Role of moesin in HMGB1-stimulated severe inflammatory responses
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Summary
Sepsis is a life-threatening condition that arises when the body’s response to infection causes systemic inflammation. High-mobility group box 1 (HMGB1), as a late mediator of sepsis, enhances hyperpermeability, and it is therefore a therapeutic target. Despite extensive research into the underlying mechanisms of sepsis, the target molecules controlling vascular leakage remain largely unknown. Moesin is a cytoskeletal protein involved in cytoskeletal changes and paracellular gap formation. The objectives of this study were to determine the roles of moesin in HMGB1-mediated vascular hyperpermeability and inflammatory responses and to investigate the mechanisms of action underlying these responses. Using siRNA knockdown of moesin expression in primary human umbilical vein endothelial cells (HUVECs), moesin was found to be required in HMGB1-induced F-actin rearrangement, hyperpermeability, and inflammatory responses. The mechanisms involved in moesin phosphorylation were analysed by blocking the binding of the HMGB1 receptor (RAGE) and inhibiting the Rho and MAPK pathways. HMGB1-treated HUVECs exhibited an increase in Thr558 phosphorylation of moesin. Circulating levels of moesin were measured in patients admitted to the intensive care unit with sepsis, severe sepsis, and septic shock; these patients showed significantly higher levels of moesin than healthy controls, which was strongly correlated with disease severity. High blood moesin levels were also observed in cecal ligation and puncture (CLP)-induced sepsis in mice. Administration of blocking moesin antibodies attenuated CLP-induced septic death. Collectively, our findings demonstrate that the HMGB1-RAGE-moesin axis can elicit severe inflammatory responses, suggesting it to be a potential target for the development of diagnostics and therapeutics for sepsis.

Keywords
Moesin, HMGB1, vascular permeability, sepsis, marker

Introduction
High-mobility group box 1 (HMGB1) is a non-histone nuclear protein that bound to DNA that stabilise nucleosome and regulates transcription (1, 2). Under inflammatory conditions, HMGB1 translocates from the nucleus to the extracellular space of cells, where it functions as a potent proinflammatory mediator by interacting with toll-like receptor (TLR)-2 or –4, or the receptor for advanced glycation end-products (RAGE) presenting on other cells (2). In cases of sepsis in humans, HMGB1 is released into circulation and participates in pathogenesis; many other proinflammatory cytokines are also released, but the kinetics of HMGB1 are distinct (3). The kinetics of HMGB1 release during sepsis are relatively slow, and it is therefore classified as a late-onset mediator of sepsis (3), making it a promising factor to be targeted as an adjunctive therapy in the treatment of sepsis (2, 4). In animal sepsis models using cecal ligation and puncture (CLP), the adminis-
tation of HMGB1-specific neutralising antibodies has been shown to increase survival rates (5).

Cytoskeletal proteins are involved in many cellular functions, including regulation of the actin cytoskeleton, control of cell shape, adhesion and motility, and modulation of signalling pathways (6). Many pathophysiological conditions are attributable in part to cytoskeletal regulation of cellular responses to external and internal cell signals. Moesin (membrane-organising extension spike protein) is a previously described cytoskeletal protein that belongs to the ezrin–radixin–moesin (ERM) family (7). Phosphorylation of moesin activates the protein and mediates its binding to surrounding cytoskeletal proteins or F-actin (8). Previous studies have suggested a role for moesin in inflammation mediator-induced immune responses (9, 10). Inhibition of moesin expression ablates lipopolysaccharide (LPS) responsiveness, and blockage of its function using an anti-moesin antibody inhibits the release of tumour necrosis factor (TNF)-α by LPS-stimulated monocytes/macrophages (10). Homozygous moesin knockout mice exhibit a three-fold reduction in neutrophil infiltration in response to injection of an inflammation mediator (9).

These previous reports demonstrated the potential effects of moesin and HMGB1 on cytoskeletal rearrangement and vascular inflammatory responses, and showed that moesin is the main ERM molecule expressed by the endothelium (11). The present study focused on the effects of moesin in mediating the HMGB1-induced interaction of signalling molecules and the cytoskeleton in HUVECs. We hypothesised that linked inflammatory mechanisms might exist between HMGB1 and moesin, by which HMGB1 could promote severe vascular inflammatory disease. Here, we report evidence that supports this hypothesis.

Materials and methods

Reagents

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan). Bacterial lipopolysaccharide (LPS; serotype: 0111:B4, L5293) was purchased from Sigma. Antibodies recognising total p38 and phosphorylated p38, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RAGE, Rho kinase (ROCK) inhibitor Y-27632, p38 inhibitor SB-203580, ERK inhibitor PD-98059, JNK inhibitor SP-600125, human moesin small interfering siRNA, and control nonsense siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody recognising phosphorylated ROCK (Thr^186) was purchased from AnaSpec Inc. (Fremont, CA, USA). Antibodies recognising total moesin (mouse anti-human antibody, sc-136268) and phosphorylated moesin (rabbit anti-human antibody, sc-12895, Thr^186) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human moesin protein was purchased from Origene (Rockville, MD, USA). Soluble RAGE (sRAGE) was purchased from AnaSpec Inc. (Fremont, CA, USA). Antibodies recognising phosphorylated ROCK (Thr^186) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody recognising phosphorylated ROCK (Thr^186) was purchased from AnaSpec Inc. (Fremont, CA, USA). Antibodies recognising total moesin (mouse anti-human antibody, sc-136268) and phosphorylated moesin (rabbit anti-human antibody, sc-12895, Thr^186) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human moesin protein was purchased from Origene (Rockville, MD, USA). Soluble RAGE (sRAGE) was purchased from AnaSpec Inc. (Fremont, CA, USA).

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as previously described (12–14). All experiments were performed using HUVECs at passage 3–5. Human neutrophils were freshly isolated from whole blood (15 ml) obtained by venipuncture from five healthy volunteers, and maintained as previously described (15, 16).

