglobulin G improves systemic inflammation in a rat model of CLP-induced sepsis

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Abstract *Objective:* Intravenous immunoglobulin therapy has been proposed as an advanced treatment for sepsis. Yet, its benefit remains unclear and the mechanism of action is poorly understood. One key mediator in the development of sepsis is high mobility group box 1 (HMGB1). Therefore, we examined the serum and lung tissue levels of HMGB1 in a rat model of sepsis. *Design and setting:* Prospective controlled animal study in a university laboratory. *Materials:* Rats received either cecal ligation and puncture-induced sepsis or had additional intravenous immunoglobulin treatment in boluses of 100, 300, or 1,000 mg/kg. *Measurements and results:* After induction of sepsis and respective treatment conditions, histopathology, wet/dry weight ratios, and signaling molecules were examined in pulmonary tissue. Serum and pulmonary levels of cytokine and HMGB1 were measured. High dose intravenous immunoglobulin (1,000 mg/kg)-treated animals demonstrated significantly improved survival and pulmonary histopathology compared to the control rats. Serum and pulmonary HMGB1 levels were lower over time among intravenous immunoglobulin-treated animals. Furthermore, administration of intravenous immunoglobulin resulted in inhibition of NF-κB activity. *Conclusions:* High-dose intravenous immunoglobulin decreased the mortality and pulmonary pathology in a rat model of sepsis. A significant reduction in HMGB1 levels was also observed, which may be mediated by inhibition of inflammation and NF-κB. *Descriptor:* 23. Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI): experimental models.

Keywords Inflammation · Endotoxemia · Sepsis · High mobility group box 1 · Immunoglobulin

Introduction

In the course of severe sepsis, inflammatory changes, coagulation, and impaired fibrinolysis are observed. The many complications inflicted by these pathological mechanisms on the major organ systems account for the high morbidity in sepsis. Indeed, the mortality rate for severe sepsis still ranges from 30 to 50%, and reaches 80–90% for septic shock and multiple organ dysfunction [1]. Despite recent advances in our understanding of the molecular underpinnings of sepsis, most of these complications remain refractory to treatment [2]. In particular, the respiratory system is severely affected and difficult to treat [3].

Physicians use intravenous immunoglobulin (IVIG), a fractionated blood product, for treating a variety of health conditions. The efficacy of polyspecific immunoglobulins or monoclonal antibodies in the treatment of patients with

severe sepsis or septic shock is still under debate after several clinical trials. A direct benefit in reducing mortality has been demonstrated in only a few studies, and sometimes only after meta-analysis [4]. On the other hand, the Score-Based Immunoglobulin Therapy of Sepsis (SBITS) study group reported that high dose IVIG does not reduce mortality in septic shock patients [5]. In theory, IVIG may be a useful adjuvant treatment during sepsis by modulating the inflammatory cascade. Previous work has suggested that its mechanism of action may involve inhibition of the Fc-receptor, cytokines, or complement activation [6]. A direct antigen–antibody reaction has also been reported, but no clear consensus on its mechanism has been achieved [7]. Such conflicting reports reflect the current lack of understanding of the exact role of IVIG in mediating sepsis.

High mobility group box protein 1 (HMGB1), formerly known as amphoterin, is a sulfoglucoronyl carbohydrate-binding member of the HMGB family [8]. HMGB1 is an intranuclear protein, originally identified to be important in the regulation of DNA-dependent activities [9]. Recent studies indicate that HMGB1 enhances inflammation [10]. Indeed, HMGB1 mediates cell-to-cell signaling by binding to several inflammation-related receptors, receptor for advanced glycosylation end product (RAGE) [11], Toll-like receptor 2 (TLR2) [12, 13], and Toll-like receptor 4 (TLR4) [12, 13]. HMGB1 also acts as a procoagulant, thereby enhancing the inflammatory response in septic shock [14]. Additionally, HMGB1 is recognized to play a key role as a late-phase mediator in the pathogenesis of sepsis [15].

We hypothesized that IVIG inhibits serum and tissue HMGB1 to prevent acute lung injury in a rat model of sepsis. To test this hypothesis, we investigated the impact of immunoglobulin administration on HMGB1 levels and pulmonary histopathology in rats with cecal ligation and puncture (CLP)-induced sepsis. To further elucidate the mechanism of this effect, we assessed the impact of IVIG by measuring the activity of nuclear factor κB (NF-κB) in pulmonary tissue.

Materials and methods

Animals

Male Wistar rats weighing 250–300 g (Kyudou, Saga, Japan) were used in all experiments. All protocols conformed to the National Institute of Health (NIH) guidelines, and the animals received humane care in compliance with the Principles of Laboratory Animal Care. All animals were housed with access to food and water ad libitum.

Anesthesia was induced with 4% sevoflurane. Animals were randomly assigned to one of five groups receiving: CLP and saline, CLP and 100 mg/kg IVIG, CLP and 300 mg/kg IVIG, CLP and 1,000 mg/kg IVIG, or sham surgery with saline ($n = 12$ for each group). CLP was performed by making a 2 cm midline incision and ligating the cecum below the ileocecal valve, taking care not to occlude the bowel. The cecum was then subjected to a single through-and-through perforation with a 21G needle. After the bowel was repositioned, the abdominal incision was closed in layers with plain gut surgical suture.

In the CLP and negative control groups, rats received intravenous infusion of 0.9% NaCl solution preoperatively. In the IVIG-treatment groups, rats were infused with different dosages of immunoglobulin (100, 300, and 1,000 mg/kg, respectively) before CLP. We provided the same volume of IVIG or saline in each condition. Human immunoglobulin was donated by Mitsubishi Pharma Co. Ltd. (Osaka, Japan). This immunoglobulin was produced from pooled human serum obtained from multiple individuals. Negative control rats underwent a sham abdominal surgery without cecal ligation and puncture, and received both an infusion and an additional bolus of 0.9% NaCl solution intravenously. Before and after surgery, animals had access to food and water ad libitum.

Wet-to-dry weight (W/D) ratio

At the end of the experimental protocol, animals were euthanized with an overdose of sodium pentobarbital. Rats were perfused with 10 ml of phosphate-buffered saline (PBS) through the right ventricle to clear the pulmonary circulation of blood cells. The lungs were removed, weighed, and then dried in an oven at 80°C for 48 h to obtain pulmonary wet/dry ratios.

Histological analysis

Lung sections were stained with hematoxylin and eosin (H&E). A pathologist blinded to the group assignment analyzed the samples and determined the levels of lung injury according to Murakami's technique [16]. Briefly, 24 areas in the lung parenchyma were graded on a scale of 0–4 for congestion, edema, infiltration of inflammatory cells, and hemorrhage (0 absent and appears normal, 1 light, 2 moderate, 3 strong, 4 intense). The mean scores for each parameter were used for analyses.

