Original Paper

Unfractionated Heparin Alleviates Human Lung Endothelial Barrier Dysfunction Induced by High Mobility Group Box 1 Through Regulation of P38–GSK3 β –Snail Signaling Pathway

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Key Words

Endothelium • Inflammation • High mobility group box protein 1 • Heparin • EndoMT

Abstract

Background/Aims: The high mobility group box 1 (HMGB1) has been regarded as an important inflammatory mediator. Previous studies showed the involvement of HMGB1 protein in the dysfunction of endothelial barrier function during acute lung injury. However, the molecular mechanism remains unclear. **Methods:** In this study, we used recombinant human HMGB1 (rhHMGB1) and HMGB1 plasmid to treat human pulmonary microvascular endothelial cell (HPMECs). We examined endothelial permeability by measuring TEER value and HRP flux. Western blot and real-time PCR were used to examine change of endothelial-to-mesenchymal transition (EndoMT) markers and related pathways. Immunofluorescence was used to examine localization and expression of ZO-1 and VE-cadherin. SB203580 was used to block p38 pathway. Unfractionated heparin (UFH) and RAGE siRNA were also used to antagonize the effect of HMGB1. **Results:** We showed that HMGB1 induced EndoMT with downregulation of ZO-1 and VE-cadherin at both mRNA and protein levels in HPMECs. We also demonstrated that HMGB1 upregulated endothelial permeability by measuring TEER value and HRP flux. Moreover, HMGB1 activated p38 / GSK3 β / Snail signaling pathway and treatment with p38 inhibitor SB203580 abolished its biological effects. In addition, we found that UFH was able to reverse the effect of HMGB1 on EndoMT and endothelial permeability through inhibition of p38 signaling in a dose-dependent manner. We discovered that RAGE, a membrane receptor of HMGB1, transduced p38 / Snail pathway to EndoMT. RAGE siRNA inhibited the effect of HMGB1 induced EndoMT in HPMECs. **Conclusion:** The present study demonstrated that HMGB1 induced EndoMT through RAGE receptor and p38 / GSK3 β / Snail pathway. While UFH antagonized HMGB1 and maintained the integrity of the endothelial barrier through p38 inhibition.

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Introduction

Endothelial cells (EC) play important roles in maintaining circulatory integrity. During septic inflammation, cytokines and inflammatory mediators induce morphological changes of EC [1, 2]. Disruption of tight junction (TJ) and adherens junction (AJ) of vascular EC often leads to increased vascular permeability, which plays an important role during the development of septic shock and acute lung injury [3, 4]. Tight junctions are composed of a branching network of sealing strands which are composed of transmembrane proteins including occludin, claudin, and ZO-1. Adherens junctions are composed of VE-cadherin and beta-catenin. Both tight junction and adherens junction play critical roles in maintaining endothelial barrier function. Disruption of TJs and AJs causes elevated permeability of endothelial cells.

The high mobility group box 1 (HMGB1) is an important chromatin protein which binds to DNA and plays a role in transcriptional regulation. Extracellular HMGB1 is secreted by immune cells (including macrophages, monocytes, dendritic cells) during inflammation process [5]. HMGB1 functions as a cytokine mediator of inflammation, which further stimulates a release of more pro-inflammatory cytokines including tumor necrosis factor- α and interleukin-1, 6, 8. Neutralizing HMGB1 by antibody confers protection against inflammatory tissue injury during arthritis, ischemia, colitis, and sepsis [5]. Our previous study demonstrated that downregulation of HMGB1 protects against the development of acute lung injury [6], the development of which has been reported to associate with increased vascular permeability caused by AJ and TJ disruption of vascular endothelial cells [7]. However, the molecular mechanism of HMGB1 induced permeability remains unclear.

Unfractionated heparin (UFH) is widely used anticoagulant drug that enhances antithrombin inhibition. In addition, UFH shows a wide range of protective function including inflammatory suppression [8], apoptosis prevention [9], reducing microvascular permeability [10]. UFH also functions as a protector of endothelial cells. UFH stabilizes endothelial surface layer [11], elevates vascular endothelial barrier function [12]. It is reported that UFH inhibits HMGB1 induced inflammation by blocking the binding of HMGB1 to macrophages surface [13]. A recent report demonstrated that UFH changes the HMGB1 conformation and decreases its affinity toward receptor [14]. Thus, we investigated the mechanism that UFH inhibits HMGB1 induced vascular permeability and stabilizes the barrier function of pulmonary microvascular endothelial cell. We hypothesized that UFH exerts its function by inhibiting p38–GSK3 β –Snail signaling activated by HMGB1.

