Expression of HMGB1 in septic serum induces vascular endothelial hyperpermeability

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Abstract. The aim of the present study was to investigate the effects of high mobility group protein B1 (HMGB1), which is expressed in the serum of patients with sepsis, on vascular endothelial permeability. Sera from patients with sepsis were used to treat endothelial cells (ECs), and the effect on endothelial permeability was evaluated using immunofluorescence. The morphologies of endothelial cytoskeletal actin and vascular endothelial (VE)-cadherin were assessed using laser scanning confocal microscopy. The protein expression levels of HMGB1, B-cell lymphoma 2 (BCL-2) and BCL-2-associated X protein (BAX) were detected using western blotting. EC apoptosis was measured using flow cytometry. The results demonstrated that HMGB1 was significantly expressed in the serum 24 h following the onset of sepsis, and the expression levels peaked at 48 h, which were sustained until 96 h post-onset. Compared with the control group, treatment of the ECs with 20% septic serum *in vitro* significantly increased endothelial monolayer permeability (P<0.01), markedly induced transcellular filamentous (F)-actin rearrangement with stress fiber formation, and resulted in the localization of VE-cadherin fragmentations at the cell borders with increased gaps between ECs. Furthermore, flow cytometry showed that the apoptotic rate of ECs was significantly increased following treatment with septic serum. In addition, the expression levels of BAX were significantly increased, whereas the expression levels of BCL-2 were significantly decreased. Pretreatment with an HMGBI inhibitor (ethyl pyruvate; 5 μM) 24 h prior to treatment with the septic serum attenuated the effects of septic serum treatment. Together, these findings suggested that treatment of ECs with sera from patients with sepsis may induce the loss of vascular endothelial monolayer integrity, elicit the formation of endothelial F-actin stress fibers and initiate VE-cadherin redistribution, which may be attributed to high levels of HMGB1 in the serum. This mechanism also appears to involve changes in the activation of BAX and BCL-2, resulting in EC apoptosis.

Introduction

Sepsis is defined as a serious, uncontrolled, systemic inflammatory response syndrome (SIRS), which is caused by infection. Infection itself is not usually the cause of damage to the body; rather, it is the abnormally high levels of infection-induced inflammation that result in injury. Furthermore, late sepsis results in systemic damage, which is often complicated by multiple organ dysfunction and septic shock (1), resulting in further deterioration of the functional status and internal environment of the patient, the mechanism of which may be associated with endothelial cell (EC) damage (2-4). The microvascular EC environment provides an important interface for the role of inflammatory cytokines (5). In addition, endothelial injury can amplify inflammatory processes, enhancing its effects (6).

It has been demonstrated that high mobility group protein B1 (HMGB1), which is released in late endotoxemia (7), is a lethal inflammatory mediator (8) that is sustained in the blood, and maintains and extends the pathological process of sepsis. High serum levels of HMGB1 are associated with mortality rates of patients with sepsis (9). Advanced glycation end product receptor (RAGE) is a receptor for HMGB1, which is expressed on the EC membrane (10). HMGB1 interacts with ECs via RAGE, and is involved in endothelial activation and injury (11). Early EC injury leads to endothelial apoptosis, and in the late stage results in endothelial death *in vivo*, which can cause increased EC permeability (12,13).

ECs possess a high degree of biological activity and are involved in various physiological processes within the body (14). Endothelial apoptosis not only destroys the barrier function of ECs, but also further aggravates the inflammatory response (14). Therefore, endothelial apoptosis significantly affects the pathological process of sepsis. When endotoxin is injected into the blood, it has been shown to cause microvascular EC damage, resulting in the shedding of ECs in animal

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experiments (15). Shedding of ECs can also be detected in the peripheral blood of patients with sepsis, and the degree of shedding is often correlated with patient mortality rates (16).

As a feature of sepsis, capillary leakage represents an endothelial barrier dysfunction, which is induced by elevated levels of inflammatory cytokines that, in turn, increase endothelial permeability. Proinflammatory cytokines, including tumor necrosis factor (TNF)-α and inflammatory mediators, including thrombin, can lead to increased EC permeability (17,18). HMGB1 has been shown to increase the permeability of ECs cultured *in vitro* (19). However, whether HMGB1 also induces endothelial apoptosis remains to be elucidated. In the present study, the expression levels of HMGB1 were detected in the serum of patients with sepsis at various time points using western blotting. EC apoptosis and permeability were also assessed following stimulation with septic serum.

Patients and methods

Subjects. The subjects involved in the present study were included healthy volunteers and patients with a diagnosis of sepsis, who were admitted to the intensive care unit (ICU) of Chongming Branch, Xin Hua Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China) within 12 h of onset. Sepsis was defined as SIRS caused by infection, which was confirmed by the presence of bacteria or clinically suspicious foci. Disease severity was classified as sepsis, severe sepsis or septic shock, according to the Surviving Sepsis Campaign (SSC) guidelines (20). The investigation period ran between August 2012 and July 2013. The inclusion criteria were also defined according to SSC guidelines (20): Patients with the following characteristics were excluded from the present study: (1) A history of coronary heart disease, chronic obstructive pulmonary disease, diabetes, hypertension, blood diseases, hyperlipidemia, autoimmune diseases, cancer, rheumatism/connective tissue disease, chronic renal insufficiency, liver cirrhosis; (2) unable to participate to the end of the clinical trial when serum samples were collected; (3) patients <18 years or >75 years of age; (4) smoking or (and) alcohol consumption; (5) patients with a medication history of >1 month. According to the above standards, 38 patients with sepsis (21 men and 17 women; age, 45±3.4 years) and 32 healthy volunteers (17 men and 15 women; age, 48±4.2 years) were enrolled in the present study, which was approved by the ethics committee of the ChongMing branch of the Xin Hua hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). All the subjects provided written informed consent prior to commencement of the investigation.