Patients and normal serum samples

Healthy volunteers served as controls. Patients were classified according to the criteria set by the Sepsis Consensus Conference Committee in 1992 (17, 18), and clinical data, diagnosis, treatment modalities, and blood samples were collected. Serum samples were isolated (by centrifugation at 2000 × g, 5 minutes [min]) within 48 hours (h) after whole blood collection, and the sera were kept at −20 °C until they were assayed. The study protocol was approved (IRB No.; KNUH 2012–01–010) by the Institutional Review Board of the Kyungpook National University Hospitals (Daegu, Republic of Korea).

Animals and husbandry

Male C57BL/6 mice (6–7 weeks old, weighing 18–20 g) were purchased from Orient Bio Co. (Sungnam, Kyungki-Do, Republic of Korea) and used after a 12-day acclimatisation period. The animals were housed five per polycarbonate cage under controlled temperature (20–25 °C) and humidity (40%–45%) under a 12:12 h light/dark cycle and fed a normal rodent pellet diet and supplied with water ad libitum. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University (IRB No.; KNU 2012–13).

RNA interference

Transfection was performed according to the protocols provided by the manufacturer, with slight modifications. Briefly, HUVECs were transfected with optimised concentrations of either human moesin siRNA, control nonsense siRNA, or with mock conditions using siRNA transfection reagent alone. At 48 h after transfection, cells were lysed, and mRNA was extracted and subjected to RT-PCR to evaluate moesin mRNA expression. Whole-cell lysates were subjected to western blot analysis with anti-moesin antibody to confirm siRNA-mediated knockdown of moesin expression.

RNA isolation and real-time PCR

Total cellular RNA was isolated using TRI-Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's suggested protocol. An aliquot (5 μg) of extracted RNA was reverse transcribed into first-strand cDNA using a PX2 Thermal Cycler (Thermo Scientific) by using 200 U/μl M-MLV reverse-transcriptase (Invitrogen) and 0.5 mg/μl of oligo(dT)-adapter primer (Invitrogen) in a 20-μl reaction mixture. Real-time PCR for
moesin and GAPDH was performed using a Mini Opticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad). The following primers were used to amplify moesin: forward 5′-GATGTCGGCGAGGAAAGC-3′ and reverse 5′-GTCTCAGCCGGCAGTAA-3′; and for GAPDH: sense 5′-TGGAGTCAAGGATT-3′ and antisense 5′-CCACGGACGTATCTAGC-3′. The PCR settings were as follows: the initial denaturation at 95 °C was followed by 30 cycles of amplification at 95 °C for 15 seconds (s) and at 58°C for 20 s, with subsequent melting curve analysis by increasing the temperature from 72°C to 98°C. The gene expression levels were quantified relative to GAPDH.

Western blotting

Total cell extracts were prepared by lysing the cells and protein concentrations were determined using a Bradford assay. Equal amounts of protein were separated by SDS-PAGE (10%) and electroblotted overnight onto Immobilon membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with 5% low-fat milk-powder in Tris-Buffered Saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 (TBS-T), followed by overnight incubation with the primary antibody of interest at 4°C. After washing three times with TBS-T, blots were incubated with horseradish-peroxidase-conjugated secondary antibody. After washing, protein bands were visualised by chemiluminescence. Densitometry analysis was performed using the ImageJ Gel Analysis tool.

Immunofluorescence staining

HUVECs were grown to confluence on glass cover slips coated with 0.05% poly-L-lysine in complete media containing 10% FBS and maintained for 48 h. Cells were then stimulated with HMGB1 (1 µg/ml) for 8 h, with or without control or moesin siRNA. For cytoskeletal staining, the cells were fixed in 4% formaldehyde in TBS (v/v) for 15 min at room temperature, permeabilised in 0.05% Triton X-100 in TBS for 15 min, and blocked in blocking buffer (5% BSA in TBS) overnight at 4°C. Then, the cells were incubated with a mouse anti-moesin monoclonal antibody (Santa Cruz), a rabbit anti-E-cadherin antibody (24E10, Cell Signalling Technology, Danvers, MA, USA), and F-actin-labelled fluorescent phalloidin (F 432; Invitrogen) overnight at 4°C. The samples were then labelled with Vybrant DiD dye and then added to previously washed and stimulated HUVECs. HUVEC monolayers were exposed to different reagents as indicated before being subjected to HMGB1 (1 µg/ml) for 8 h. After HUVECs were washed with PBS three times, isolated human neutrophils were allowed to adhere, and non-adherent neutrophils were removed by washing with PBS. The percent adherence of neutrophils was calculated using the formula: % adherence = (adherent signal/total signal) × 100. Results are expressed as the mean of at least three independent experiments.

MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma) was used as an indicator of cell viability as previously described (20–21). HUVECs were seeded at a density of 5 × 10^4 cells in 96-well dishes. Cells were treated with HMGB1 (0.01–5 µg/ml) for 8 or 24 h. Subsequently, MTT was added (final concentration, 1 mg/ml) to each well. Finally, DMSO (150 µl) was added in order to solubilize the formazan salt formed and the amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (Tecan Austria GmbH, Grödig, Austria).

Cell viability assay

Adherence of human neutrophils to HUVECs was evaluated by fluorescent labelling of neutrophils, as previously described (12). Briefly, isolated human neutrophils (1.5 × 10^6/ml, 200 µl/well) were labelled with Vybrant DiD dye and then added to previously washed and stimulated HUVECs. HUVEC monolayers were exposed to different reagents as indicated before being subjected to HMGB1 (1 µg/ml) for 8 h. After HUVECs were washed with PBS three times, isolated human neutrophils were allowed to adhere, and non-adherent neutrophils were removed by washing with PBS. The percent adherence of neutrophils was calculated using the formula: % adherence = (adherent signal/total signal) × 100. Results are expressed as the mean of at least three independent experiments.