Materials and Methods

Cell culture, treatment, and transfection

The Human pulmonary microvascular endothelial cell (HPMEC) was purchased from ScienCell Research Laboratories. Cells were cultured in DMEM (Gibco, USA) containing 10% serum (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂. HPMECs were incubated with rhHMGB1 at the concentration of 50, 100 and 500 ng/ml for 1, 6 and 24 hours respectively. rhHMGB1 was trypsinized to demonstrate that the roles of rhHMGB1 were not mediated by micro-amounts of endotoxin mixed in the protein [14]. RAGE, TLR4 siRNA and negative control siRNA were obtained from Santa Cruz (CA, USA). DharmaFECT 1 transfection reagent was used for siRNA transfection (GE Healthcare, USA). pCMV6-HMGB1 plasmid and control plasmid was obtained from Origene company (Rockville, USA). Attractene reagent (Qiagen, Hilden, Germany) was used for plasmid transfection. Recombinant human HMGB1 (rhHMGB1) was obtained from Primegene Company (Shanghai, China). Unfractionated heparin was obtained from Shanghai No.1 Biochemical & Pharmaceutical Company (Shanghai, China). SB203580 was obtained from Sigma (St. Louis, MO, USA).

Western blot and ELISA

Protein extraction and quantification were performed by the Bradford method. Whole cell lysates were used for western blot analysis. About 50 μ g protein was transferred to PVDF membranes after separated by

SDS-PAGE. The membranes were incubated at 4°C overnight with VE-cadherin (1:1000, Abcam, USA), ZO-1 (1:800, Abcam, USA), Claudin 5, Occludin, RAGE, TLR4 (1:800, Proteintech, USA), DDK (1:1000, Origene, USA), p-p38, p38, p-ATF2, ATF2, p-GSK3 β , GSK3 β , Snail (1:1000, Cell Signaling, USA) and Actin (1:3000; Santa Cruz, USA). The membranes were incubated with HRP-coupled anti-mouse/rabbit IgG antibody (1:2000 dilution, Cell Signaling Technology, USA). Proteins were visualized using ECL kit (Pierce, USA) and images were captured using DNR BioImaging System (DNR, Israel). Western blot quantification was performed using ImageJ software. ELISA was performed using HMGB1 ELISA kit from CUSABIO biotech (Wuhan, China) according to the manufacturer's protocol.

Real-time PCR

RNA extraction was performed using RNAiso (TAKARA, China). Reverse transcription of 1 μ g of RNA was performed using the PrimeScript RT Mastermix (TaKaRa, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green Master Mix from ABI (Applied Biosystem, USA). Real-time PCR was carried out using ABI 7500 (Applied Biosystems, USA). Relative quantification of target genes was calculated using the 2- $\Delta\Delta$ Ct method.

Immunofluorescence

Cells on Lab-Tek Chamber Slides were fixed with ice-cold methanol, permeabilized using 0.1% Triton X-100, blocked with serum, and then incubated with primary antibodies (ZO-1 and VE-cadherin, 1:400, Abcam, USA) and Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, USA). The image was taken using Olympus BX53 microscope (Olympus, Japan).

Transendothelial electrical resistance (TEER) measurements

HPMECs were seeded on a Transwell chamber and to reach 100% confluence. The transendothelial electrical resistance value of HPMECs monolayers on Transwell filters was examined by Millicell-ERS instrument (Millipore, MA, USA). Electrical resistance unit was expressed in Ohms per cm² by calculating the area of Transwell filter.

Horseradish Peroxidase (HRP) Flux Measurement

HPMECs were seeded on a Transwell chamber and to reach 100% confluence. Then horseradish peroxidase (HRP, Sigma, USA) was added to the upper chamber of the Transwell filter at a concentration of 0.5 μ mol/L in DMEM without serum. After indicated treatment, the medium of lower transwell chamber was collected, visualized by 3, 3',5, 5'-Tetramethylbenzidine and measured at the wavelength of 370 nm by microplate reader (Tecan, USA). The HRP flux was expressed as picomoles passed per square centimeter surface area.