Measurement of cytokine and HMGB1 secretion

Cytokine enzyme-linked immunosorbent assays were performed as recommended by the manufacturer. HMGB1 (measurement range 0–80 ng/ml), IL-6 (measurement range 0–2,000 pg/ml), and TNF-alpha (measurement range 0–1,000 pg/ml) levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (HMGB1: Shino-Test Corporation, Tokyo, Japan; IL-6 and

TNF-alpha: R&D Systems Inc., Minneapolis, MN). All samples and standards were measured in duplicate. The absorbance at 450 nm was determined using an automated plate reader (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemical analysis

Lung tissue samples were fixed upon resection in 4% paraformaldehyde at 4°C overnight, embedded in Optimal Cutting Temperature (O.C.T) Compound (Sakura Finetechnical Co., Tokyo Japan), and cut into 5 μm sections. Immunohistochemistry was performed after blocking endogenous peroxidase activity with 0.3% H_2O_2 and sodium azide (1 mg/ml) for 10 min, and non-specific protein binding with 10% sheep serum for 10 min. Sections were then incubated with anti-HMGB1 polyclonal antibody (1:1,000 dilution; Shino-Test Corporation, Tokyo, Japan) overnight at 4°C. After three rinses of 5 min with PBS, sections were incubated with peroxidase-conjugated anti-mouse IgG antibody. Following three rinses of 5 min with PBS, the slides were then stained utilizing the biotin–avidin peroxidase method with the LSAB2 kit (Dako, Via Real Carpinteria, CA). After development, slides were counterstained with Mayer's hematoxylin, and mounted.

Western blotting

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA.). The membranes were incubated with polyclonal antibodies to HMGB1 (Shino-Test Corporation, Tokyo, Japan) or beta-actin (1:1,000 dilution; Abcam, Cambridge, UK) for 1 h at room temperature followed by HRP-conjugated secondary antibody (1:1,000 dilution; Invitrogen, Carlsbad, CA) for 1 h at room temperature. Blots were subsequently developed using an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm (Amersham, Buckinghamshire, UK).

NF-κB binding assay

The DNA binding activity of NF-κB (p50/p65) was determined using an ELISA-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA). The absorbance at 450 nm was determined using an automated plate reader (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

All data were presented as the mean ± SE. Data were compared using nonparametric tests. The Kruskal–Wallis test for comparisons between all groups was used. Survival curves were derived by the Kaplan–Meier method and compared by log-rank test. A P value of less than 0.05 was considered to be statistically significant.

Results

Mortality

Survival of rats was analyzed at 72 h after surgical procedures. At this time point, only 3 of 12 rats survived in the groups pretreated with saline only or with low-dose IVIG (100 mg/kg) before induction of CLP. In contrast, at 72 h, 7 of 12 rats receiving 300 mg/kg of IVIG and 9 of 12 rats receiving prophylactic high-dose IVIG (1,000 mg/kg) before CLP survived, indicating a significant increase in survival rate ($P < 0.05$) (Fig. 1). All saline-treated negative control animals survived.

Effect of IVIG on the lung after CLP-induced septic shock

In the negative control group, pulmonary histology did not change (Fig. 2a). In septic rats, lung tissue observed 12 h after the induction of CLP demonstrated interstitial edema, neutrophilic infiltration, and reduced alveolar spaces (Fig. 2b). IVIG (1,000 mg/kg) treatment significantly reduced the interstitial edema and inflammatory cell infiltration in comparison to the CLP-alone group (Fig. 2c). Histology scores, based on the number of areas with congestion, edema, inflammation, and hemorrhage, were all significantly higher in the CLP-only group than in the negative control or the high-dose IVIG group ($P < 0.05$) (Fig. 2d). The wet/dry ratio (W/D) of the lungs in the CLP group was also significantly higher in comparison to the W/D high-dose IVIG rats but comparable to the control sham-treated animals ($P < 0.05$) (Fig. 3).

Effect of IVIG on serum levels of IL-6, TNF-alpha, and HMGB1

Administration of IVIG significantly decreased the concentrations of TNF-alpha and IL-6 at 3–6 h after induction of CLP in rats ($P < 0.05$; Fig. 4a, b). Serum levels of HMGB1 at 12 h also increased after CLP. However, this increase was less prominent in rats treated with IVIG ($P < 0.05$; Fig. 4c). The decline in IL-6, TNF-alpha, and HMGB1 levels also demonstrated a dependence on dose of immunoglobulin, with high-dose IVIG (1,000 mg/kg) being the most effective. Serum levels of IL-6, TNF-alpha, and HMGB1 did not increase in negative control rats at any time point assayed (data not shown). A western blot was performed, coincubating

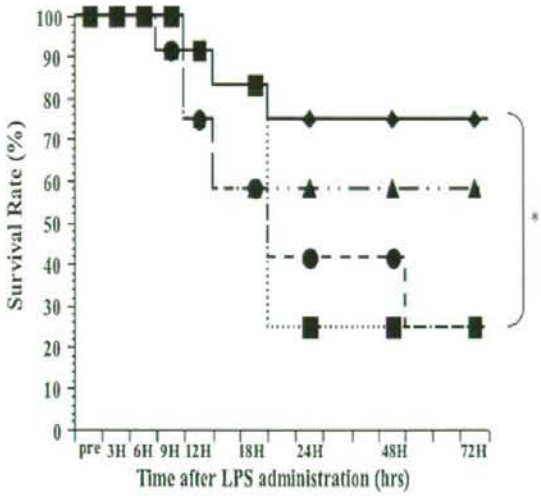

Fig. 1 Survival of CLP-induced septic shock rats with IVIG treatment. Survival rates of animals treated with a bolus of saline into the tail vein (CLP group, $n = 12$) versus animals receiving immunoglobulin (100, 300, and 1,000 mg/kg, respectively) before CLP (IVIG treated CLP groups, $n = 12$/group) were compared. Animals treated with high-dose IVIG (1,000 mg/kg) had significantly improved survival. *Squares* represent the CLP group, *circles* represent the 100 mg/kg IVIG-treated CLP group, *triangles* represent the 300 mg/kg IVIG-treated CLP group, and *diamonds* represent the 1,000 mg/kg IVIG-treated CLP group. * Significant difference compared with the CLP-only group ($P < 0.05$)

human IVIG with recombinant HMGB1. The IVIG had no effect on the anti-HMGB1 antibody recognizing recombinant HMGB1 (data not shown).

Effect of IVIG on lung tissue

The expression of HMGB1 in pulmonary tissue increased as a result of CLP. This increase was less pronounced among rats receiving IVIG (1,000 mg/kg) compared with untreated animals (Fig. 5a). Furthermore, immunohistochemical analysis also revealed that the increase in cellular expression of HMGB1 after CLP decreased dramatically after high-dose IVIG (1,000 mg/kg) administration (Fig. 5c, d). The negative control group showed only slight expression of HMGB1 in pulmonary tissue (Fig. 5b). CLP also led to a robust activation of the NF-κB transcription factor p50/p65, which was partially suppressed by IVIG ($P < 0.05$; 1,000 mg/kg; Fig. 6).