In vitro permeability assay

Endothelial cell permeability in response to HMGB1 was quantified by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers using a modified two-compartment chamber model, as previously described (19). HUVECs were plated (5 × 10^4/well) in 12-mm diameter transwells with a pore size of 3 µm for three days. Confluent monolayers of HUVECs were exposed to different reagents as indicated before being subjected to HMGB1 (1 µg/ml) for 8 h. Transwell inserts were then washed with TBS (pH 7.4), followed by the addition of 0.5 ml of Evans blue (0.67 mg/ml) diluted in a growth medium containing 4% BSA. Fresh growth medium was then added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min, the optical density of the sample in the lower chamber was measured at 650 nm.

Cell viability assay

Migration assays were performed in 6.5-mm diameter Transwell plates containing 8-µm pore size filters. HUVECs (6 × 10^4) were cultured for three days to obtain confluent endothelial monolayers. Before the addition of isolated human neutrophils to the upper compartment, HUVECs were exposed to different reagents, as indicated, before being exposed to HMGB1 (1 µg/ml) for 8 h. Transwell plates were then incubated at 37°C, 5% CO2, for 2 h. After HUVECs were washed with PBS three times, isolated human neutrophils in the upper chamber were then aspirated and non-migrating cells that remained on the surface of the filter were removed using a cotton swab. Isolated human neutrophils on the lower side of the filter were fixed with 8% glutaraldehyde and stained with 0.25% crystal violet in 20% methanol (w/v). Each
Expression of cell adhesion molecules (CAMs)

Expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin on HUVECs was determined by whole-cell enzyme-linked immunosorbent assay (ELISA), as previously described (12). Briefly, confluent monolayers of HUVECs were exposed to different reagents, as indicated, before being exposed to HMGB1 (1 µg/ml) for 8 h. The medium was removed, and cells were washed with TBS and fixed with 50 µl of 1% paraformaldehyde for 15 min at room temperature. After washing, 100 µl of mouse anti-human monoclonal antibodies (VCAM-1, ICAM-1, E-selectin, Temecula, CA, USA; 1:50 each) was added. After 1 h (37°C, 5% CO₂), the cells were washed three times, followed by addition of 100 µl of 1:2,000 peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 1 h. The cells were washed another three times and developed using o-phenylenediamine substrate (Sigma). Colourimetric analysis was performed by measuring the absorbance at 490 nm. All measurements were performed in triplicate.

Measurement of F-actin/G-actin ratio

Confluent monolayers of HUVECs were exposed to different reagents, as indicated, before being exposed to HMGB1 (1 µg/ml) for 8 h. Cells were lysed with lysis buffer (50 mM HEPES, pH 6.4, 1 mM MgCl₂, 10 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Sigma)) on ice for 10 min. Cells were harvested, and the cell extracts were centrifuged at 4°C for 1 h at 16,000 x g. The supernatants were recovered (Triton-soluble fraction, containing G-actin), and the pellets were resuspended (Triton-insoluble fraction, containing F-actin) in the same lysis buffer with vigorous agitation.

ELISA for RAGE

To determine the expressions of RAGE, whole-cell ELISA was performed, as previously described (22). Briefly, confluent monolayers of HUVECs were exposed to different reagents, as indicated, before being exposed to HMGB1 (1 µg/ml) for 8 h and then fixed in 1% paraformaldehyde. After washing three times, mouse anti-human monoclonal antibody for RAGE was added, followed by incubation of the cells for 1 h (37°C, 5% CO₂). Cells were then washed, treated with peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 1 h, and washed a further three times, followed by development using o-phenylenediamine substrate (Sigma).

Competitive ELISA for moesin

Ninety-six-well flat plastic microtitre plates (Corning, NY, USA) were coated with moesin protein in 20 mM carbonate/bicarbonate buffer (pH 9.6) containing 0.02% sodium azide, and maintained at 4°C overnight. Plates were rinsed three times in TBS-T and kept at 4°C. Standard moesin, conditioned culture media, or mouse serum was pre-incubated with anti-moesin antibody (diluted 1:500 in TBS-T) in 96-well plastic round microtitre plates for 90 min at 37°C. Pre-incubated samples were transferred to pre-coated plates, and incubated for 30 min at room temperature. Plates were rinsed three times in TBS-T, and then incubated for 90 min at room temperature with peroxidase-conjugated anti-mouse IgG antibodies (diluted 1:1,000 in TBS-T). Plates were rinsed three times in TBS-T and incubated for 60 min at room temperature in the dark with 200 µl substrate solution (100 µg/ml o-phenylenediamine and 0.003% H₂O₂). After stopping the reaction with 50 µl of 8 N H₂SO₄, absorbances were read at 490 nm.

ELISA for interleukin (IL)-1β, -6, and TNF-α

To determine the concentrations of IL-1β, IL-6, and TNF-α, commercially available ELISA kits were used according to the manufacturer's supplied protocol (R&D Systems). Values were measured using an ELISA plate reader (Tecan Austria GmbH).

Cecal ligation and puncture (CLP)

For induction of sepsis, male mice were anesthetized with the anesthetic agents zoletil 50 (Vibac Laboratories, Carros, France) and rompun (Bayer Korea, Seoul, Korea). The CLP-induced sepsis model was prepared as previously described (23). In brief, a 2-cm midline incision was made to expose the cecum and adjoining intestine. The cecum was then tightly ligated using a 3.0 silk suture at 5.0 mm from the cecal tip, punctured once using a 22-gauge needle, gently squeezed to extrude feces, and returned to the peritoneal cavity. The laparotomy site was then stitched using 4.0-silk. In sham controls, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. This protocol was approved in advance by the Animal Care Committee at Kyungpook National University (IRB No.; KNU 2012–13).

In vivo permeability and leukocyte migration assays

CLP-operated mice were administered normal IgG or moesin blocking antibody; the mice were anesthetized with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg/kg) and Rompun (xylazine, 10 mg/kg), which were administered via intraperitoneal injection. For the permeability assay, Evans Blue Dye solution (1%) in phosphate-buffered saline (PBS) was infused intravenously into each mouse. Peritoneal exudates were collected by washing cavities with 5 ml of PBS and were centrifuged at 200 × g for 10 min. The absorbance values of supernatants were read at 650 nm by using a Sunrise ELISA Analyzer (Tecan). Vascular permeability was indicated as the amount (µg) of leaked dye per mouse. In vivo permeability levels were calculated using a standard curve, as described previously (24, 25). For the leukocyte migration assay, peritoneal exudates were collected by washing cavities with 5 ml of PBS. Peritoneal fluid (20 µl) was mixed with 0.38 ml of Türk's solution.
(0.01 % crystal violet in 3 % acetic acid), and the number of leukocytes was determined using a light microscope.