Statistical analysis

Data are expressed as means $\pm$ SD. SPSS 17 for Windows was used for all statistical analyses. Student's t-test was used to examine the difference between groups. $P < 0.05$ was considered as statistically significant.

Results

HMGB1 treatment disrupts barrier function of HPMECs through downregulation of tight junction / adherens junction related proteins

First, we assessed whether HMGB1 induces tight junction and adherens junction disruption in human pulmonary microvascular endothelial cell by checking protein expression of VE-cadherin, ZO-1, Claudin 5 and Occludin. rhHMGB1 was given at the concentration of 50, 100 and 500 ng/ml for 1, 6 and 24 hours. As shown in Fig. 1A, rhHMGB1 treatment reduced protein expression of VE-cadherin, ZO-1, Claudin 5 and Occludin at a dose-dependent manner. 100 and 500 ng/ml for 24 hours treatment of rhHMGB1 exhibited the most significant effect. mRNA change of VE-cadherin and ZO-1 was examined by real-time PCR analysis. The results showed that rhHMGB1 downregulated mRNA expression of VE-cadherin and ZO-1 (Fig. 1B). To clarify further the mechanism of tight junction / adherens junction disruption, the

Fig. 1. HMGB1 disrupts microvascular endothelial barrier function via down-regulation of VE-Cadherin and ZO-1. (A) HPMECs were treated with different doses of rhHMGB1 for 1, 6, and 24 hours and protein expression of VE-cadherin, ZO-1, Claudin 5 and Occludin was examined by western blot. Relative intensity was indicated in each lane. (B) HPMECs were treated with different doses of rhHMGB1 for 1, 6, and 24 hours and mRNA of VE-cadherin and ZO-1 was examined by realtime PCR. (C) HPMECs were treated with different doses of rhHMGB1 for 1, 6, and 24 hours and localization of VE-cadherin and ZO-1 was examined by immunofluorescence. Original magnification 400X. (D) HPMECs were grown on a transwell membrane and treated with different concentration of rhHMGB1 for different period. Transendothelial electrical resistance (TEER) values were measured after treatment. (E) HPMECs were

grown on a transwell membrane and treated with different concentration of rhHMGB1 for different period. HRP was added to the upper chamber of the Transwell filter and HRP flux was calculated. Data are presented as mean $\pm$ SD of three separate experiments. * indicates $p < 0.05$ compared with control.

ZO-1 and VE-cadherin were examined by immunofluorescence microscopy. VE-cadherin, the major component of adherens junction and ZO-1, the major component of tight junction mainly localized in the cell periphery and cell-cell contact. After treatment of rhHMGB1, expression of VE-cadherin and ZO-1 decreased depending on concentration. ZO-1 protein also slightly relocalized from cell border into cytoplasm (Fig. 1C). We further checked the effect of rhHMGB1 on endothelial permeability by measuring TEER value and HRP flux. As shown in Fig. 1D & e, rhHMGB1 treatment downregulated TEER value and upregulated the rate of HRP flux in a dose-dependent manner. Taken together, these results demonstrated that rhHMGB1 increased the permeability of human pulmonary microvascular endothelial cell by downregulation of VE-cadherin and ZO-1 mRNA and protein expression.

HMGB1 downregulates VE-cadherin and ZO-1 through regulation of p38-GSK3β-Snail signaling in HPMECs

Then, we employed pCMV6-HMGB1 plasmid to upregulate its endogenous expression in HPMECs. By checking DDK1 protein tag transfection of HMGB1 plasmid significantly upregulated its expression (Fig. 2A). We performed ELISA to examine HMGB1 release

in the culture medium. As shown in Fig. 2A, after 24, 48 and 72 hours of transfection, the concentration of HMGB1 protein in the medium was increased. Thus the HMGB1 plasmid mimics the effect of exogenous rhHMGB1 stimulation. Western blot results showed that HMGB1 overexpression downregulated ZO-1, VE-cadherin, claudin 5 and occludin. Immunofluorescence showed that HMGB1 overexpression downregulated membrane localization of ZO-1 and VE-cadherin (Fig. 2B). mRNA expression of VE-cadherin and ZO-1 was also downregulated (Fig. 2C), which was similar to the effects of rhHMGB1 treatment (Fig. 1A). To find out the potential mechanism of HMGB1 induced downregulation of ZO-1 and VE-cadherin, we checked several related signaling pathways. We found that HMGB1