Human serum. Peripheral venous blood (5 ml) was collected from the subjects and healthy volunteers at 6, 12, 18, 24, 36, 48 and 96 h following hospital admission. All blood samples were subjected to natural clotting at room temperature, followed by centrifugal serum separation (2,000 x g for 8 min) and complement inactivation at 56°C. The aliquots were stored at -70°C until further use.

Chemicals and reagents. A human umbilical vein EC (HUVEC) line was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cell line was originally isolated from human embryonic umbilical vein ECs. Ethyl pyruvate (EP), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and anti-rabbit secondary antibodies, and tetramethylrhodamine (TRITC)-phalloidin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-vascular endothelial (VE)-cadherin antibodies (polyclonal goat anti-human; cat. no. sc-6458), anti-B-cell lymphoma 2 (BCL-2; monoclonal mouse anti-human; cat. no. sc-7382) and anti-BCL-2-associated X protein (BAX; monoclonal mouse anti-human; cat. no. sc-70407) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Polyclonal rabbit anti-human HMGB1 antibody (cat. no. LS-C31535-100) was purchased from LifeSpan Biosciences, Inc. (Seattle, WA, USA). Invitrogen Annexin V-FITC/propidium iodide (PI) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. The HUVECs (1x10^6 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 150 U/ml penicillin (Beyotime Institute of Biotechnology, Hangzhou, China) and 150 U/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. Cell morphology was observed under a microscope (IX70; Olympus Corporation, Tokyo, Japan) and identified using factor VIII immunofluorescence staining (2%) with anti-factor VIII rabbit polyclonal primary antibody (Hua Yi Biotechology, Shanghai, China; cat. no. hy032974). Briefly, ECs were seeded onto slides and cultured for 24 h, and the slides were subsequently fixed with 4% paraformaldehyde at room temperature for 15 min. The slides were then washed with phosphate-buffered saline (PBS) and treated with 0.2% Triton X-100 (Liansuo Biological Technology Co., Ltd., Shanghai, China), prior to being blocked with 1% bovine serum albumin (BSA; LiRui Biological Technology Co., Ltd., Shanghai, China) at room temperature for 30 min, and incubated with rabbit anti-human factor VIII polyclonal primary antibody (100 μl; 1:50) at 4°C for 12-14 h. Following washing with PBS, the slides were incubated with PE-labeled mouse anti-rabbit IgG 100 μl (1:50) in the dark at room temperature for 1 h. Images of the slides were captured using a IX71 fluorescence microscope (Olympus, Tokyo, Japan). Once grown to 80% confluence, the cells were pretreated with EP (5 μM) in DMEM with 10% FBS for 24 h at 37°C. The culture medium was subsequently removed by washing twice with PBS (pH 7.4), and the cells were exposed to 20% septic serum obtained from patient blood samples, diluted in culture medium for 6 h at 37°C. The present study included the following experimental groups: A 20% septic serum-treated group and 20% septic serum + EP (5 μM) pre-treated group; and the following control groups: A 20% control serum-treated (healthy volunteers) group and a 20% control serum + EP (5 μM)-treated group. Cell viability was determined using trypan blue staining (40%; Beyotime Institute of Biotechnology).

Endothelial monolayer permeability. The ECs (1x10^5 cells) were grown on 3 μm pore Transwell filters (Costar®, Corning Incorporated, Corning, NY, USA) until confluent, and were then transferred to starvation medium (Biochrom AG) containing 1% FBS for 2 h. FITC-dextran (Mr, 42,000; Sigma-Aldrich) was applied apically at 1 μg/ml, and allowed to equilibrate for 30 min at 25°C prior to 200 μl sample of the

medium being removed from the lower chamber, to measure basal permeability. The monolayers were pretreated with EP (5 μM) for 24 h at 37°C, and were then either untreated (control) or were stimulated in triplicate with 20% septic serum for 6 h at 37°C. Samples (200 μl) were removed from the lower chamber for fluorescence measurements, which were compared with those from the control monolayers. The fluorescence intensity of FITC was detected using a fluorescence spectrometer (LS-50B; PerkinElmer, Waltham, MA, USA), operating at an excitation wavelength of 492 nm and a detection wavelength of 520 nm.

VE-cadherin and filamentous (F)-actin immunofluorescence staining. The ECs (1x10^{4} cells) were serum-starved for 2 h, pretreated with EP for 24 h, and then either remained untreated or were stimulated with 20% septic serum for 6 h. For F-actin staining, the ECs were fixed in 3.7% formaldehyde (Liansuo Biological Technology Co., Ltd.) at 4°C for 10 min. The cells were permeabilized in 0.2% Triton-X-100 for 5 min and then blocked in 1% BSA in PBS, prior to being incubated with TRITC-phalloidin (2 kμ/l) at room temperature in the dark for 1 h. For VE-cadherin staining, following dewaxing, samples were heated to 92-98°C for 15-20 min in 0.01 M sodium citrate buffer solution (pH 6.0), and then cooled to room temperature for 20-30 minutes. Finally, the slice was rinsed with distilled water and PBS, respectively. The ECs were fixed and blocked, followed by incubation with mouse anti-VE-cadherin antibody (1:100 dilution) overnight at room temperature. The culture medium was removed by washing twice with PBS (pH 7.4) and the ECs were subsequently exposed to FITC-labeled goat anti-mouse IgG secondary antibody (1:200 dilution; cat. no. sc-2010; Santa Cruz Biotechnology, Inc.) at room temperature in the dark for 1 h. Images were captured using a confocal laser scanning microscope (LSM410; Carl Zeiss AG, Oberkochen, Germany).