Isolation of mouse neutrophils and FACS analysis

For assessment of neutrophils migration, CLP operated mice or sham mice were treated with moesin antibody or control IgG antibody (300 µg/kg, i.v.) in normal saline for 12 h after CLP surgery. After the mice were then sacrificed, the peritoneal cavities were washed with 5 ml of normal saline and then aspirating the fluid 2 min later. The peritoneal washings were then filtered through a 100-µM filter to remove debris. Mouse neutrophils were isolated from peritoneal fluid and maintained for assay as described before (26). Isolated neutrophils were incubated with predetermined optimal concentrations of fluorochrome-conjugated antibodies to cell surface antigens and/or isotype control antibodies and then washed in staining buffer (PBS without calcium and magnesium with 1 % FBS (Hyclone, Logan, UT, USA) and 0.09 % sodium azide). In this study, Ly6G, mouse neutrophils marker, was used to stain purified neutrophils (27). Neutrophils were then incubated with FITC conjugated Ly6G antibody (Abnova, MAB 5968) for 1 h at 4°C. Stained cells were resuspended in 1 mL of PBS, and the fluorescence quantified using a FACS Aria III (BD).

Figure 1: Knockdown of moesin expression in HUVECs with siRNA and effects of moesin siRNA or kinase inhibitors on HMGB1-mediated cytoskeletal changes and permeability. A, B) HUVECs were either untreated (Un), treated with control siRNA (Cont), or treated with siRNA targeting moesin. Cells were harvested 48 h after transfection and then lysed, and total protein or mRNA was extracted and subjected to western blotting (A) or RT-PCR (B) to evaluate moesin protein (A) or mRNA (B) expression. C) The effects of moesin siRNA on the distribution of F-actin, moesin and E-cadherin before or after HMGB1 treatment were tested by staining for F-actin, moesin and E-cadherin. HUVEC monolayers grown on glass coverslips were transfected with either control or moesin siRNA, followed by stimulation with HMGB1 (1 µg/ml) for 8 h, and immunofluorescence staining of F-actin, moesin and E-cadherin was performed. Arrows indicate intercellular gaps. Images are representative of three independent experiments. D) HUVECs were pretreated with either siRNA (control or moesin), antibody (control IgG or anti-RAGE, 100 µg/ml for 1 h), sRAGE (15 µg/ml, for 1 h), or 25 µM kinase inhibitors (the p38 inhibitor SB-203580 (SB), the ERK inhibitor PD-98059 (PD), the JNK inhibitor SP-600125 (SP), or the ROCK inhibitor Y-27632 (Y)) for 30 min. HUVECs were then stimulated with HMGB1 (1 µg/ml for 8 h). Permeability was monitored by measuring the flux of Evans blue-bound albumin across HUVECs. E) The effects of HMGB1 (incubated for 8 or 24 h) on cellular viability were measured using MTT assays. Values were expressed as mean ± SEM of three separate experiments and student t test was used (B, D, E).
Immunohistochemistry

For analysis of the expression pattern of moesin, aortas from CLP-induced septic (day 4) and sham-operated mice were removed and fixed in 4% formaldehyde solution (Junsei, Japan) in PBS for 20 h at 4°C. Following fixation, the aortas were dehydrated through an ethanol series, embedded in paraffin, and cut into 3-µm sections. Deparaffinised sections were quenched in 3% H2O2 in methanol, washed in PBS, placed in boiled 1 mM Tris solution (pH 9.0), supplemented with 0.5 mM EGTA solution to reveal the antigens, and blocked in PBS supplemented with 1% bovine serum albumin, 0.2% gelatin, and 0.05% saponin for 1 h at room temperature. Sections were incubated with anti-moesin antibody diluted 1:100 in PBS, and supplemented with 0.1% BSA and 0.3% Triton X 100 for 16 hours at 4°C in a humidified chamber. After washing in PBS, supplemented with 0.1% BSA, 0.2% gelatin, and 0.05% saponin, the sections were incubated with peroxidase-conjugated anti-goat IgG antibody (Santa Cruz Biotechnology) for 1 h at room temperature and then developed using the Liquid DAB+ Substrate-Chromogen System (DAKO, Glostrup, Denmark). Counterstaining was performed with 0.5% methyl green in ddH2O. Non-immune goat IgG (Santa Cruz Biotechnology, at the same concentration as the moesin antibody) and anti-CD31 antibody (1:200, Abcam, Cambridge, UK) was used as the negative and positive controls for immunohistochemistry, respectively.

Statistical analysis

All experiments were independently repeated at least three times. Values were expressed as mean ± SEM and Student t-test or ANOVA with Bonferroni corrections was used. Kaplan-Meier survival analysis was performed for evaluation of overall survival rates. All statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS, Inc., Chicago, IL). A p-value < 0.05 was considered statistically significant.