Fig. 2. HMGB1 downregulates VE-cadherin and ZO-1 through p38 / ATF-2 / GSK3 β / Snail signaling. (A) HPMECs were transfected with pCMV6-HMGB1 plasmid and changes of downstream proteins were examined by western blot. Relative intensity was indicated in each lane. After plasmid transfection (24, 48 and 72 hours) and ELISA was performed to examine HMGB1 release in the culture medium. The concentration of HMGB1 protein in the medium was shown in the bar chart. HMGB1

existed in culture medium of HPMEC cells transfected with plasmid. (B) HMGB1 transfection downregulated membrane expression of VE-cadherin and ZO-1. Original magnification $\times 400$. (C) HMGB1 transfection downregulated mRNA expression of VE-cadherin and ZO-1. (D) HPMEC cells were treated with p38 inhibitor together with rhHMGB1 treatment and HMGB1 transfection. Expression of p38, ATF2 and GSK3 β (Thr390) phosphorylation and VE-cadherin, ZO-1, Snail protein was examined by western blot. (E) HPMEC cells were treated with p38 inhibitor together with rhHMGB1 treatment and HMGB1 transfection. Membrane expression of VE-cadherin and ZO-1 was examined by immunofluorescence. Original magnification $\times 400$. (F, G) HPMEC cells were treated with p38 inhibitor together with rhHMGB1 treatment and HMGB1 transfection. mRNA expression of VE-cadherin and ZO-1 was examined by realtime PCR. (H, I) Examination of TEER value and HRP flux of HPMECs treated with p38 inhibitor together with rhHMGB1 treatment and HMGB1 transfection. Data are presented as mean $\pm$ SD of three separate experiments. * indicates $p < 0.05$ compared with control.

upregulated phosphorylation of p38, ATF2 and GSK3 β (Thr390) and Snail protein. Activation of p38 signaling has been reported to induce downstream ATF2 and GSK3 β (Thr390) phosphorylation, which in turn upregulates Snail protein. Snail is a well known mediator of epithelial-mesenchymal transition, which could induce downregulation of VE-cadherin and ZO-1. To validate the involvement of p38 signaling in HMGB1 induced biological effects, we employed p38 inhibitor SB203580. As shown in Fig. 2D, p38 inhibitor treatment significant blocked downstream ATF2 and GSK3 β (Thr390) phosphorylation. In addition, p38 inhibitor treatment abolished the effects of rhHMGB1 treatment and HMGB1 plasmid on ZO-1, VE-cadherin and Snail (Fig. 2D). Immunofluorescence showed that pre-treatment with p38 inhibitor maintained membrane expression of both ZO-1 and VE-cadherin protein (Fig. 2E), which was in accord with real-time PCR results (Fig. 2F & G). The effect of p38 inhibition on endothelial permeability was also checked by measuring TEER value and HRP flux. As shown in Fig. 2H & I, p38 inhibitor significant blocked the effect of HMGB1 on TEER value.

Fig. 3. UFH dose-dependently antagonizes the effect of HMGB1 on barrier function and VE cadherin / ZO-1 through downregulation of p38 signaling pathway. (A) HPMECs were incubated with rhHMGB1 and transfected with pCMV6-HMGB1 plasmid. Different dose of UFH (5 or 10 U/L) was used to treated cells and protein expression was examined for ZO-1, VE-cadherin, claudin 5, Occludin, p-p38, p-ATF2, p-GSK3 β (Thr390) and Snail. Relative intensity was indicated in each lane. (B) Immunofluorescence staining of VE-cadherin and ZO-1 was performed and membrane expression of both proteins was examined by fluorescence microscopy. Original magnification $\times 400$. (C, D) The effect of UFH and HMGB1 on mRNA expression of VE-cadherin and ZO-1 was examined by realtime PCR. (E) Examination of TEER value of HPMECs treated with HMGB1 and UFH. (F) Assessment of HRP flux of HPMECs treated with HMGB1 and UFH. * $p < 0.05$. UFH, unfractionated heparin.

p38 inhibitor also abolished the effect of HMGB1 on HRP flux. Taken together, these results indicated that HMGB1 upregulates endothelial permeability and downregulates VE-cadherin and ZO-1 through activation of p38 signaling pathway.