Western blotting. The ECs (3x10^{6} cells) were serum-starved for 2 h, pretreated with EP for 24 h, and either remained untreated or were stimulated with 20% septic serum for 6 h. The ECs were subsequently lysed with SDS sample buffer (Beijing Taize Technology Development Co., Ltd., Beijing, China). Protein concentration was determined using a Bicinchoninic acid assay method, and the supernatants (100 μl protein) were separated by 10% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes (Mai Bio Co., Ltd., Shanghai, China), which were blocked with 10% non-fat dry milk in Tris-buffered saline and Tween-20 (Double Helix Biotechnology Co., Ltd., Shanghai, China), containing 20 mmol/l Tris (pH 8.0); 137 mmol/l NaCl and 10% Tween-20, and incubated for 12 h at 4°C with monoclonal mouse anti-human BAX, monoclonal mouse anti-human BCL-2 antibody, and polyclonal rabbit anti-human HMGB1 primary antibodies, and then incubated for 2 h at 25°C with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (cat. no. B1706516; ShangHaiyelibio) and HRP-conjugated goat anti-rabbit IgG (cat. no. 96692; Baijibio Beijing). Bound proteins were detected using enhanced chemiluminescence (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. The chemiluminescence signal was detected using a Quantitative Gel and Western Blot Imaging system, and the results were analyzed using FluorChem Q software (ProteinSimple, San Jose, CA, USA).

Flow cytometry. The ECs were serum-starved for 2 h, pretreated with EP (5 μM) for 24 h, and then either remained untreated or were stimulated with 20% septic serum for 6 h. The ECs were then diluted to a suspension of 1x10^{6} cells/ml in 1X binding buffer (Ebioeasy Co., Ltd., Shanghai, China) and were lysed twice with cold PBS buffer. Purified recombinant Annexin V (5-15 μg) was mixed gently with the cell suspension (100 μl) at room temperature for 15 min. Subsequently, Annexin V-FITC (5 μl) and PI (10 μl) were added to the cell suspension and mixed gently for 15 min at room temperature in the dark, prior to adding 1X binding buffer (400 μl). The rate of EC apoptosis was measured using flow cytometry (Epics XL; Beckman Coulter, Inc., Brea, CA, USA) within 1 h.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Statistically significance differences between groups were identified using one-way analysis of variance, and a least significant difference test was used for inter-group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Serum expression levels of HMGB1 in patients with sepsis. The serum expression levels of HMGB1 in patients with sepsis were detected using western blot analysis. As shown in Fig. 1, HMGB1 was detected in the sera of the patients with sepsis; however, it was not expressed until 24 h post-onset (Fig. 1), following which its expression gradually increased, peaking at 48 h (Fig. 1). The expression levels of HMGB1 remained elevated for a long period of time, prior to gradual weakening. The elevated expression levels were ultimately sustained for ~96 h. These results suggested that HMGB1 was not released until sepsis is fairly well established (24 h), and continued to be expressed from that point, maintaining and extending the pathological process of sepsis.

Effects of septic serum on the permeability of HUVECs in vitro. Treatment of ECs with septic patient sera increased cell permeability, as determined using immunofluorescence. As shown in Fig. 2, septic serum induced EC permeability, compared with control serum, after 18 and 24 h; however, the difference was only significant after 36 h (Fig. 2; $P<0.05$), and peaked at 48 h (Fig. 2; $P<0.01$). These results suggested that HMGB1 was released in late sepsis and was involved in the activation and injury of ECs, predominantly by inducing endothelial permeability. In addition, as shown in Fig. 3, treatment with septic serum significantly increased endothelial monolayer permeability after 6 h, compared with cells treated with control serum ($P<0.01$). However, pretreatment with 5 μM EP (an inhibitor of HMGB1) for 24 h prior to treatment with serum significantly reduced serum-induced permeability. HMGB1 was detected readily in the sera from the patients with sepsis, and its inhibition decreased permeability. These results
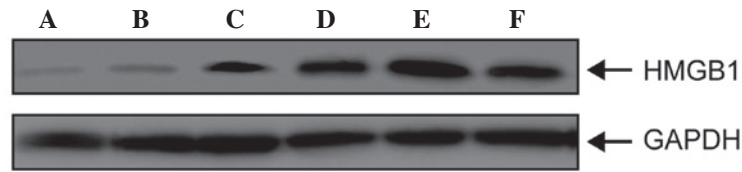
Figure 1. Expression of HMGB1 in the serum of patients with sepsis at various time points. Expression of HMGB1 in septic serum (A) 6 h, (B) 12 h, (C) 24 h, (D) 36 h, (E) 48 h and (F) 96 h following hospital admission. HMGB1, high mobility group protein B1.
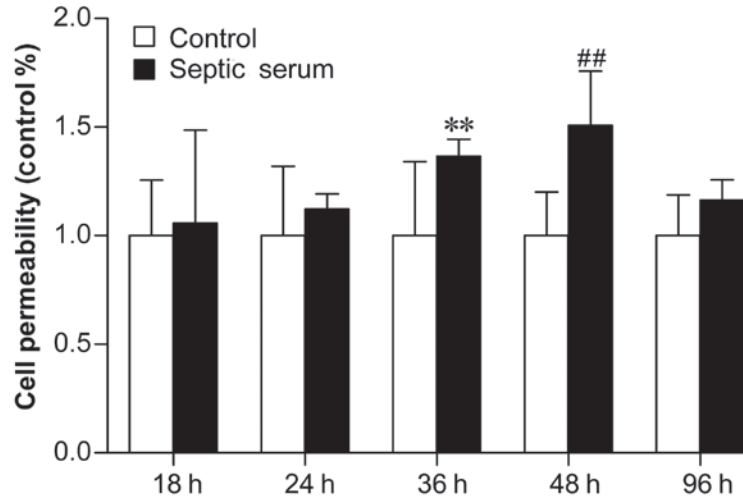