Discussion

In a CLP model of sepsis in rats, we demonstrated that treatment with IVIG improved the survival rate in a dose-dependent manner, with a maximal effect at a dose of 1,000 mg/kg. Surprisingly, prophylactic treatment with human immunoglobulin, in the absence of antibiotics, improved the outcome in the CLP model of rats. In

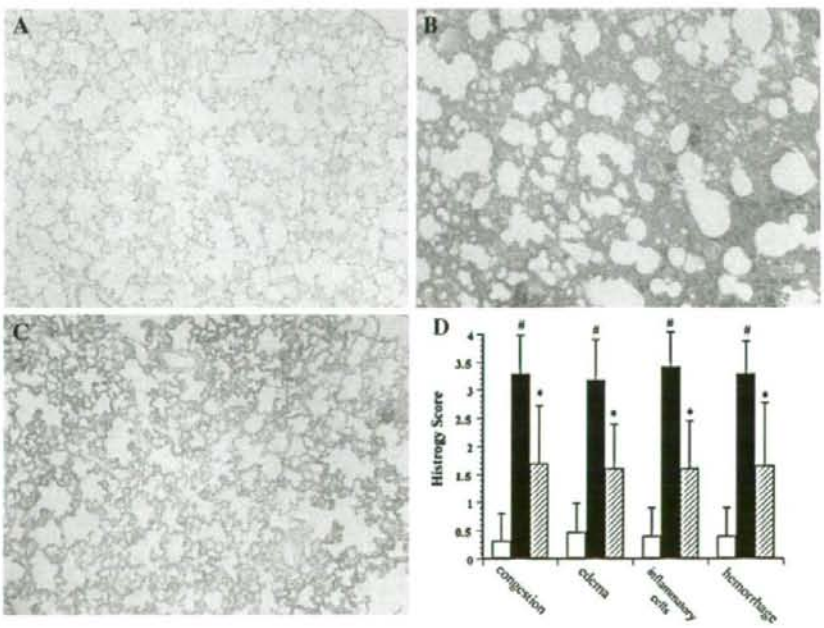

Fig. 2 Effect of IVIG on lung histopathology in rats with CLP-induced sepsis. Lung tissue was obtained from rats undergoing: **a** sham operation, **b** CLP, or **c** CLP along with IVIG (H&E, ×40 magnification). **d** Histological changes were scored based on findings of congestion, edema, inflammation, and hemorrhage, 12 h after CLP. *White bars* represent the sham-operated control animals, *black bars* represent animals receiving only CLP, and *slashed bars* represent animals injected with IVIG before undergoing CLP. The data are expressed as the mean ± SE. # Significant difference compared with the negative control group ($P < 0.05$). * Significant difference compared with the CLP group ($P < 0.05$)

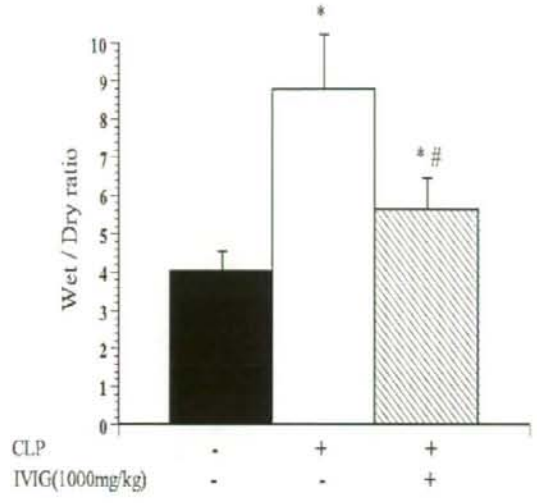

Fig. 3 Effect of IVIG on wet-to-dry weight (W/D) ratio of CLP-treated rats. Rats were sham-operated (*black bar*), induced with CLP alone (*white bar*), or received IVIG along with CLP (*hatched bar*). The W/D ratio was examined after 3 h for each group. The data are expressed as the mean ± SE. # Significant difference compared with the sham-operated negative control group ($P < 0.05$). * Significant difference compared with the CLP-only group ($P < 0.05$)

addition, increased levels of HMGB1 were demonstrated in serum and pulmonary tissue after induction of sepsis by CLP. Furthermore, we demonstrated that IVIG inhibited activation of NF-κB in lung tissue of septic rats. Our findings suggest that infusion of high-dose IVIG at 1,000 mg/kg may prevent acute lung injury, possibly due to inhibition of sepsis-induced HMGB1.

Mechanistically, we demonstrated that immunoglobulin treatment exerts an anti-inflammatory effect and significantly improves acute lung injury in a CLP-induced shock model. Acute lung injury is characterized by noncardiogenic edema, pulmonary inflammation, and severe systemic hypoxemia. It is now widely accepted that the formation of inflammatory mediators plays an important role in the pathophysiology of inflammation in acute lung injury. Indeed, many sequelae associated with acute lung injury result from the excessive production of the cytokine mediators, TNF-α and IL-6, by activated monocytes [17]. Our results extend these studies by suggesting that immunoglobulin may improve septic shock-related lung injury by inhibiting cytokine secretion.

Intravenous immunoglobulin therapy has been proposed as an adjuvant treatment for sepsis. Yet, clinical studies indicate potential differences in the efficacy of immunoglobulin preparations in patients with sepsis. Thus, its benefit remains unclear. Indeed, Werdan et al. [5] indicated that IVIG treatment was ineffective in septic shock patients. On the other hand, Laupland et al. [18] suggested that IVIG treatment might reduce mortality in adults with septic shock. Still other studies demonstrate that IVIG produces an anti-inflammatory effect [19, 20]. In concordance with some of the literature, we showed that IVIG reduced mortality and inflammation in a

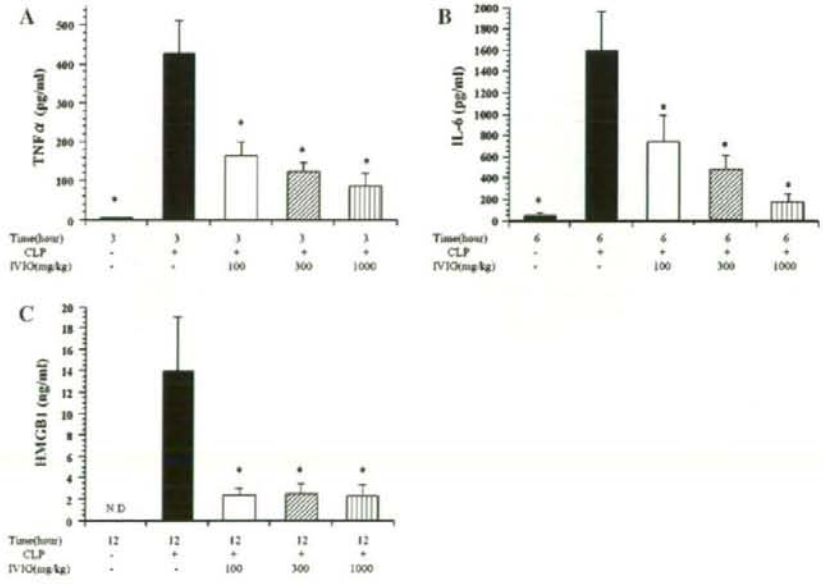

Fig. 4 Temporal changes in serum TNF-alpha, IL-6, and HMGB1 concentrations after CLP in rats. Rats were sham-operated ($n = 6$), induced with CLP-only ($n = 6$), or received various doses of IVIG along with CLP ($n = 6$/group). After induction of sepsis with CLP, the serum levels of **a** TNF-alpha after 3 h, **b** IL-6 after 6 h, and **c** HMGB1 after 12 h, were measured and shown for each group. All data are expressed as the mean ± SE. * Significant difference compared with the CLP-only group ($P < 0.05$)