UFH blocks HMGB1-induced downregulation of ZO-1 and VE-cadherin in HPMECs

Since UFH has been reported to stabilize endothelial barrier function and antagonize HMGB1 induced inflammatory response, we explored if UFH could regulate expression of junction molecules in HMGB1-treated and transfected HPMEC cells. 5 or 10 U/L UFH was used to treat HPMECs together with HMGB1 transfection or treatment. As shown in Fig. 3A & B, HMGB1 transfection, and treatment reduced protein levels of ZO-1, VE-cadherin, claudin 5 and Occludin, while UFH partly reduced the effects of HMGB1 on these proteins in a dose-dependent manner. Membrane localization, protein and mRNA expression changes of ZO-1 and VE-cadherin were also validated by immunofluorescence and real-time PCR (Fig. 3B, C, D). As shown in Fig. 3E & F, UFH treatment reversed the role of HMGB1 in endothelial permeability. Furthermore, we explored if the antagonizing role of UFH was dependent on p38 signaling. As shown in Fig. 3A, UFH dose-dependently downregulated p-p38, p-ATF2, p-GSK3 β (Thr390) and Snail, suggesting UFH could partly block HMGB1-induced downregulation of ZO-1 and VE-cadherin, possibly through regulation of p38 / GSK3 β / Snail signaling pathway.

Fig. 4. Blockage of p38 pathway diminished the effect of UFH on HMGB1 induced EndoMT. (A) HPMEC cells were treated with HMGB1, different concentration of UFH and p38 inhibitor SB203580. Protein expression of ZO-1, VE-cadherin, claudin 5, Occludin, p-ATF2, p-GSK3 β (Thr390) and Snail was detected by western blot analysis. Relative intensity was indicated in each lane. (B, C) mRNA expression of VE-cadherin and ZO-1 was examined by realtime PCR. (D) Examination of TEER value of HPMECs. (E) Examination of HRP flux of HPMECs. (F) Immunofluorescence staining of VE-cadherin and ZO-1 was performed and membrane expression of both proteins was examined by fluorescence microscopy. Original magnification $\times 400$. * $p < 0.05$. UFH, unfractionated heparin.

UFH blocks HMGB1-induced endothelial permeability through the p38 signaling pathway

To demonstrate the involvement of p38 signaling in UFH induced effect, we employed p38 inhibitor together with UFH in HPMECs. As shown in Fig. 4A, SB203580 effectively reduced ATF2 and GSK3 β (Thr390) phosphorylation. In addition, in cells treated with p38 inhibitor, UFH failed to reduce Snail expression or upregulate ZO-1 / VE-cadherin protein expression and membrane localization. p38 inhibitor and UFH showed a synergistic effect on the expression of VE-cadherin, ZO-1, and Snail. Real-time PCR analysis also showed that the blocking effect of UFH on HMGB1 was significantly reduced in the presence of p38 inhibitor (Fig. 4B & C). As shown in Fig. 4D & E, in SB203580, treated cells, UFH failed to reverse the role of HMGB1 in endothelial permeability. The above results demonstrated that UFH antagonizes the effect of HMGB1 through modulation of p38 activity.

HMGB1-induced ZO-1 / VE-cadherin loss in HPMECs involves RAGE

Previous reports indicated that HMGB1 exerts its biological effects through binding to membrane receptors including RAGE, TLR2, and TLR4, which leads to activation of

Fig. 5. HMGB1 activates p38 and EndoMT through RAGE. (A) HPMECs were transfected with RAGE siRNA and then treated with rhHMGB1 and HMGB1 plasmid. Western blot was used to examine the protein expression of RAGE, p-p38, p-GSK3 β (Thr390), ZO-1, VE-cadherin, claudin 5, Occludin and Snail. Relative intensity was indicated in each lane. (B) HPMECs were transfected with RAGE siRNA and then treated with rhHMGB1 and HMGB1 plasmid. Immunofluorescence staining of VE-cadherin and ZO-1 was examined by fluorescence microscopy. Original magnification $\times 400$. (C) HPMECs were transfected with TLR4 siRNA and then treated with rhHMGB1. TLR4 depletion did not influence the role of rhHMGB1 on ZO-1 and VE-cadherin. (D, E) mRNA expression of VE-cadherin and ZO-1 was examined by realtime PCR. (F, G) Examination of TEER value and HRP flux of HPMECs. * $p < 0.05$.