Figure 2. Effects of sera from patients with sepsis on the permeability of human umbilical vein endothelial cells *in vitro*. Data are presented as the mean ± standard deviation (n=6). $^{**}P<0.05$, compared with the control group; $^{##}P<0.01$, compared with the control group.
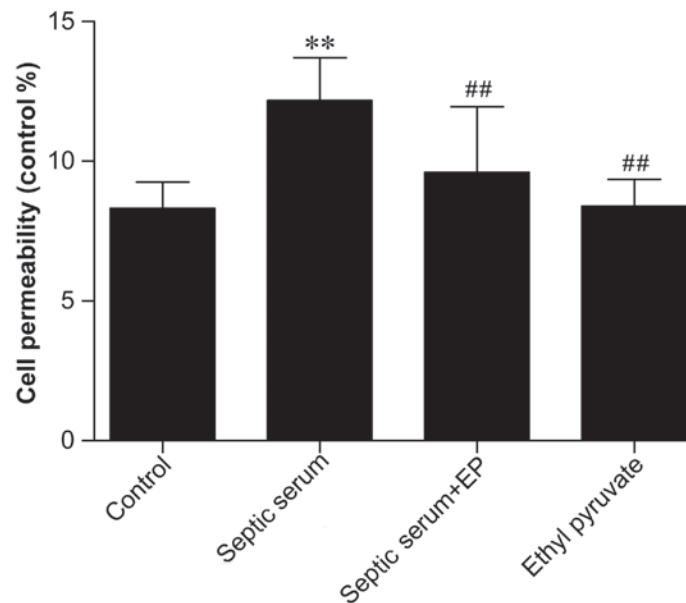

Figure 3. Effects of pretreatment with EP on the permeability of human umbilical vein endothelial cells *in vitro*. Data are presented as the mean ± standard deviation (n=6). $^{**}P<0.01$, compared with the control group; $^{##}P<0.01$, compared with the septic serum-treated group. EP, ethyl pyruvate.

indicated that the functions of HMGB1 were likely responsible for the increased EC permeability

Effects of septic serum on the morphologies of endothelial cytoskeletal actin and VE-cadherin. In the control cells, the majority of F-actin was localized to the cell periphery, parallel to cell-cell junctions, with no clear gaps between the cells

(Fig. 4A). When the ECs were exposed to septic serum for 6 h, almost all of cells became elongated, with thick actin stress fibers that traversed the cells in the direction of cell elongation (F-actin reorganization), increasing the intercellular gap distances (Fig. 4B). However, pretreatment of the cells with EP (5 μM) for 24 h inhibited F-actin reorganization, as shown in Fig. 4C. Treatment with EP alone did not cause F-actin
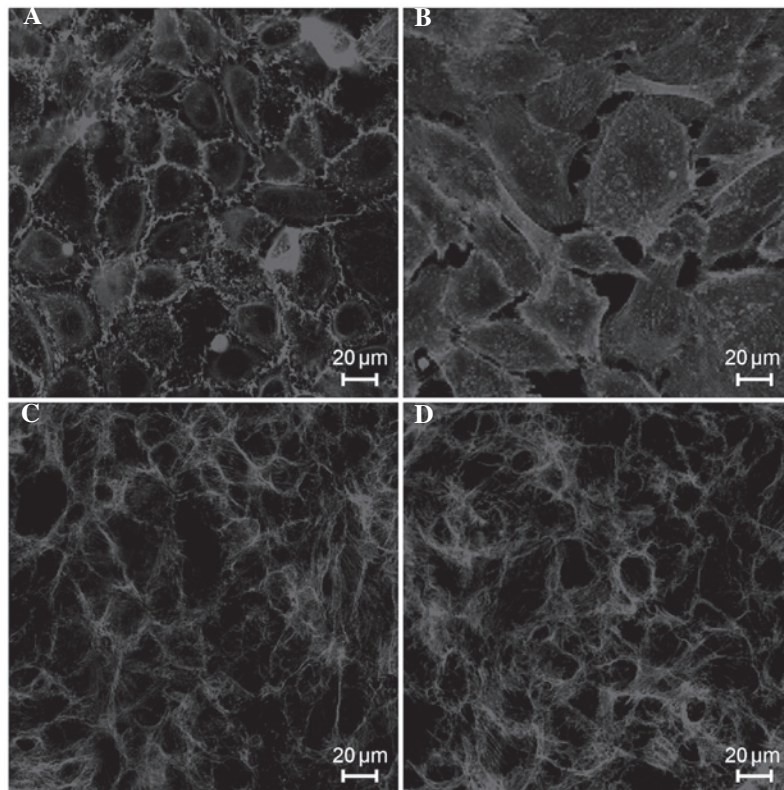
Figure 4. Septic serum induces morphological changes in endothelial cytoskeletal F-actin. Endothelial cells were pretreated with EP for 24 h prior to incubation with 20% septic serum for 6 h. The morphology of endothelial cytoskeletal F-actin was detected under a confocal microscope. (A) Control group; the majority of F-actin is localized to the cell periphery, with no clear gaps between the cells. (B) septic serum (20%)-treated group; the majority of cells are elongated, with thick actin stress fibers that traverse the cells in the direction of cell elongation (F-actin reorganization), increasing the intercellular gap distances. (C) EP (5 μM)-pretreated + 20% septic serum-treated group; inhibition of actin reorganization. (D) EP (5 μM)-treated group; no actin reorganization and morphology of endothelial F-actin similar to that observed in normal cells. Scale bar=20 μm. F-actin, filamentous actin; EP, ethyl pyruvate.
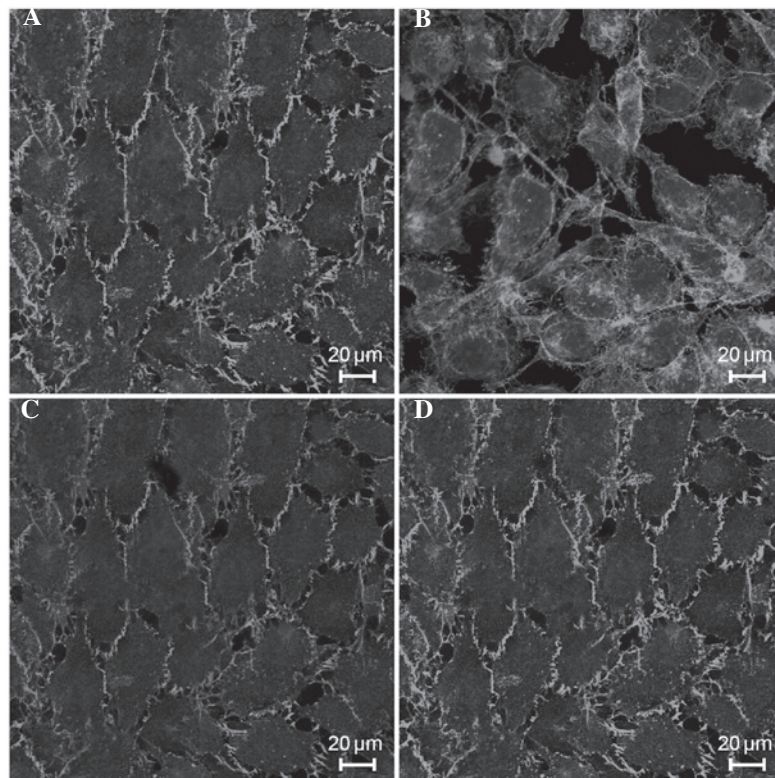