Results

Knockdown of moesin using siRNA suppressed HMGB1-induced vascular barrier disruption

To evaluate the role of moesin in HMGB1-mediated barrier disruptive responses, siRNA targeting moesin and control siRNA were administered to HUVECs. Treatment with siRNA against moesin inhibited moesin protein and mRNA expression in HUVECs but had no effect on the expression of GAPDH (Figure 1A, B). The role of moesin in modulating HMGB1-induced endothelial cell (EC) responses was examined by comparing 1) the distribution of F-actin-labeled fluorescein phalloidin in HUVEC monolayers and 2) the fluxes of albumin across the EC monolayer, between control siRNA and moesin siRNA treatments. Without stimulation with HMGB1, the organisation of F-actin in moesin siRNA-treated HUVECs was similar to that observed in control HUVECs (Figure 1C). Exposure of control siRNA-treated HUVECs to 1 µg/ml HMGB1 for 8 h caused vascular disruption by the formation of paracellular gaps (indicated by arrows) in HUVECs (Figure 1C). However, this change was prevented in HUVECs treated with siRNA against moesin (Figure 1C). These results were confirmed by using tight junction marker, E-cadherin. These results indicate that moesin protein is required for this HMGB1-mediated F-actin rearrangement process. To further address the role of moesin in HMGB1-mediated increases in endothelial permeability, a transwell system was used to examine the fluxes of albumin across the HUVEC monolayer. In each group, HUVECs were treated with either control buffer or 1 µg/ml HMGB1 for 8 h before Evans blue-labeled albumin was added to the upper wells. EC monolayers treated with control siRNA or moesin siRNA alone showed similar permeability to albumin (Figure 1D). In response to HMGB1 stimulation, HUVECs treated with control siRNA showed a significant increase in permeability to albumin, whereas this increase was notably attenuated by moesin siRNA treatment. These data indicate that moesin is essential for the HMGB1-induced increase in EC monolayer permeability.

To assess the cytotoxicity of HMGB1, cell viability assays were performed in HUVECs treated with HMGB1 (0.01–5 µg/ml) for 8 h or 24 h. Although high concentration HMGB1 decreased cell viability, used HMGB1 condition (1 µg/ml for 8 h) in this study did not affect HUVECs viability (Figure 1E).

Effects of moesin on HMGB1-induced inflammatory responses

Next, we investigated whether suppression of moesin could modulate HMGB1-mediated inflammatory responses. The results indicated that HMGB1 enhanced vascular inflammatory responses, such as adhesion and migration of leukocytes toward endothelial cells and upregulation of CAMs such as VCAM-1, ICAM-1, and E-selectin; however, HMGB1-mediated vascular inflammatory responses were significantly inhibited by moesin siRNA treatment (Figure 2A–D). To quantify the effects of moesin siRNA on the actin dynamics of HMGB1-activated HUVECs, we measured the relative amounts of F-actin and G-actin, and the relative abundance of F-actin and G-actin (the F/G-actin ratio) which is the marker for barrier function. As shown in Figure 2E, the relative F/G-actin ratio was increased by HMGB1 treatment; this result was reversed by treatment with moesin siRNA. In addition, HMGB1 enhanced the production of IL-1β, IL-6, and TNF-α, which were suppressed by moesin siRNA (Figure 2F and G). We also investigate the potential effects of exogenously added moesin on the production of IL-1β, IL-6, and TNF-α. Data showed that exogenously added moesin enhanced the proinflammatory cytokine or chemokine production (Figure 2F and G).

HMGB1 induced threonine phosphorylation of moesin in HUVECs

After the essential role of moesin in HMGB1-induced EC responses was confirmed by suppressing moesin expression, the effect of HMGB1 on the threonine phosphorylation of moesin in
confluent HUVECs was examined. Treatment of HUVECs with 1 µg/ml HMGB1 resulted in a significant increase in moesin phosphorylation (Figure 3A).

Involvement of the HMGB1 receptor RAGE in HMGB1-induced moesin phosphorylation

To determine whether moesin is the upstream or downstream target of HMGB1-RAGE axis, HUVECs were pretreated with moesin siRNA. HMGB1 significantly increased the expression of RAGE, but moesin did not affect HMGB1-mediated RAGE expression (Figure 3B). In addition, to test the role of the HMGB1 receptor (RAGE) in HMGB1-induced moesin phosphorylation, HUVECs were pretreated with 100 µg/ml RAGE antibody 1 h before exposure to 1 µg/ml HMGB1 for another 8 h. RAGE antibody blocked HMGB1-induced moesin phosphorylation (Figure 3C, D). Therefore, these results indicate that moesin is the downstream target of the HMGB1-RAGE axis. Furthermore, pretreatment of HUVECs with RAGE antibody for 1 h or soluble RAGE (sRAGE) for 1 h also attenuated HMGB1-induced vascular disruption.

Figure 2: Effects of moesin siRNA or kinase inhibitors on HMGB1-mediated inflammatory responses. HUVECs were pretreated with either siRNA (control or moesin), antibody (control IgG or anti-RAGE, 100 µg/ml for 1 h), or 25 µM kinase inhibitors (the p38 inhibitor SB-203580 (SB), the ERK inhibitor PD-98059 (PD), the JNK inhibitor SP-600125 (SP), or the ROCK inhibitor Y-27632 (Y)) for 30 min. HUVECs were then stimulated with HMGB1 (1 µg/ml for 8 h). A-E) HUVECs were pretreated with control siRNA, moesin siRNA, or with mock conditions using siRNA transfection reagent alone. Then, HUVECs were stimulated with HMGB1 (1 µg/ml for 8 h). HMGB1-mediated RAGE expression was determined by whole-cell ELISA, as described in Methods and expressed in fold increase compared to control. C) HUVECs were pretreated with antibody (control IgG or anti-RAGE, 100 µg/ml for 1 h), and then HUVECs were stimulated with HMGB1 (1 µg/ml for 8 h). Phosphorylation of moesin, ROCK, and p38 was examined by western blot analysis. D) The graphs show the densitometric intensities of phosphorylated moesin, ROCK, and p38 normalised to total levels. n=3 blots. Images are representative of three independent experiments.