downstream signaling cascades. We silenced these receptors using siRNA and found that only depletion RAGE reversed the effect of HMGB1 on ZO-1 and VE-cadherin. Silencing TLR4 expression in HPMECs by siRNA could not reverse the role of rhHMGB1 on ZO-1 and VE-cadherin (Fig. 5C). As shown in Fig. 5A, RAGE knockdown blocked HMGB1-induced downregulation of VE-cadherin and ZO-1 and upregulation of Snail. Moreover, RAGE knockdown downregulated p38 and GSK3 β (Thr390) phosphorylation, blocking HMGB1 on p38 signaling. Immunofluorescence demonstrated that RAGE knockdown restored VE-cadherin and ZO-1 membrane expression, making HPMECs irresponsive to HMGB1 treatment (Fig. 5B), which was in accord with real-time PCR results (Fig. 5C & D). Then we checked the effect of RAGE knockdown on endothelial permeability. RAGE knockdown reversed the inhibitory role of HMGB1 on TEER value and the upregulation of HRP flux. In HPMECs with RAGE depletion, the effect of HMGB1 was abolished.

Discussion

This study demonstrated that HMGB1 induces endothelial-to-mesenchymal transition (EndoMT) and upregulates endothelial permeability through RAGE receptor and p38 signaling pathway, which could be blocked by UFH treatment. During the progression of acute lung injury, release of inflammatory mediators often occurs in lung tissues with infiltration of immune cells. These processes lead to microvascular dysfunction and local inflammatory response, which in turn causes microvascular disorder, alveolar capillary barrier leakage, alveolar edema, and apoptosis. HMGB1 is one of the pro-inflammatory cytokines during acute lung injury. Our previous study demonstrated that blocking HMGB1 rescues barrier function and inhibits inflammation response of endothelial cells [15-17]. In this study, we further investigated its underlying mechanism. Using cultured human pulmonary microvascular endothelial cell, we were able to demonstrate that HMGB1 treatment/transfection increased endothelial permeability, disrupted endothelial barrier function and induced endothelial-to-mesenchymal transition. In addition, a similar function of HMGB1 on endothelial permeability has been reported in various type endothelial cells [18-20].

Mechanism study revealed that elevated permeability is accompanied by downregulation of ZO-1 and VE-cadherin, which serve as the key component of tight junction (TJ) and adherens junction (AJ). Through immunofluorescence staining, we found that ZO-1 was expressed in the cytoplasm and the plasma membrane, while VE-cadherin were predominantly localized to the plasma membrane. Decreased membrane VE-cadherin expression was observed following HMGB1 treatment. HMGB1 also decreased both cytoplasm and membrane ZO-1 protein expression. Thus our results demonstrated that HMGB1 directly induced EndoMT with downregulation of both TJ and AJ, which plays important barrier function such as material transport and maintenance of osmotic balance. Our result was in accord with previous reports showing that recombinant HMGB1 could induce endothelial dysfunction in mice [21] and downregulated ZO1/2 and VE-cadherin in mouse endothelial cells [22].

Furthermore, we showed that HMGB1 downregulates TJ and AJ components through activation of the p38 / GSK3 β / Snail signaling pathway. p38 / GSK3 β signaling has been reported to induce an epithelial-to-mesenchymal transition in peritoneal mesothelial cells and cancer cells [23, 24]. It has been reported that HMGB1 could activate GSK3 β through PI3K-dependent AKT phosphorylation in bronchial epithelial cells. However, we did not observe a change of AKT phosphorylation in HPMECs, suggesting HMGB1 induced signaling may be cell-type dependent. Snail is a zinc-finger transcription factor that can repress E-cadherin transcription and cause epithelial-mesenchymal transition in epithelial cells [25]. In endothelial cells. Snail also downregulates the activity of the VE-cadherin promoter, inhibits its mRNA / protein expression and induces EndoMT [26]. Phosphorylation of Snail by GSK3 β regulates its stability, cellular localization, and function. Taken together, our results demonstrated a link between HMGB1 and p38 / GSK3 / snail signaling, which is responsible for disruption of barrier function.