Figure 5. Septic serum induces morphological changes in VE-cadherin. Endothelial cells were pretreated with EP for 24 h prior to incubation with 20% septic serum for 6 h. The morphology of endothelial VE-cadherin was detected under a confocal microscope. (A) Control group; (B) 20% septic serum-treated group; (C) EP (5 μM)-pretreated + 20% septic serum-treated group; (D) EP (5 μM)-treated group. Scale bar=20 μm. VE, vascular endothelial; EP, ethyl pyruvate.
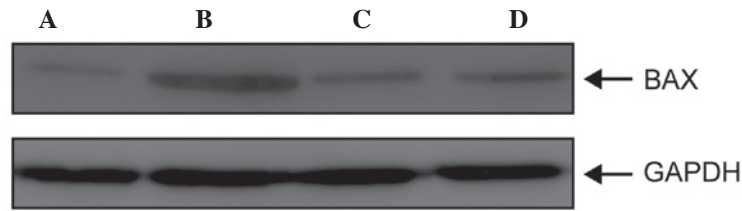
Figure 6. Effects of sera from patients with sepsis on the protein expression levels of endothelial BAX. Endothelial cells were pretreated with EP for 24 h and exposed to 20% septic serum for 6 h. (A) Control group; (B) septic serum-treated group; (C) EP (5 μM)-pretreated and septic serum-treated group; (D) EP (5 μM)-treated group. BAX, B-cell lymphoma 2-associated X protein; EP, ethyl pyruvate.
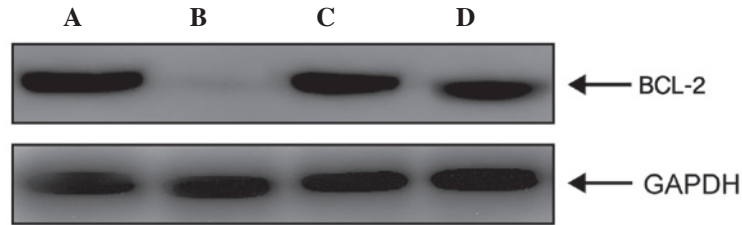

Figure 7. Effects of sera from patients with sepsis on the protein expression levels of endothelial BCL-2. (A) Control group; (B) septic serum-treated group; (C) ethyl pyruvate (5 μM)-pretreated and septic serum-treated group; (D) EP (5 μM)-treated group. BCL-2, B-cell lymphoma 2.
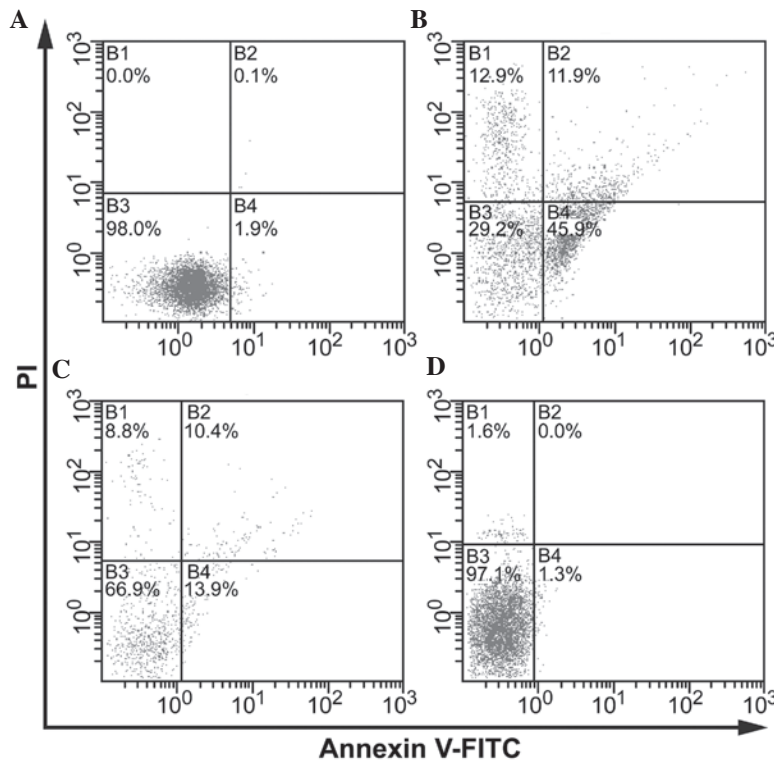

Figure 8. Effects of sera from patients with sepsis on endothelial apoptosis. Endothelial cells were pretreated with EP for 24 h and then exposed to 20% septic serum for 6 h. (A) Control group; (B) septic serum-treated group; (C) EP (5 μM)-pretreated and septic serum-treated group; (D) EP (5 μM)-treated group. EP, ethyl pyruvate; FITC, fluorescein isothiocyanate; PI, propidium iodide.