Figure 3: Effects of HMGB1 on phosphorylation of moesin, effects of moesin siRNA on HMGB1-mediated RAGE expression, and effects of RAGE on HMGB1-mediated phosphorylation of moesin, ROCK, and p38. A) HUVECs were stimulated with 0, 0.5, or 1 µg/ml HMGB1 for 8 h. The effect of HMGB1 on the expression of phosphorylated or total moesin protein was examined using western blot analysis. B) HUVECs were pretreated with either control siRNA, moesin siRNA, or with mock conditions using siRNA transfection reagent alone. Then, HUVECs were stimulated with HMGB1 (1 µg/ml for 8 h). HMGB1-mediated RAGE expression was determined by whole-cell ELISA, as described in Methods and expressed in fold increase compared to control. C) HUVECs were pretreated with antibody (control IgG or anti-RAGE, 100 µg/ml for 1 h), and then HUVECs were stimulated with HMGB1 (1 µg/ml for 8 h). Phosphorylation of moesin, ROCK, and p38 was examined by western blot analysis. D) The graphs show the densitometric intensities of phosphorylated moesin, ROCK, and p38 normalised to total levels. n=3 blots. Images are representative of three independent experiments. Values were expressed as mean ± SEM of three separate experiments and Student t-test was used (B, D).
Involvement of the ROCK and p38 pathways in HMGB1-induced moesin phosphorylation

To elucidate the signal pathways that might be involved in HMGB1-induced moesin phosphorylation, HUVECs were pre-treated with the ROCK inhibitor Y-27632, the p38 inhibitor SB-203580, the ERK inhibitor PD-98059, and the JNK inhibitor SP-600125, respectively, before being exposed to HMGB1. In HUVECs, HMGB1-induced vascular barrier disruption was strongly prevented by the ROCK inhibitor and the p38 inhibitor, whereas ERK or JNK inhibition did not attenuate this HMGB1-induced alteration (Figure 1D). Inhibition of the ROCK and p38 pathways also clearly attenuated HMGB1-induced increases in inflammatory responses (Figure 2). This evidence confirms the specific effects of the kinases ROCK and p38 on HMGB1 signalling pathways. The roles of ROCK and p38 were further confirmed by results showing that HMGB1 treatment significantly induced phosphorylation of ROCK and p38 proteins via interactions with RAGE, because RAGE antibody blocked HMGB1-induced phosphorylation of ROCK and p38 in HUVECs (Figure 3C, D).

Moesin levels in septic patients, CLP-induced septic mice, and HMGB1-activated HUVECs

We next investigated whether moesin is secreted under septic conditions. First, we determined the concentration of moesin in septic patients. The median serum moesin concentration of 21 healthy volunteers was 0.90 ng/ml (0.84–1.05 ng/ml). In contrast, the mean concentration of moesin increased to 1.68 ng/ml (0.83–9.05 ng/ml, n = 72) in patients with sepsis, 4.23 ng/ml (4.37–12.31 ng/ml, n = 19) in patients with severe sepsis, and 8.76 ng/ml (4.14–19.37 ng/ml, n = 41) in patients with septic shock (Figure 4A). The differences between the groups were statistically significant (p < 0.0001), indicating that moesin is a potentially useful marker for the diagnosis and determination of the severity of sepsis.
of the 132 patients, eight died within 15 days after admission (2 with sepsis, 1 with severe sepsis, and 5 with septic shock). The mean concentration of moesin was significantly higher in non-survivors than in survivors: 12.74 ng/ml (5.82–19.37 ng/ml) vs 3.71 ng/ml (0.83–11.87 ng/ml; p < 0.0001; ▶ Figure 4 B). Thus, in septic patients, the blood level of moesin is related to the severity of illness and patient outcome, and may represent a novel EC dysfunction marker.

Receiver-operating characteristics (ROC) curve analysis for moesin differentiating between sepsis patients (n=72) and those with control (n=21) gave an area under the curve (AUC) of 1.000 (95 % confidence interval [CI] 1.000–1.000) and 1.000 for IL-6 (95 % CI 1.000–1.000). The AUC for moesin differentiating severe sepsis (n=19) and sepsis (n=72) was 0.837 (95 % CI 0.721–0.952) compared with 0.782 (95 % CI 0.667–0.887) for procalcitonin and 0.735 (95 % CI 0.642–0.828) for IL-6. And the AUC for moesin differentiating septic shock (n=41) and all other septic patients (n=91) was 0.996 (95 % CI 0.992–1.000) compared with 0.856 (95 % CI 0.777–0.935) for procalcitonin and 0.835 (95 % CI 0.742–0.928) for IL-6.

In addition, we examined serum concentrations of moesin in the mouse CLP model of sepsis, because this model closely resembles human sepsis (11). At 24 h after the CLP operation, the animals manifested signs of sepsis, including shivering, bristled hair, and weakness. Serum moesin levels increased in CLP-induced septic mice in a time-dependent manner (▶ Figure 4 C). In addition, immunohistochemical analysis showed that moesin expression in endothelium was lower in CLP-induced septic mice than in normal controls (▶ Figure 4 D). Therefore, we investigated whether moesin is secreted in HUVECs under septic conditions. To investigate the inducible release of moesin in resting and activated HUVECs, HUVECs were incubated with or without HMGB1, LPS (gram-negative organism), or Staphylococcus aureus (gram-positive organism) for 0 h to 24 h. HUVEC-released supernatants were separated and analysed using western blotting (▶ Figure 4 E, upper panel) and ELISA (▶ Figure 4 E, lower panel). The data indicated that after activation by HMGB1 or LPS but not S. aureus (data not shown), moesin was secreted into the supernatant, consistent with the in vitro data. This result indicated that moesin is released or shed by septic mediators in the late stage of sepsis. However, we did not find the phosphorylated moesin in patient’s serum with sepsis and HMGB1– or LPS-treated HUVECs culture supernatants (data not shown).