ELISA result confirmed that HMGB1 transfection upregulates HMGB1 release in the culture medium. Cytosolic HMGB1 and exogenous HMGB1 stimulation induce different biological effects. Intracellular HMGB1 mainly translocates to the nucleus where it functions as an architectural chromatin-binding protein and regulates gene expression [27]. It has been reported that cytoplasmic HMGB1 can interact with beclin-1 and function as an important regulator of autophagy [28]. Extracellular HMGB1 functions as a signaling molecule during inflammation, cell differentiation, cell migration, and tumor metastasis [27]. Based on our ELISA results, we believed that HMGB1 transfection not only upregulated HMGB1 inside the cells but also increased HMGB1 release, which activated downstream signaling through RAGE receptor.

Unfractionated heparin (UFH) has been used as an anticoagulant for more than 5 decades. UFH has been shown to reduce inflammatory response through reducing the release of inflammation-related cytokines. Our previous study showed treatment of low molecular weight heparin inhibits systemic inflammation and prevents endotoxin-induced acute lung injury in rats [29]. It is reported that heparin blocks thrombin-induced endothelial permeability [30]. UFH also reduced LPS-induced endothelial barrier dysfunction [31]. However, the molecular mechanism of UFH on the barrier function of human pulmonary vascular endothelial cells are still not fully understood. In this study, we showed that UFH could maintain barrier function of HPMECs by reversing the effects of HMGB1 on AJ and TJ. We also found that UFH could downregulate the p38 phosphorylation induced by HMGB1. Inhibition of lipopolysaccharide-induced p38 activation by heparin has also been reported in endothelial cells [32]. Moreover, we observed there is a synergistic effect of UFH and p38 inhibitor in stabilizing barrier function of HPMECs. Combination of UFH and SB203580 is more effective than UFH alone, suggesting UFH stabilizes barrier function in both p38 dependent and independent manner. It has been reported that UFH regulates lung vascular leak by regulating Rho GTPase [33], which could preserve endothelial permeability through biosynthesis of specific lipid metabolites. Therapeutically, a combination of UFH and p38 inhibiting agents such as simvastatin [34] may confer better protection against HMGB1 in lung endothelial cells. Taken together, our data demonstrated heparin suppresses p38 signaling to exert barrier-stabilizing function. The mechanism associated with heparin-induced p38 inhibition requires future study.

HMGB1 interacts with its receptors such as RAGE, TLR2, and TLR4 to exert its downstream function. Here we found that HMGB1 induced endothelial barrier disruption and EndoMT was mainly mediated by RAGE receptor, not TLR2 / 4. RAGE is reported to be involved in HMGB1-induced barrier dysfunction and epithelial-mesenchymal transition [35, 36]. RAGE-HMGB1 interaction also takes part in Rac, MAPK, NF- κ B and AKT pathways, all of which are involved in mesenchymal transition. In the present study, we found that RAGE transduced p38 / Snail pathway to EndoMT. Further investigation is needed to determine the involvement of other potential pathways.

In conclusion, this study elucidated the effect of Heparin / HMGB1 interaction in EndoMT of human pulmonary microvascular endothelial cell. Our study proposed a novel mechanism by which HMGB1 promotes EndoMT by p38 / GSK3 / Snail signaling via RAGE, providing a potential therapeutic target for severe septic lung injury. Our study demonstrated a potential protective effect of UFH, which may reduce lung injury to prevent vascular leak and maintain the integrity of the endothelial barrier through p38 inhibition.

Abbreviations

HMGB1 (high mobility group box 1); EndoMT (endothelial-to-mesenchymal transition); HPMEC (human pulmonary microvascular endothelial cell); ZO-1 (zonula occludens 1); VE-cadherin (vascular endothelial cadherin); TEER (transendothelial electrical resistance); HRP (horseradish peroxidase); UFH (unfractionated heparin); RAGE (receptor for advanced glycation end products); siRNA (small interfering RNA); TJ (tight junction); AJ (adherens junction); GSK3 β (glycogen synthase kinase 3 β); ATF2 (activating transcription factor 2).

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Disclosure Statement

The authors declare that they have no competing interests.

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