reorganization, and the morphology of endothelial F-actin in these cells was similar to the normal morphology (Fig. 4D). Similarly, in the control cells VE-cadherin was located at the cell periphery and predominantly formed a continuous line at the cell-cell junctions with occasional gaps (Fig. 5A). However, upon exposure to septic serum for 6 h, the junctions became segmented and discontinuous, despite the fact that VE-cadherin remained localized to areas of cell-cell contact, indicating that the adhesive connection integrity was disrupted (Fig. 5B). However, pretreatment with EP partially inhibited the diffuse redistribution of VE-cadherin (Fig. 5C), whereas EP treatment alone had no effect on the morphology of VE-cadherin (Fig. 5D).

Effects of septic serum on the protein expression levels of endothelial BAX and BCL-2. As shown in Fig. 6A, BAX, a

pro-apoptotic protein, was not expressed in the ECs treated with control serum; however, its expression was markedly upregulated following treatment with septic serum (Fig. 6B). In addition, the upregulation in the expression of BAX was inhibited by pretreatment with 5 μM EP (Fig. 6C), whereas treatment with EP alone had no effect on the expression levels of BAX (Fig. 6D). As shown in Fig. 7, the protein expression levels of BCL-2, an inhibitor of apoptosis, were markedly reduced when the ECs were stimulated with septic serum, compared with those treated with control serum (Fig. 7A and B). However, the protein expression levels of BCL-2 were restored following EP pretreatment (Fig. 7C). These results suggested that HMGB1-rich serum induced EC apoptosis by downregulating the expression of BCL-2 and upregulating the expression of BAX.

Effects of septic serum on endothelial apoptosis. The effects of septic serum on endothelial apoptosis were evaluated using flow cytometry. In Fig. 8, the lower left quadrant represents normal cells, whereas the upper left quadrant represents dead cells, and the lower right quadrant represents early apoptotic cells, whereas the upper right quadrant represents late apoptotic cells. Treatment with septic serum markedly increased the rate of endothelial apoptosis; with early apoptotic cells accounting for 45.9% of the total cells and late apoptotic cells accounted for 11.9%. In addition, compared with the control group (Fig. 8A), necrotic cells accounted for 12.9% of the cells (Fig. 8B). However, endothelial apoptosis was markedly reduced following EP pretreatment, particularly the early apoptotic cells, which accounted for only 13.9% of the cells (Fig. 8C). However, treatment with EP alone had no effect on endothelial apoptosis (Fig. 8D). These results indicated that high levels of HMGB1 in the septic serum may be involved in inducing endothelial apoptosis and EC permeability, particularly early apoptosis. However, with prolonged exposure, EC death occurs.

Discussion

Among critical illnesses, sepsis accounts for the highest mortality rate of non-cardiac diseases in the ICU (21), which is often complicated by acute respiratory distress syndrome, septic shock, multiple organ dysfunction syndrome (MODS) and/or multiple organ failure (MOF) (1). The reason for this is that the uncontrolled release of inflammatory cytokines leads to SIRS and compensatory anti-inflammatory response syndrome, which ultimately results in the patient succumbing to mortality. Proinflammatory cytokines, including early stage TNF-α and interleukin-1β, and late stage HMGB1, interact with other inflammatory effector cells, including polymorphonuclear neutrophils and ECs, to form the inflammatory cascade effect, which causes damage to body organs and affects their functions (22). It has been demonstrated that EC dysfunction is an important factor that contributes to the mortality rates of patients with sepsis (3).

As a late release proinflammatory cytokine, compared with early release inflammatory cytokines, HMGB1 exhibits distinct kinetic effects due to its relatively late release and sustained duration in the blood (8,23). The expression of HMGB1 is often associated with the mortality rates of patients

with sepsis; and high levels of plasma HMGB1 are positively correlated with mortality rate (9,24). HMGB1 interacts with ECs via RAGE, which is expressed on ECs (10,25). The present study demonstrated that the expression of HMGB1 was not detected until 24 h following the onset of sepsis; however, serum expression levels were sustained until 96 h. Furthermore, ECs treated with septic serum, containing highly expression levels of HMGB1, exhibited increased endothelial permeability. When the function of HMGB1 was inhibited by EP pretreatment, the effects of septic serum were significantly attenuated. These results indicated that HMGB1 is a pro-inflammatory cytokine secreted in late sepsis, which may be involved in activating and injuring ECs, and maintaining and extending the pathological process of sepsis.

As a feature of sepsis, capillary leakage represents an endothelial barrier dysfunction, which can be induced by the secretion of inflammatory cytokines that increase endothelial permeability (26). The dangers of capillary leakage include increased tissue edema, cell hypoxia and the excessive local accumulation of inflammatory cytokines, which directly lead to tissue and cell damage and can induce MODS or MOF (27). The pathological basis of increased endothelial permeability is the loss of or damage to EC barrier function integrity (28). The key factor in maintaining integrity is the state of the actin cytoskeleton and connexin (29,30). Under normal conditions, F-actin appears in the form of dense bands around the periphery, and ECs appear relaxed enabling their barrier functions to maintain integrity (31). When ECs are subjected to inflammatory stimuli, F-actin can undergo rearrangement and aggregate into thick stress fibers across the cell, resulting in increased EC contraction and the formation of gaps between the ECs (17). In turn, the paracellular permeability of the ECs increase, and macromolecules, which usually cannot pass through, will leak outside the capillary through gaps between the ECs, resulting in capillary leakage. Cadherin is a calcium-dependent endothelial cell-specific transmembrane adhesion protein, which connects to the actin cytoskeleton via catenin (32). VE-cadherin undergoes morphological changes when the tension generated by F-actin rearrangement passes to VE-cadherin through catenin (33). As a result, VE-cadherin is endocytosed or degraded, causing ECs to lose integrity.