Figure 5: Effects of moesin blocking antibodies on CLP-induced septic lethality. A) Effects of moesin-blocking antibodies on levels of moesin after CLP. Serum was obtained from CLP-operated mice (white bars), CLP-operated mice treated with control IgG (gray bars), and CLP-operated mice treated with moesin-blocking antibodies (black bars) on the days indicated (n = 10). Moesin levels were measured by ELISA. B) Effects of moesin-blocking antibodies on CLP-induced septic lethality. Wild-type male C57Bl/6 mice (n = 20) were treated with moesin-blocking antibodies (●, 12 h; ○, 12 h and 60 h) or control IgG (□) 12 h and/or 60 h after CLP (300 µg/kg, i.v.). Animal survival was monitored every 6 h for 132 h after CLP. Control CLP-operated (●) or sham-operated (○) mice were treated with sterile saline (n = 20). C) Effects of moesin-blocking antibodies on CLP-induced hyperpermeability. Wild-type male C57BL/6 mice were subjected to CLP and treated with moesin-blocking antibodies or control IgG 12 h after CLP (300 µg/kg, i.v.). Mice were then injected intravenously with 1 % BSA-bound Evans blue dye and sacrificed after 6 h, and peritoneal exudates were collected. Vascular permeability was determined based on the amount of Evans blue dye extravasation into the peritoneal cavity. D, E) In addition, CLP-induced migration of leukocytes (D) and mouse neutrophils (E) into the peritoneal cavity of mice was analysed by counting (D) except that CLP-induced migration of leukocytes (D and mouse neutrophils (E) into the peritoneal cavity of mice was analysed by counting (D) or FACS analysis (E). Values were expressed as mean ± SEM of three separate experiments and Student t-test was used (A, C, D). Kaplan-Meier survival analysis was performed for evaluation of overall survival rates (B).
Effects of anti-moesin blocking antibody on CLP-induced septic mortality

Prompted by our clinical and experimental findings, we hypothesized that treatment with anti-moesin blocking antibodies would ameliorate the deleterious effects of CLP on the mouse survival rate. To demonstrate the effects of anti-moesin blocking antibodies treatment on serum levels of moesin and the survival of CLP-induced septic mice, anti-moesin blocking antibodies (300 µg/kg, 12 h after CLP i.v.) were administered. Data showed that the anti-moesin blocking antibodies reduced serum moesin levels significantly (Figure 5A) and lowered CLP-induced mortality (Figure 5B, p < 0.0001). In a subsequent experiment, anti-moesin blocking antibodies were administered once (12 h after CLP) or twice (once 12 h and a second time 60 h after CLP), and Kaplan-Meier survival analysis revealed a significant increase in this treatment group (p < 0.0001, Figure 5B). The final survival rate 132 h after CLP was 30% for once and 40% for twice injection. However, there was no further benefit in the progress of CLP induced sepsis by administering anti-moesin blocking antibodies more often (data not shown). To define the molecular mechanism and assess the role of anti-moesin blocking antibodies in sepsis, we evaluated the effects of anti-moesin blocking antibodies on vascular permeability and leukocytes migration to the vascular endothelium. CLP increased in vivo vascular permeability (Figure 5C) and migration of leukocytes (Figure 5D) and neutrophils (Figure 5E), and anti-moesin blocking antibodies reduced CLP-induced vascular permeability and migration of leukocytes. Thus, these results suggest that reducing moesin levels protects against polymicrobial septic mortality and blocking moesin may provide a therapeutic strategy for the managements of sepsis and septic shock.

Discussion

To the best of our knowledge, our study is the first to confirm the participation of moesin in the HMGB1-induced EC response by knockdown of moesin expression with siRNA. The inhibition of moesin expression attenuated the formation of F-actin stress fibres, and the induction of hyperpermeability and the inflammatory response in HMGB1-stimulated HUVECs. In addition, HMGB1 induced the phosphorylation of moesin at the conserved threonine residue. Moesin phosphorylation requires HMGB1-induced signalling pathways that include the binding of HMGB1-RAGE and activation of ROCK and p38 (Figure 6).

Previous studies (28, 29) have shown that endothelial cells responded to HMGB1 stimulation by reorganisation of F-actin, disruption of vascular-endothelial-cadherin-composed adherence junctions, and increased monolayer permeability through mechanisms including HMGB1-RAGE binding and activation of the ROCK and p38 pathways. Data from the present study confirmed that moesin acts as a linker between HMGB1-mediated inflammatory signalling pathways. The results showed that silencing of moesin expression inhibited HMGB1-mediated disruption of F-actin, hyperpermeability (Figure 1), adhesion and transmigration of human neutrophils, and expression of CAMs such as VCAM, ICAM, and E-selectin, and alteration of the F-actin/G-actin ratio (Figure 2) which all play pivotal roles in vascular inflammatory responses. We also revealed that moesin is phosphorylated following HMGB1 application, but that the phosphorylation is inhibited by RAGE antibody (Figure 3 A, C, and D). These data are the first to demonstrate the involvement of moesin phosphorylation in HMGB1-induced endothelial responses. It is well known that HMGB1 exerts its cellular effects by binding with specific receptors to trigger subsequent signalling pathways (2). In this study, blocking HMGB1-RAGE binding by anti-RAGE IgG inhibited HMGB1-induced moesin phosphor-
ylation and subsequent hyperpermeability and septic responses in HUVECs (Figure 1D and Figure 2), showing the importance of HMGB1-receptor binding in this sustained cellular activation process. This study confirmed that RAGE expression was enhanced after exposure to HMGB1 and showed that the inhibition of moesin expression by moesin siRNA did not affect HMGB1-induced RAGE expression. However, the phosphorylation of moesin was strongly inhibited by anti-RAGE antibody. These results indicate that moesin is the downstream target of HMGB1 (Figure 6).

The kinases that phosphorylate moesin have been well characterised, and the participants include ROCK (30, 31), p38 (11), and PKC (11), etc. Rho GTPases play a central role in the function of the endothelial cytoskeleton, and moesin proteins act both upstream and downstream of Rho GTPases (32). Mitogen-activated protein kinases (MAPKs) represent the major signalling system that transduces a variety of extracellular signals through a cascade of intracellular protein phosphorylation processes (33). p38 is an important downstream signal triggered by HMGB1-RAGE binding (34, 35). HMGB1 is known to induce hyperpermeability by promoting phosphorylation of p38 (34, 36), and our recent studies have shown that HMGB1 application causes the activation of p38 and subsequently increases permeability in HUVECs (37–39). Because the threonine phosphorylation of moesin proteins has also been shown to be related to MAPK activation (11), it is reasonable to estimate that HMGB1-induced phosphorylation of moesin is mediated by MAPKs. Several studies have shown that there is cross-talk between the ROCK and p38 pathways, and most of the reports have demonstrated that ROCK is the upstream regulator of p38 activation (40). We specified p38 and ROCK as the critical subgroup of MAPKs by screening for the effects of MAPKs on HMGB1-triggered inflammatory responses, using inhibitors of the p38, ERK, and JNK pathways (Figure 1D and Figure 2). The resultant HMGB1-triggered inflammatory and hyperpermeability responses to HMGB1 stimulation that we observed in HUVECs were attenuated by downregulation of p38 and MOCK with their respective inhibitors (Figure 1D). Therefore, we wish to emphasise that the effects of ROCK and p38 are important in HMGB1-induced moesin phosphorylation and inflammatory responses (Figure 6).