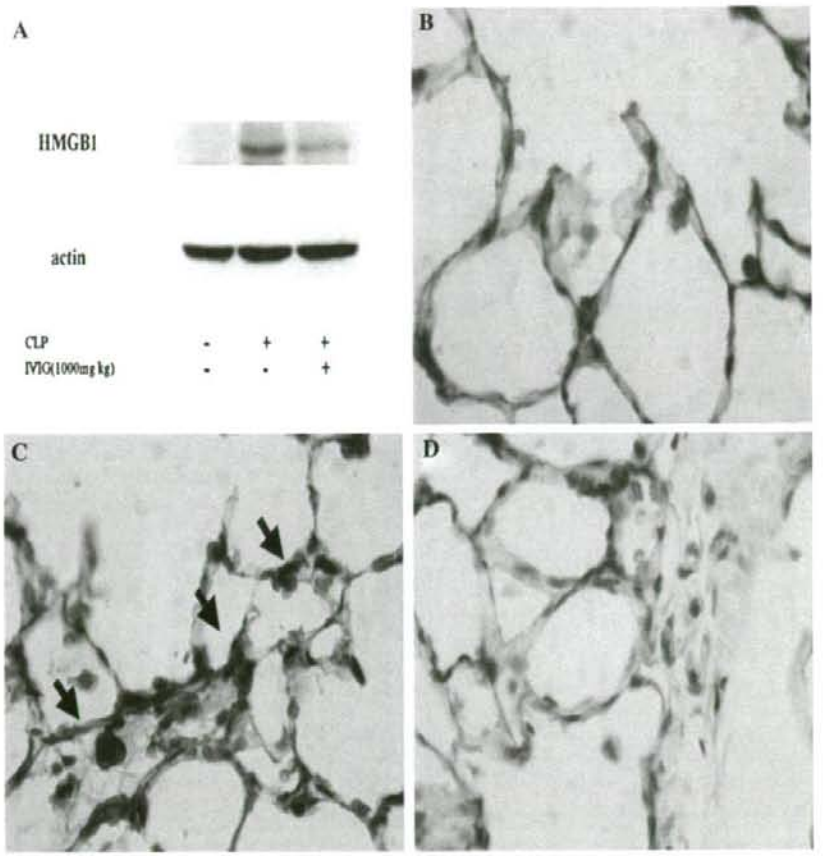

Fig. 5 Changes in HMGB1 protein expression in lung tissue after CLP in rats. Rats were sham-operated ($n = 6$), induced with CLP-only ($n = 6$), or received high-dose IVIG along with CLP ($n = 6$). **a** Pulmonary expression of HMGB1 from respective animal groups was examined 12 h after CLP by Western blot. Immunohistochemistry of HMGB1 expression in pulmonary tissue 12 h after CLP induction of sepsis in: **b** sham-operated, **c** CLP-only, and **d** IVIG-treated animals. ×400 magnification

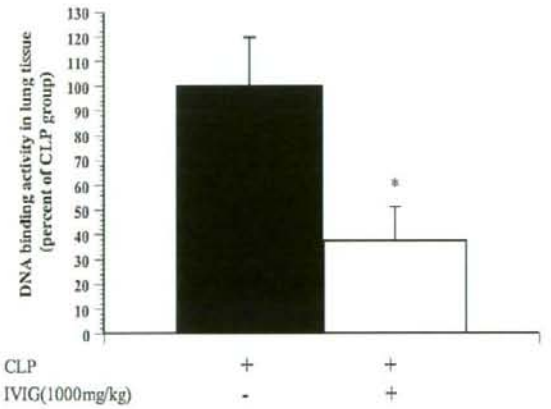

Fig. 6 Effect of IVIG on the CLP-induced increase in the specific binding of p50 and p65 to DNA. Rats were treated with or without IVIG before undergoing CLP. Nuclear fractions were harvested 6 h after CLP induction for DNA binding activity analysis. All data are expressed as the mean ± SE. # Significant difference compared with the CLP group ($P < 0.05$)

CLP-induced septic shock model. These results suggest that a well-designed, adequately powered, and transparently reported new clinical trial may be necessary in septic shock patients. As a caveat, in this study, the control group was given saline, which does not control for the absence of a similar protein load. Therefore, the control data may fail to reflect the influence of protein load such as albumin or a nonspecific IVIG preparation.

In this study, we demonstrated that IVIG reduced the serum levels of HMGB1 and improved mortality. Recent work has shown that HMGB1 contributes to the pathology and mortality of sepsis, presumably as an inflammatory mediator [21]. HMGB1 is present in the serum of sepsis patients, with higher levels associated with increased mortality [22, 23]. However, the relationship between HMGB1 and adverse outcomes in sepsis is controversial. One study reported that HMGB1 concentrations do not differ between survivors and nonsurvivors and do not correlate to either disease severity or concurrently measured cytokine levels in patients with severe infections [24]. In contrast, HMGB1

is elevated in almost all community-associated pneumonia patients, and higher circulating HMGB1 is associated with mortality [25]. Moreover, specific inhibition of endogenous HMGB1 by anti-HMGB1 antibody reverses the lethality of established sepsis in mice [26]. Hence, improvement in mortality rates by the administration of IVIG in CLP-induced sepsis might be related to reduction of HMGB1.

Additionally, our data showed that cytokines and HMGB1 were significantly inhibited even by low doses of IVIG. The demonstration that IVIG inhibits HMGB1 in both serum and lung tissue of septic rats represents the first such report in the literature, to our knowledge. These results build on the growing body of evidence suggesting the importance of HMGB1 in the initiation of acute lung injury [21] and as a late mediator of lethality in sepsis [27]. In previous work, IVIG administration, in combination with adequate antibiotics, improved the survival of surgical ICU patients with intra-abdominal sepsis [28]. Similarly, high-dose IVIG led to a remarkable decrease in mortality among septic rats.

To investigate the mechanisms underlying the effect of IVIG on sepsis, the inflammatory mediator HMGB1 was examined and found to be decreased with IVIG. Various pathways have been implicated in the mechanism of HMGB1 secretion, including stimulation by a number of cytokines [29]. In the present study, IVIG significantly decreased serum TNF-alpha and IL-6 levels TNF-alpha, in particular, has been found to regulate LPS-induced HMGB1 release [29]. Our results support the hypothesis that the inhibition of cytokine secretion by IVIG in sepsis mediates the inhibition of HMGB1.

Many of the signaling events leading to cytokine synthesis and release following exposure to a toxin are now well established in septic shock [30, 31]. Of note, septic shock and stress responses activate the transcription factor, NF-κB, in many cell types [30, 32, 33]. Enhanced NF-κB activity is also observed in alveolar macrophages of patients with septic lung injury [34]. Furthermore, increase in NF-κB in peripheral mononuclear cells correlates with the severity of sepsis as quantified by the Acute Physiology and Chronic Health Evaluation score [35]. NF-κB activity is significantly higher in moribund patients than those who survive [35, 36]. In addition, activation of NF-κB contributes to the secretion of HMGB1 after endotoxin administration [37]. The present data revealed greater activation of NF-κB following CLP when not treated with IVIG. Furthermore, IVIG inhibition of CLP-induced NF-κB in lung tissue may in turn lead to the observed decrease in HMGB1. However, the present study could not elucidate the mechanisms underlying the anti-inflammatory effect of immunoglobulin, which require further investigation.

In conclusion, our results suggest that high-dose IVIG might therapeutically benefit septic patients through a number of mechanisms. IVIG improves pulmonary pathology and overall survival in sepsis by inhibiting the inflammatory response, in particular HMGB1.

As a caveat, we demonstrated that pretreatment with IVIG improved CLP-induced septic shock in a rat model. In the clinical situation, patients cannot be pretreated. Thus, the effect of delayed administration of IVIG on clinical outcome and HMGB1 mRNA expression in septic shock remains to be elucidated. In addition, further study on the relationship of IVIG treatment in septic shock patients is required to extend the results illuminated in our animal model.