The present study demonstrated that EC permeability increased significantly (P<0.01) when the ECs cultured *in vitro* were stimulated with septic serum, compared with control serum. In addition, endothelial F-actin rearrangement occurred; in which the dense filaments disappeared at the periphery and thick stress fibers formed across the ECs, creating gaps between the ECs and altering VE-cadherin morphology. Green fluorescence intensity was significantly reduced, suggesting that VE-cadherin underwent degradation or endocytosis to account for the loss of EC integrity, however, these effects were largely inhibited by EP pretreatment. These results suggested that HMGB1 affected cell permeability, and may be considered a therapeutic target to reduce the detrimental effects of sepsis and subsequent inflammation.

Previous studies have demonstrated that apoptosis is an important pathological change, which occurs during sepsis (34,35). ECs are important in the host response to sepsis, and endothelial system dysfunction is an important feature of sepsis-associated organ failure and mortality (4). At

the cellular level, the loss of endothelial function in the present study was predominantly due to the increased apoptosis of ECs. EC apoptosis is an important part of the pathological process of sepsis. Vascular ECs (VECs) encounter various inflammatory cytokines and toxins, the exposure to which may result in VEC activation and damage. If the stimulus cannot be removed, the VECs will inevitably move toward apoptosis and, once EC apoptosis begins, apoptotic cells enter the blood-stream and form circulating endothelial cells (36). Animal experiments have previously demonstrated that the injection of endotoxin into the blood can damage microvascular ECs and lead to EC shedding (16). In addition, EC shedding can be detected in the peripheral blood of patients with sepsis, and the extent of shedding is associated with mortality rates (16). VEC apoptosis can lead to the loss of endothelial barrier function, resulting in vascular leakage and edema in patients with sepsis. The present study demonstrated that the number of apoptotic ECs in the peripheral blood of patients with late sepsis was significantly increased, indicating that the apoptosis of ECs in late sepsis may be an important characteristic contributing to multiple organ dysfunction and sepsis-associated conditions. However, EP pretreatment reduced apoptosis, suggesting that HMGB1 is also likely to be associated with apoptosis in patients with sepsis.

The relative expression levels of BCL-2 and BAX, which are encoded by genes upstream of apoptotic signaling pathways, are important factors, which determine whether apoptosis takes place in a cell (37). For this reason, these proteins have increased in interest. The results of the present study revealed that when ECs were stimulated with septic serum, the expression levels of BCL-2 were significantly decreased, whereas the expression levels of BAX were significantly increased. However, these expression patterns were restored to normal levels following EP pretreatment, suggesting that HMGB1 is involved in inducing apoptosis by affecting the expression levels of BCL-2 and BAX. Additional investigations are required to elucidate the precise molecular mechanism underlying the expression of apoptotic genes/proteins.

The results of the present require caution in interpretation, as sera from patients with sepsis, which occurs at different time points, may have different compositions of inflammatory mediators affecting EC permeability (38). In addition, HMGB1, which is a major proinflammatory cytokine in late sepsis, can induce the secretion of other inflammatory cytokines (39). Therefore, it is difficult to distinguish the direct effect of HMGB1 alone, compared with the effects of other cytokines/mediators present in whole serum.

In conclusion, the present study demonstrated that HMGB1 was released in the sera of patients with late sepsis, which increased the permeability of ECs cultured *in vitro*. The underlying mechanism of action may be associated with the induction of EC apoptosis by altering the expression levels of BAX and BCL-2.

Acknowledgements

References

1. Yao YM and Sheng ZY: Progress of basis research on traumatic sepsis in China. Chin J Trauma 19: 9-12, 2003.
2. Koh IH, Menchaca-Diaz JL, Koh TH, Souza RL, Shu CM, Rogerio VE and Liberatore AM: Microcirculatory evaluation in sepsis: A difficult task. Shock 34 (Suppl 1): 27-33, 2010.
3. Aird WC: The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101: 3765-3777, 2003.
4. Lee WL and Liles WC: Endothelial activation, dysfunction and permeability during severe infections. Curr Opin Hematol 18: 191-196, 2011.
5. Lee WL and Slutsky AS: Sepsis and endothelial permeability. N Engl J Med 363: 689-691, 2010.
6. Schouten M, Wiersinga WJ, Levi M and van der Poll T: Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol 83: 536-545, 2008.
7. Wang H, Yang H and Tracey KJ: Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med 255: 320-331, 2004.
8. Wang H, Yang H, Czura CJ, Sama AE and Tracey KJ: HMGB1 as a late mediator of lethal systemic inflammation. Am J Respir Crit Care Med 164: 1768-1773, 2001.
9. Karlsson S, Pettilä V, Tenhunen J, Laru-Sompa R, Hynninen M and Ruokonen E: HMGB1 as a predictor of organ dysfunction and outcome in patients with severe sepsis. Intensive Care Med 34: 1046-1053, 2008.
10. Kokkola R, Andersson A, Mullins G, Ostberg T, Treutiger CJ, Arnold B, Nawroth P, Andersson U, Harris RA and Harris HE: RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. Scand J Immunol 61: 1-9, 2005.
11. Mullins GE, Sunden-Cullberg J, Johansson AS, Rouhiainen A, Erlandsson-Harris H, Yang H, Tracey KJ, Rauvala H, Palmblad J, Andersson J and Treutiger CJ: Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. Scand J Immunol 60: 566-573, 2004.
12. Gill SE, Taneja R, Rohan M, Wang L and Mehta S: Pulmonary microvascular albumin leak is associated with endothelial cell death in murine sepsis-induced lung injury in vivo. PloS One 9: e88501, 2014.
13. Hornburger MC, Mayer BA, Leonhardt S, Willer EA, Zahler S, Beyerle A, Rajalingam K, Vollmar AM and Fürst R: A novel role for inhibitor of apoptosis (IAP) proteins as regulators of endothelial barrier function by mediating RhoA activation. FASEB J 28: 1938-1946, 2014.
14. Aird WC: Endothelium as an organ system. Crit Care Med 32 (5 Suppl): S271-S279, 2004.
15. Leclerc J, Pu Q, Corseaux D, Haddad E, Decoene C, Bordet R, Six I, Jude B and Vallet B: A single endotoxin injection in the rabbit causes prolonged blood vessel dysfunction and a proco-agulant state. Crit Care Med 28: 3672-3678, 2000.
16. Mutunga M, Fulton B, Bullock R, Batchelor A, Gascoigne A, Gillespie JI and Baudouin SV: Circulating endothelial cells in patients with septic shock. Am J Respir Crit Care Med 163: 195-200, 2001.
17. McKenzie JA and Ridley AJ: Roles of Rho/ROCK and MLCK in TNF-alpha-induced changes in endothelial morphology and permeability. J Cell Physiol 213: 221-228, 2007.
18. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG and van Hinsbergh VW: Activation of RhoA by thrombin in endothelial hyperpermeability: Role of Rho kinase and protein tyrosine kinases. Circ Res 87: 335-340, 2000.
19. Zheng YJ, Zhou B, Song ZF, Li L, Wu J, Zhang RY and Tang YQ: Study of *Astragalus mongholicus* polysaccharides on endothelial cells permeability induced by HMGB1. Carbohydr Polym 92: 934-941, 2013.
20. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, *et al*: Surviving Sepsis Campaign: International guidelines for management of severe sepsis and septic shock, 2012. Intensive Care Med 39: 165-228, 2013.