Effective treatment of sepsis requires prompt diagnosis of the syndrome, as sepsis can lead to severe sepsis or septic shock during the “golden hour” in the emergency department (41). Markers for the prognosis of septic patients are also needed. The utility of a sepsis biomarker would be further enhanced if it could both indicate the severity of sepsis and act as a guide to the potential efficacy of therapy. Pathophysiological markers that could illuminate the diagnosis, severity, and/or prognosis of the disease have been sought (42). Various molecules, including C-reactive protein, procalcitonin, interleukin-6, von Willebrand factor protein, high-density lipoprotein cholesterol, and N-terminal pro-brain natriuretic peptide have been identified in the blood of patients with septic shock and have been proposed as markers of the severity of sepsis; however, none have gained wide clinical acceptance (42). Here, we propose moesin as a novel biomarker of sepsis, because we found that circulating levels of moesin were increased in septic patients and that the degree of moesin elevation was linked to the severity of illness. Thus, in septic patients, blood levels of moesin are related to the severity of illness and the patient outcome, and may represent a novel marker of EC dysfunction, although further clinical studies are necessary (Figure 6).

Based on the current findings that 1) moesin is upregulated and secreted by HMGB1 or LPS in cell culture media; 2) plasma concentrations of moesin in sepsis patients were significantly higher than healthy controls, which was strongly correlated with disease severity; 3) treatment of HUVECs with HMGB1 enhanced moesin phosphorylation in cell lysates; 4) extracellular moesin is not phosphorylated by HMGB1 or LPS; 5) moesin is not phosphorylated in serum of septic patients or CLP-induced septic mice, we conclude that the potential mechanism of HMGB1-mediated extracellular moesin secretion might be the de-phosphorylation of moesin and in the cell, moesin is phosphorylated by HMGB1 which resulted in the septic responses. Therefore, when moesin is secreted to the extracellular environment, moesin is used for diagnostic marker for sepsis (Figure 6).

A previous report (43) showed that moesin antibody was found to inhibit LPS-induced responses. And, that inhibition was specific for the LPS-moesin interaction, because inflammatory responses by unrelated receptors were not inhibited by moesin antibody. The response to the S. aureus was also not abolished by moesin antibody, confirming that the effects were unique to LPS-elicited responses (43). We also investigated moesin secretion stimulated of gram-negative organism (LPS) or gram-positive organism (S. aureus); LPS, similar to HMGB1, stimulated moesin secretion but S. aureus had no effect on moesin secretion which is consistent with previous report (43).

Over the last three decades, our understanding of the pathophysiology of sepsis has progressed considerably. Since TNF and IL-1 mimic septic shock in animal models of sepsis, several clinical trials have focused on neutralisation of these inflammatory mediators (44). These trials, however, have had very disappointing results. During the 1990s, many scientists and companies regarded TNF and IL-1 as the ‘bad guys’ in sepsis despite the fact that important experimental and clinical data contradicting this viewpoint were already available. For example, murine anti-TNF monoclonal antibody preparations (CB0006, Celltech; BAYx1351, Nereliomomab, Bayer) were the first anti-TNF agents to be tested for treatment of human septic shock, but, despite some early positive result (45), a large RCT (NORASEPT II) found no beneficial effect on mortality, the duration of septic shock, or the resolution of sepsis-induced organ failure, even in a subgroup of patients who had elevated TNF levels on study entry (46). And IL-1 acts synergistically with TNF to produce the haemodynamic features of septic shock (47). Studies of patients with sepsis have focused on the IL-1 receptor antagonist (IL-1RA). Although the survival benefits of IL-1RA in a phase 2 clinical (48), a second, phase 3 trial was terminated after an interim analysis showed no significant differences in mortality rates (49). The results of clinical trials of immunomodulatory therapies have been more disappointing than encouraging, and many reasons have been put forward to explain the
What is known about this topic?

- Sepsis is a life-threatening condition that arises when the body’s response to infection.
- HMGB1 is a late mediator of sepsis and a therapeutic target for sepsis.
- Moesin is a cytoskeletal protein involved in cytoskeletal changes and paracellular gap formation.

What does this paper add?

- To the best of our knowledge, here we have demonstrated for the first time that moesin was found to be required in HMGB1-induced F-actin rearrangement, hyperpermeability.
- HMGB1-treated human endothelial cells exhibited an increase in phosphorylation of moesin.
- Circulating levels of moesin in sepsis patients were significantly higher than healthy controls, which was strongly correlated with disease severity.
- Administration of blocking moesin antibodies attenuated CLP-induced septic death.

Conflicts of interest

None declared.

References


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apparent “failures” (50–51); The experimental agents are ineffective, Doses of experimental agents are inadequate, Timing of intervention is inadequate, Patient population is too heterogeneous, and Single therapies may be ineffective.

In summary, the present study demonstrates that by binding to its specific receptor, RAGE, HMGB1 elicits a complex signalling system that includes the activation of ROCK and p38. These kinases then phosphorylate the threonine residues of moesin and lead to the linking of activated moesin and HMGB1 with F-actin, resulting in reorganisation of the cytoskeleton, disruption of endothelial barrier function, and septic responses. This study is the first to elucidate the important role of moesin in HMGB1-induced morphological and functional alterations in HUVECs. We also suggest moesin as a novel biomarker of sepsis and therapeutic target, because circulating levels of moesin were found to be elevated in patients with sepsis and to be correlated with disease severity and Administration of blocking moesin antibodies attenuated CLP-induced septic death.

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