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Research

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Danaparoid sodium inhibits systemic inflammation and prevents endotoxin-induced acute lung injury in rats

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Abstract

Introduction Systemic inflammatory mediators, including high mobility group box 1 (HMGB1), play an important role in the development of sepsis. Anticoagulants, such as danaparoid sodium (DA), may be able to inhibit sepsis-induced inflammation, but the mechanism of action is not well understood. We hypothesised that DA would act as an inhibitor of systemic inflammation and prevent endotoxin-induced acute lung injury in a rat model.

Methods We used male Wistar rats. Animals in the intervention arm received a bolus of 50 U/kg of DA or saline injected into the tail vein after lipopolysaccharide (LPS) administration. We measured cytokine (tumour necrosis factor (TNF)α, interleukin (IL)-6 and IL-10) and HMGB1 levels in serum and lung tissue at regular intervals for 12 h following LPS injection. The mouse macrophage cell line RAW 264.7 was assessed following stimulation with LPS alone or concurrently with DA with identification of HMGB1 and other cytokines in the supernatant.

Results Survival was significantly higher and lung histopathology significantly improved among the DA (50 U/kg) animals compared to the control rats. The serum and lung HMGB1 levels were lower over time among DA-treated animals. In the *in vitro* study, administration of DA was associated with decreased production of HMGB1. In the cell signalling studies, DA administration inhibited the phosphorylation of IκB.

Conclusion DA decreases cytokine and HMGB1 levels during LPS-induced inflammation. As a result, DA ameliorated lung pathology and reduces mortality in endotoxin-induced systemic inflammation in a rat model. This effect may be mediated through the inhibition of cytokines and HMGB1.

Introduction

Despite extensive investigation of strategies for treating acute lung injury (ALI), the overall mortality still remains high at approximately 30 to 50% [1]. One of the mechanisms of sepsis-induced acute lung injury involves bacterial endotoxin release into the circulation that activates interconnected inflammatory cascades in the lung, ultimately leading to lung damage [2,3]. The production of inflammatory mediators plays an important role in the pathophysiology of inflammation in lung injury.

High mobility group box 1 (HMGB1) protein is an intranuclear protein that was originally identified as a DNA-binding protein, [4], but is also a late-phase mediator in the pathogenesis of sepsis [5]. HMGB1 acts as a pro-coagulant [6], thereby enhancing the inflammatory response in septic shock [7,8]. The timing of its release and action is typically later than other cytokines, such as TNFα and IL-1β [5]. Inhibitors of HMGB1 might therefore be beneficial in the treatment of various inflammatory diseases.

The role of clotting factors as inflammatory mediators has attracted close attention. Initiation of the coagulation cascade and the subsequent production of proinflammatory cytokines (particularly in response to factor Xa (FXa)) are central to the pathogenesis of sepsis [9,10]. Danaparoid sodium (DA) is a low molecular weight heparinoid consisting of heparan sulfate, dermatan sulfate and chondroitin sulfate that has both anticoagulant and anti-inflammatory effects. DA inhibits of FXa and factor IIa (FIIa) at ratios greater than heparin, while enacting minimal effects on platelet function [11-13]. Anti-inflammatory and anticoagulant agents have thus become a focus of new treatments for sepsis [14,15].

We hypothesised that DA would act as an inhibitor of systemic inflammation and prevent acute lung injury in a rat model. To

ALI = acute lung injury; ARDS = acute respiratory distress syndrome; DA = danaparoid sodium; FIIa = factor IIa; FXa = factor Xa; HMGB1 = high mobility group box 1; IKK = IkB kinase; LPS = lipopolysaccharide; NF-kB = nuclear factor kB.

test this hypothesis, we investigated the impact of DA administration on serum and lung levels of HMGB1, serum cytokine levels and on lung histopathology in rats with lipopolysaccharide (LPS)-induced systemic inflammation. To further elucidate the mechanism of action of these effects, we assessed the impact of DA on HMGB1 and cytokine secretion by RAW264.7 cells.

Materials and methods

In vivo study

Materials

Danaparoid sodium was purchased from Organon Co. Ltd. (CC, Oss, The Netherlands). Lipopolysaccharide (LPS, O127:B8) was obtained from Sigma (St Louis, MO, USA). Antibodies to rabbit polyclonal IgE anti-HMGB1 were purchased from Becton Dickinson and Company (Franklin Lakes, NJ, USA). Antibodies to β-actin were obtained from Abcam PLC (Cambridge, UK).

Treatment protocol

The study was approved by the Ethical Committee of Animal Research at the College of Medicine, Oita University, Oita, Japan. Male Wistar rats weighing 250 to 300 g (Kyudou, Saga, Japan) were used. Anaesthesia was induced by 4% sevoflurane. The animals were randomly assigned to one of three groups: (1) untreated LPS group: rats received a bolus of a 0.9% NaCl solution (1.0 ml/kg) and LPS (7.5 mg/kg) into the tail vein; (2) DA-treated LPS group: rats received a bolus of DA (50 U/kg), and LPS (7.5 mg/kg) into the tail vein; (3) Negative control group: rats received a bolus of 0.9% NaCl solution (1.0 ml/kg) into the tail vein. Before and after surgery, animals had unlimited access to food and water.

Histological analysis

A pathologist blind to group assignment analysed the samples and determined levels of lung injury according to Murakami's technique [16]. Briefly, 24 areas in the lung parenchyma were graded on a scale of 0 to 4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, oedema, infiltration of inflammatory cells, and haemorrhaging.

Measurements of cytokine and HMGB1 secretion

HMGB1, Il-6 and TNFα levels were determined using a commercial enzyme-linked immunosorbent assay kit. HMGB1 was from Shino-Test Corporation, Tokyo, Japan; IL-6, IL-10 and TNFα were from R&D Systems Inc, Minneapolis, MN, USA.

Western blotting

Proteins were subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibody (1:1,000 dilution). After incubation with secondary antibody, blots were developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham). We used the NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA) to quantitate protein band concentrations.

Cell culture study

The murine macrophage cell line, RAW264.7, was maintained in RPMI 1640 medium containing 5% heat-inactivated foetal bovine calf serum and antibiotics at 37°C under 5% CO_2. The medium was removed and replaced with RPMI 1640 containing 5% fetal bovine serum (FBS) for most experiments, or Opti-MEM (Sigma) for experiments designed to measure HMGB1 in conditioned media.

Nuclear factor (NF)-κB binding assay

The DNA binding activity of NF-κB (p50/p65) was determined using an ELISA-based non-radioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA).

Statistical analysis

For descriptive purposes, all continuous data were presented as mean ± SD. The data were analysed by Mann-Whitney U test for comparison between two independent groups. A p value of less than 0.05 was considered to be statistically significant. Survival data were analysed with the Kaplan-Meier program included in the Prism 4.0 software package (San Diego, CA, USA). p Values less than 0.05 were considered statistically significant.