21. Lagu T, Rothberg MB, Shieh MS, Pekow PS, Steingrub JS and Lindenauer PK: Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007. Crit Care Med 40: 754-761, 2012.
22. Bilgin YM, van de Watering LM, Versteegh MI, van Oers MH, and Brand A: Effects of allogeneic leukocytes in blood transfusions during cardiac surgery on inflammatory mediators and postoperative complications. Crit Care Med 38: 546-552, 2010.
23. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, et al: HMG-1 as a late mediator of endotoxin lethality in mice. Science 285: 248-251, 1999.
24. Bitto A, Barone M, David A, Polito F, Familiari D, Monaco F, Giardina M, David T, Messina R, Noto A, et al: High mobility group box-1 expression correlates with poor outcome in lung injury patients. Pharmacol Res 61: 116-120, 2010.
25. Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH and Suffredini AF: Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood 101: 2652-2660, 2003.
26. Liu MW, Wang YH, Qian CY and Li H Xuebijing exerts protective effects on lung permeability leakage and lung injury by upregulating Toll-interacting protein expression in rats with sepsis. Int J Mol Med. 34: 1492-1504,2014.
27. Bannerman DD and Goldblum SE: Direct effects of endotoxin on the endothelium: Barrier function and injury. Lab Invest 79: 1181-1199, 1999.
28. Goldenberg NM, Steinberg BE, Slutsky AS and Lee WL: Broken barriers: A new take on sepsis pathogenesis. Sci Transl Med 3: 88ps25, 2011.
29. Jones TJ, Adapala RK, Geldenhuys WJ, Bursley C, AbouAlaiwi WA, Nauli SM and Thodeti CK: Primary cilia regulates the directional migration and barrier integrity of endothelial cells through the modulation of hsp27 dependent actin cytoskeletal organization. J Cell Physiol 227: 70-76, 2012.
30. Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, Lampugnani MG, Martin-Padura I, Stoppacciaro A, Ruco L, et al: Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. Proc Natl Acad Sci USA 96: 9815-9820, 1999.
31. Ng CT, Fong LY, Sulaiman MR, Moklas MA, Yong YK, Hakim MN and Ahmad Z: Interferon-gamma increases endothelial permeability by causing activation of p38 MAP kinase and actin cytoskeleton alteration. J Interferon Cytokine Res 35: 513-522, 2015.
32. Muradashvili N, Tyagi N, Tyagi R, Munjal C and Lominadze D: Fibrinogen alters mouse brain endothelial cell layer integrity affecting vascular endothelial cadherin. Biochem Biophys Res Commun 413: 509-514, 2011.
33. Wesche-Soldato DE, Swan RZ, Chung CS and Ayala A: The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets 8: 493-500, 2007.
34. Schmid FX, Vudattu N, Floerchinger B, Hilker M, Eissner G, Hoenicka M, Holler E and Birnbaum DE: Endothelial apoptosis and circulating endothelial cells after bypass grafting with and without cardiopulmonary bypass. Eur J Cardiothorac Surg 29: 496-500, 2006.
35. Fu H, Wang QS, Luo Q, Tan S, Su H, Tang SL, Zhao ZL and Huang LP: Simvastatin inhibits apoptosis of endothelial cells induced by sepsis through upregulating the expression of Bcl-2 and downregulating Bax. World J Emerg Med 4: 291-297, 2014.
36. Kluz J, Kopeć W, Jakobsche-Policht U and Adamiec R: Circulating endothelial cells, endothelial apoptosis and soluble markers of endothelial dysfunction in patients with systemic lupus erythematosus-related vasculitis. Int Angiol 28: 192-201, 2009.
37. Xiao D and Zhang L: Upregulation of Bax and Bcl-2 following prenatal cocaine exposure induces apoptosis in fetal rat brain. Int J Med Sci 5: 295-302, 2008.
38. Liang Y, Li X, Zhang X, Li Z, Wang L, Sun Y, Liu Z and Ma X: Elevated levels of plasma TNF-α are associated with microvascular endothelial dysfunction in patients with sepsis through activating the NF-κB and p38 mitogen-activated protein kinase in endothelial cells. Shock 41: 275-281, 2014.
39. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H and Tracey KJ: High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 192: 565-570, 2000.