Results

In vivo study

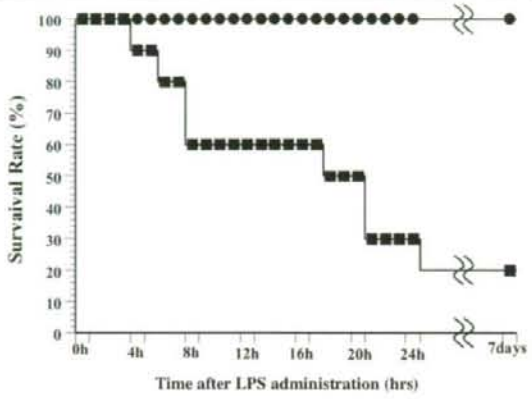
Mortality

A total of 40% of the rats in the untreated LPS group died within 12 h, and an additional 30% died within 24 h, while all rats in the DA-treated LPS group (50 U/kg) survived (Figure 1). In addition, only 20% of rats treated with 1 U/kg DA and 50% of rats treated with 10 U/kg DA survived for 24 h, suggesting a dose-dependent effect of DA on the survival rate of LPS-treated rats (data not shown). All of the saline-treated control animals survived for 7 days. Kaplan-Meier analysis revealed a significantly shorter time-to-death among the untreated LPS group compared to the DA (50 U/kg)-treated LPS group ($p < 0.05$).

Effect of DA on lung tissue specimens

In the negative control group, no histological alterations were observed (Figure 2a,d,g). Among the LPS group with sepsis, the microscopic changes in the lung tissue specimens observed 12 h after LPS administration showed oedema-like formation, and interstitial infiltration by neutrophils (Figure 2b,e,h). The interstitial oedema and inflammatory cell infiltration were markedly reduced in the DA-treated group; DA treatment reduced each of these parameters. All of the scores were significantly lower in the DA (50 U/kg) group than in the LPS group ($p < 0.05$) (Figure 3).

Figure 1

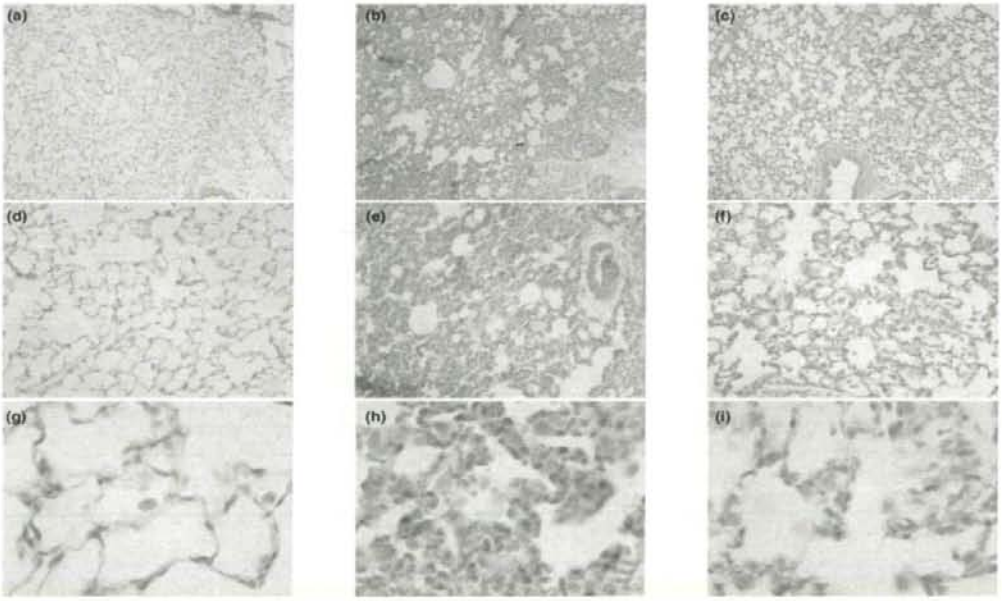


Effect of danaparoid sodium (DA) on the survival rate of lipopolysaccharide (LPS)-treated rats. The survival rate of animals treated with a bolus of LPS (7.5 mg/kg) into the tail vein (LPS group, n = 10) is represented by black squares. The survival rate of animals that received DA (50 U/kg) in addition to the intravenous injection of LPS (7.5 mg/kg) into the tail vein (DA treated LPS groups, n = 10) is represented by black circles.

Effects of DA on the serum levels of IL-6, TNFα, IL-10 and HMGB1

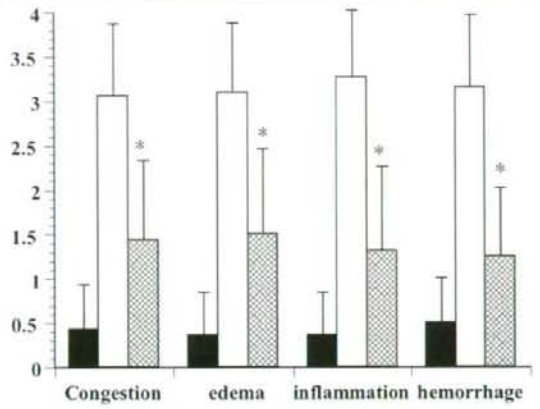
Prior to LPS administration, IL-6, TNFα, IL-10 and HMGB1 in the serum were below levels detectable by the assays. Subsequent to LPS infusion, serum levels of IL-6 increased, with a peak value observed at 3 h in both groups. Treatment with DA following LPS administration led to a significantly decreased concentration of IL-6 at all assay times ($p < 0.05$) (Figure 4a). Likewise, serum levels of TNFα peaked 3 h post-LPS-infusion, with the DA treatment group showing significantly decreased levels at this time point ($p < 0.05$). During the investigation period, TNFα levels of DA-treated LPS group were lower than the LPS group at all assay times (Figure 4b). Serum levels of HMGB1 increased over time following LPS infusion. This increase was less prominent in DA-treated rats compared to the untreated ones. At 6, 9 and 12 h following LPS administration, HMGB1 was significantly lower in the DA-treated LPS group compared to the untreated LPS group ($p < 0.05$) (Figure 4c). By contrast, serum levels of IL-10 peaked 3 h post-LPS-infusion, with the DA-treatment group showing increased levels at all assay times. At 6, 9 and 12 h following LPS administration, IL-10 was significantly higher in the DA-treated LPS group compared to the untreated LPS group ($p < 0.05$) (Figure 4d).

Figure 2



Effects of danaparoid sodium (DA) on lung histopathology in lipopolysaccharide (LPS)-administered rats. Rats were intravenously infused with either saline (control group), 7.5 mg/kg LPS (LPS group), or 7.5 mg/kg LPS with 50 U/kg DA (DA+LPS group). Lung tissue specimens were obtained from the negative control **(a)** magnification ×40, **(d)** magnification ×100, **(g)** magnification ×400); LPS **(b)** magnification ×40, **(e)** magnification ×100, **(h)** magnification ×400; and DA+LPS **(c)** magnification ×40, **(f)** magnification ×100, **(i)** magnification ×400 groups, respectively. Haematoxylin and eosin staining was used.

Figure 3



Effects of danaparoid sodium (DA) on lung histopathology score in lipopolysaccharide (LPS)-administered rats. The histological changes identified included congestion, oedema, inflammation, and haemorrhaging 12 h after the administration of LPS. White bars represent the non-injected control animals, black bars represent the animals injected with LPS, and slashed bars represent animals injected with DA and LPS. The data are expressed as the mean ± SD. *Denotes a significant difference compared with the LPS group ($p < 0.05$).

Effect of DA on the HMGB1 levels in the lung

HMGB1 expression in lung tissue increased following LPS injection. This increase was less pronounced among DA-treated rats compared to the untreated LPS group (Figure 5a,b). In an immunohistochemical analysis, cells expressing HMGB1 increased following LPS administration (Additional file 1a). By contrast, the percentage of cells expressing HMGB1 decreased dramatically in the LPS-administered rats treated with DA (Additional file 1b).

***In vitro* study**

Effect of DA on the culture supernatant and cell protein of HMGB1

The secretion of HMGB1 was measured in the culture supernatant at 20 h after the administration of LPS. The HMGB1 level of the culture supernatant increased after the administration of LPS, but the secretion of HMGB1 was inhibited by the administration of DA. In addition, the inhibition of HMGB1 by DA was minimal at a dose of 1 U/ml, was intermediate at a dose of 15 U/ml, and was maximal at a dose of 50 U/ml (Figure 6). We therefore used a concentration of 50 U/ml DA for subsequent experiments.

Effect of DA on the culture supernatant of cytokines

The TNFα level in the culture supernatant increased 3 h following the administration of LPS. The administration of DA significantly inhibited the secretion of TNFα. The IL-6 level in the culture supernatant also increased after the administration of LPS. The administration of DA was thus found to significantly inhibit the secretion of IL-6 in a manner similar to TNFα (Figure 7).

DA inhibits the IKK pathway and modulates NF-κB

Since the NF-κB pathway plays a critical role in the secretion of cytokines, we measured the quantity of p50 and p65 in the nucleus. Treatment with LPS led to a robust activation of the NF-κB transcription factor p50/p65. This activation was partially blocked by DA (Figure 8).

We subsequently examined the IκB kinase (IKK) system as another activation agent of NF-κB. Treatment with LPS resulted in the degradation of IκB alpha and this degradation was inhibited by DA (Additional file 2). In addition, the phosphorylation of p-IκB alpha in RAW264.7 cells increased after LPS administration, and was also inhibited by DA (Additional file 2).

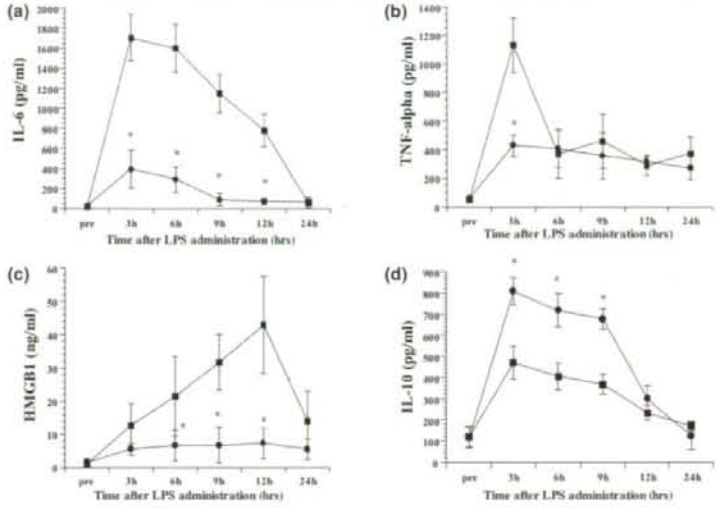
Discussion

This is the first study to demonstrate the anti-inflammatory actions of DA in a rat model of endotoxin-induced lung injury. Acute inflammatory events, such as those that occur in ALI, lead to dysregulation of the coagulation cascade. Indeed, ALI is characterised by profound alterations in both systemic and intra-alveolar coagulation and fibrinolysis [17]. Activation of coagulation with resultant fibrin deposition also has proinflammatory consequences, serving to further amplify the inflammatory cascade [18]. Lung damage may result not only from the release of inflammatory mediators, but also from coagulation. These results suggest that coagulation and inflammation are related and therefore, anticoagulant therapy, such as treatment with DA, will benefit patients with ALI.

In this study, we demonstrated that treatment with the anticoagulant DA significantly improved acute lung injury and mortality in a rat model. Acute lung injury is characterised by non-cardiogenic oedema, pulmonary inflammation and severe systemic hypoxemia. Many sequelae associated with ALI result from excessive production of cytokine mediators (such as TNFα and IL-6) by activated monocytes [19]. In addition, studies have shown that HMGB1 is an important late mediator of inflammation and acute lung injury in sepsis [20-22]. This study adds to the previous findings by suggesting that DA may prevent LPS-induced lung injury by inhibiting cytokine and HMGB1 secretion.

We demonstrated that IL-10 increased following the administration of DA during endotoxin-induced systemic inflammation. A previous study showed that IL-10 inhibited the action of inflammatory cytokines [23] and had profound negative effects on macrophage activation [24]. In particular, IL-10 was closely related to the secretion of TNFα [25]. IL-10 has been identified in the lungs of patients with ARDS, where it was correlated with improved survival [26]. Based on our results, the inhibition of cytokines and prevention of lung injury might be related to

Figure 4



Temporal changes in the tumour necrosis factor (TNF)α, interleukin (IL)-6, IL-10, and high mobility group box 1 (HMGB1) serum concentrations following LPS administration. The IL-6 **(a)**, TNFα **(b)**, HMGB1 **(c)** and IL-10 **(d)** serum concentrations at the indicated times are shown for the lipopolysaccharide (LPS) (n = 6; squares) and danaparoid sodium (DA)-treated (n = 6; circles) groups. All data are expressed as mean ± SD. *Denotes a significant difference compared with the LPS group ($p < 0.05$).

increased serum levels of IL-10 resulting from administration of DA at LPS-induced systemic inflammation.

NF-κB-dependent genes are related to the development of septic shock and to septic lethality. Studies using an LPS model of septic shock have consistently demonstrated that blocking the NF-κB pathway improves outcome [27,28]. Following LPS stimulation, NF-κB is phosphorylated and coordinates the induction of several genes encoding the production and secretion of pro-inflammatory cytokines [29]. Therefore, inhibiting NF-κB activation is crucial for treating inflammation. Here, we showed that DA inhibits LPS-induced NF-κB activation, and may in turn inhibit the secretion of inflammatory mediators and improve survival rate.

Recent studies have demonstrated that coagulation, particularly the generation of thrombin, FXa, and the tissue factor-factor VIIa complex, is related to acute inflammatory responses [30]. Indeed, Riewald M *et al.*, reported that FXa activates NF-κB [31]. DA is a strong inhibitor of FXa. Binding of DA to AT III leads to an accelerated inhibition of FXa, resulting in the antithrombotic effect of DA. [32]. These results suggest that the inhibitory effects of DA on NF-κB may be partially due to inhibition of FXa. Further studies are needed to clarify the signalling mechanisms that mediate the beneficial anti-inflammatory effects of DA.

Recent studies have elucidated how LPS is recognised by monocytes and macrophages of the innate immune system. LPS stimulation of murine macrophages activates several intracellular signalling pathways, including the IκB kinase (IKK)-NF-κB pathway [33,34]. We used a murine macrophage cell line to show that DA suppresses the activation of NF-κB by preventing the phosphorylation of IκB. Accordingly, the inhibition of IκB phosphorylation following DA administration in sepsis may lead to the inhibition of NF-κB activation. As a limitation of this study, the mechanisms that mediate these effects of DA in the LPS-induced systemic inflammatory model are not understood, and we need to further investigate the mechanisms of DA on the inhibition of NF-κB activation.

Materials and methods

Antibodies to phosphorylated IkB and IkB-alpha were obtained from Cell Signaling Technology (Beverly, MA).

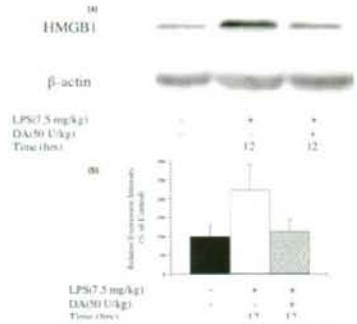
Immunohistochemical analysis

Immunohistochemistry was performed after blocking endogenous peroxidase activity. Blocked sections were incubated with anti-HMGB1 polyclonal antibody (1:1000 dilution). Primary antibody binding was visualized with horseradish peroxidase conjugate and diaminobenzidine.

Western blotting

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford. MA.). The membranes were incubated with primary antibody

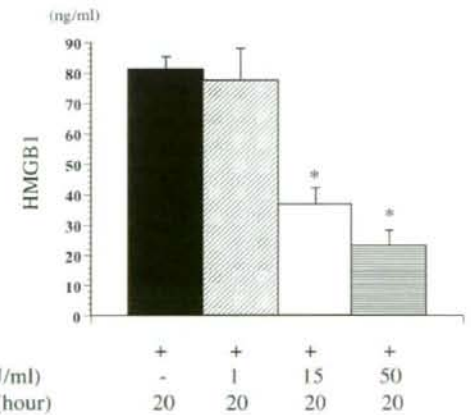
Figure 5



Changes in high mobility group box 1 (HMGB1) protein expression in lung tissue after lipopolysaccharide (LPS) administration in rats. **(a)** The expression of HMGB1 protein in the lung 12 h following administration of LPS in untreated LPS and danaparoid sodium (DA)-treated LPS groups was detected by Western blot. Representative blots from three separate experiments are shown. **(b)** Signal intensities for HMGB1 expression in lung tissue were quantified using an image analyser. Black bars represent the negative control group, white bars represent the LPS group, mesh bars represent the DA-treated LPS group. The expression intensity of HMGB1 protein relative to that of the negative control group was calculated for each group.

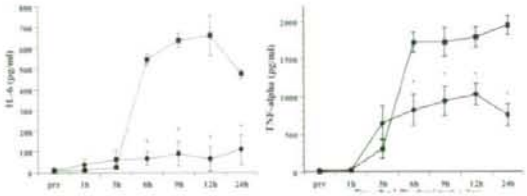
(1:1000 dilution). After incubation with secondary antibody, blots were developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham, Buckinghamshire, UK).

Figure 6



Effect of danaparoid sodium (DA) on high mobility group box 1 (HMGB1) production by lipopolysaccharide (LPS)-stimulated murine macrophages. Murine macrophages treated without or with DA (1, 15, 50 U/ml) were stimulated with LPS (100 ng/ml) for 20 h. Supernatants and cell protein were prepared and examined by enzyme linked immunosorbent assay (ELISA). All data are expressed as means ± SD. *Denotes a significant difference compared with the LPS group ($p < 0.05$).

Figure 7

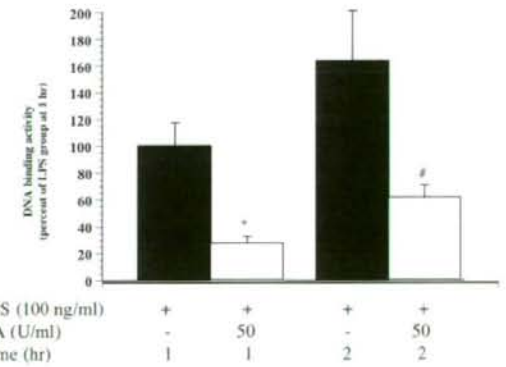


Effect of danaparoid sodium (DA) on interleukin (IL)-6 and tumour necrosis factor (TNF)α production by lipopolysaccharide (LPS)-stimulated murine macrophages. Murine macrophages treated with or without DA (50 U/ml) were stimulated with LPS (100 ng/ml) for the indicated time. Supernatants were collected and IL-6 and TNFα levels were determined by enzyme linked immunosorbent assay (ELISA). All data are expressed as mean ± SD. *Denotes a significant difference compared with the LPS-treated cells ($p < 0.05$).

Conclusion

Using an LPS-induced systemic inflammation model in rats, we demonstrated that danaparoid sodium (50 U/kg) can reduce pulmonary histopathology, decrease mortality, and diminish systemic inflammatory mediators. To our knowledge, this is the first *in vivo* study that has shown such an effect. In a companion tissue culture experiment, we also demonstrated that LPS-induced secretion of cytokines can be decreased by inhibiting the IKK system. Our results suggest that DA may play a role in reducing the pathology of systemic inflammation, and that the potential mechanism of action is through the adjustment of various inflammatory mediators. Given our results, it is possible that DA may have a therapeutic effect on

Figure 8



Effect of danaparoid sodium (DA) on the lipopolysaccharide (LPS)-induced increase of p50/p65 binding to DNA. The DNA binding activity assay showed a marked decrease in the p50/p65 binding activity in nuclear fractions from RAW264.7 cells. All data are expressed as the mean ± SD. *Denotes a significant difference compared with LPS group at 1 h ($p < 0.05$). #Denotes a significant difference compared with LPS group at 2 h ($p < 0.05$).

patients with systemic inflammation, such as septic shock, ARDS, and so on. DA has low toxicity and it is approved for the treatment of systemic inflammatory diseases.

Competing interests

The authors declare that they have no competing interests.

Key messages

Using a lipopolysaccharide (LPS) sepsis model in rats, we demonstrate that danaparoid sodium (50 U/kg) can reduce pulmonary histopathology, decrease mortality, and diminish inflammatory mediators and high mobility group box 1 (HMGB1) serum and lung levels.

In a companion tissue culture experiment, we also demonstrate that LPS-induced secretion of HMGB1 and cytokine can be decreased by inhibiting the IκB kinase (IKK) system.

Our results indicate that danaparoid sodium may play a role in reducing the pathology of sepsis, and that the potential mechanism of action is through the inhibition of systemic inflammation.

Authors' contributions

SH participated in the study design, performed animal, cell culture, biochemical and histological studies, and drafted the manuscript. HI planned the experimental design and performed biochemical and histological studies. SH participated in the study design and performed animal studies. SH performed animal, cell culture study and biochemical analysis. TN participated in the study design, helped to draft the manuscript and coordinated the research group. All authors read and approved the final manuscript.

Additional files

The following Additional files are available online:

Additional file 1

Changes in the HMGB1 protein expression in lung tissue specimens after LPS administration in rats. (A) Immunohistochemcal analysis to detect HMGB1 in lung from animals killed twelve hours after 7.5 mg/kg LPS intravenous administration. The arrows indicate cells stained positive for HMGB1; ×400. (B) An immunohistochemical analysis to detect HMGB1 in the lung from animals treated with 50 units/kg DA and killed twelve hours after 20 mg/kg LPS intravenous administration; ×400.
See http://www.biomedcentral.com/content/supplementary/cc6851-S1.tiff

Additional file 2

Effect of DA on the LPS-induced phosphorylation of IkB. Murine macrophages treated with or without DA (50 units/ml) were stimulated with LPS (100 ng/ml) for 1 hr. The cytoplasmic levels of phosphorylated IkB were determined by Western blot analysis using phosphorylated IkB alpha, IkB alpha, and beta-actin antibodies. Representative blots from three separate experiments are shown.
See http://www.biomedcentral.com/content/supplementary/cc6851-S2.tiff

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