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Original Article

Protective effects of heparin on endothelial cells in sepsis

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Abstract: Objective: This study aims to observe the protective effects of heparin on endothelial cells in sepsis and explore the involved signal pathway regulated by heparin. Methods Human vascular endothelial cells were treated by TNFα in vitro to simulate the inflammatory environment when sepsis occurred. They were intervened by heparin and the expression levels of soluble thrombomodulin (sTM) and serum activated protein C (APC) were detected by ELISA, the regulatory mechanism of heparin improving vascular endothelial cells injury induced by TNFα was detected by Western Blotting method, the methylation of histone in the gene promoter region of endothelial nitric oxide synthase (eNOS) and monocyte chemotactic protein-1 (MCP-1) were detected using chromatin immunoprecipitation method. Results Heparin could inhibit the secretion of sTM and APC protein and the expression of MCP-1 gene which involved in NF-κB signal pathway. Conclusions Heparin could protect vascular endothelial cells from injury induced by TNFα and sepsis, the mechanisms were related with the effects of heparin on the histone methylation of promoter region and the regulation of heparin on the MAPK and NF-κB signal pathways. These results provide a theoretical basis for the application of heparin in the prevention and treatment of vascular disease related with sepsis.

Keywords: Heparin, vascular endothelial cells, inflammation, signal regulating mechanism

Introduction

Sepsis is often accompanied by chronic inflammation. Inflammation could lead to vascular endothelial injury and was also an important reason for the occurrence and development of vascular complications of sepsis. Heparin is an anticoagulant and a polymer composed of two kinds of polysaccharides connected alternately. The application of heparin expands along with the progress of pharmacology and clinical medicine.

Studies showed that it had many pharmacological activities such as antioxidant, anti-inflammatory, anti-atherosclerosis, blood pressure-lowering, lipid-lowering and hypoglycemic functions and was widely used in the prevention and treatment of sepsis and cardiovascular disease [1, 2]. The methylation of histone related to epigenetics was directly involved in the process of vascular endothelial cell inflammatory injury in sepsis as regulation of gene expression, which became a new way for the prevention of vascular disease in sepsis [3].

In this study, human vascular endothelial cells were treated by TNFα in vitro to simulate the inflammatory environment when sepsis occurred. They were intervened by heparin and the expression of proteins related with vascular function and inflammation in culture supernatant were detected. The methylation of histone in the gene promoter region of endothelial nitric oxide synthase (eNOS) and monocyte chemotactic protein-1 (MCP-1) were detected using chromatin immunoprecipitation method [4, 5], which could provide a theoretical basis for the application of heparin in the prevention and treatment of vascular disease related with sepsis.

Materials and methods

Materials

Vascular endothelial cells were purchased from American ATCC; RT-PCR kits (TaKaRa Biotech (Dalian) Co., Ltd., Dalian, China); RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China); BAC protein assay kit, PBS,

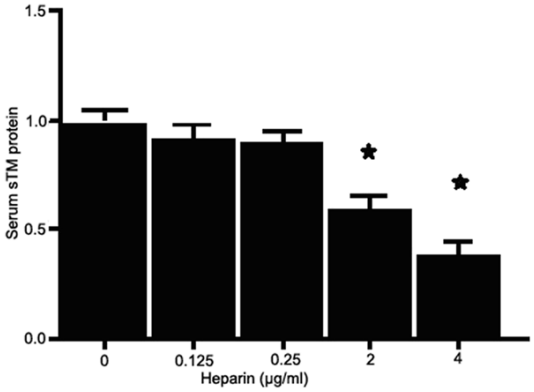

Figure 1. The serum sTM protein levels in different concentration of heparin intervention group. *Compared with control group, P<0.05.

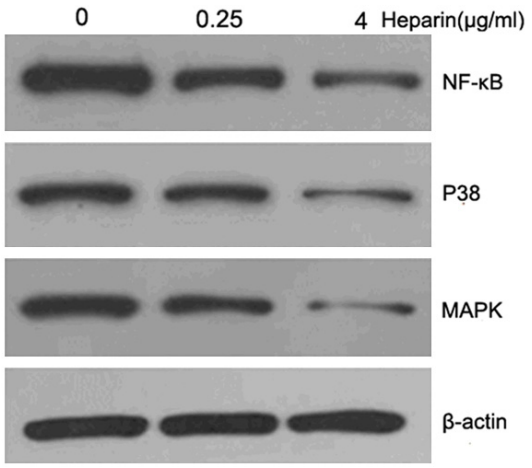

Figure 2. The expression levels of APC gene in different concentration of heparin intervention group. The expression levels of NF-kB, P38 and MAPK in high dose heparin group were lower than that of control group.

PMSF and RNase A (Sangon Biotech (Shanghai) Co.,Ltd, Shanghai, China); 0.25% Pancreatin-EDTA and APOAV (Boster Biotech Co.,Ltd, Wuhan, China); Antibodies of β-actin, PPARα, LXRα, Goat anti-mouse IgG(H+L)-HRP, Goat anti-rabbit IgG(H+L)-HRP (Proteintech Inc, Chicago, IL, USA).

The serum sTM and APC proteins were detected by ELISA

The standard sample was diluted according to the manual. The buffered solution of the antigen to be tested for and standard sample were added to ELISA coated plate. Then they were incubated at 37°C for 2 h and washed with PBS for 5 times after that.

A secondary antibody was added and incubated at 37°C for 1 h, washed with PBS after that. The substrate and chromogenic agents were added and incubated at 37°C for 15 min avoid light. The terminated solution was added into them after that. The optical density (OD) values were measured at 450 nm wavelength. The standard curve was drawn according to the serial dilutions of standard sample and the concentration of samples was calculated compared to the standard curve.

The expression levels of NF-κB and P38-MAPK in brain tissue detected by Western-blotting

Total proteins were lysed by RIPA lysis buffer and extracted to quantify using BAC protein assay kit according to the manual. They were analyzed with SDS-PAGE electrophoresis. Then it was electro-transferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with required antibodies. The first antibodies were anti-NF-κB, anti-p38-MAPK and anti-β-actin (1: 1000). The second antibodies were anti-rabbit and anti-mouse antibody with Horseradish Peroxidase. The protein bands were scanned and quantified as a ratio to β-actin.

Chromatin immunoprecipitation (CHIP)

The methylation of histone in the gene promoter region of endothelial nitric oxide synthase (eNOS) and monocyte chemotactic protein-1 (MCP-1) were detected using chromatin immunoprecipitation method. They were performed according to the protocol of CHIP kit.

Statistical analysis

The data were analyzed using SPSS13.0 software. The variance analysis ANOVA was conducted for comparison among groups. A value of $P<0.05$ and $P<0.01$ was considered statistical significance.

Results

The effects of heparin on the serum sTM protein

The results of ELISA indicated that heparin could inhibit the expression of sTM gene. **Figure 1** showed that the effects of heparin on vascu-

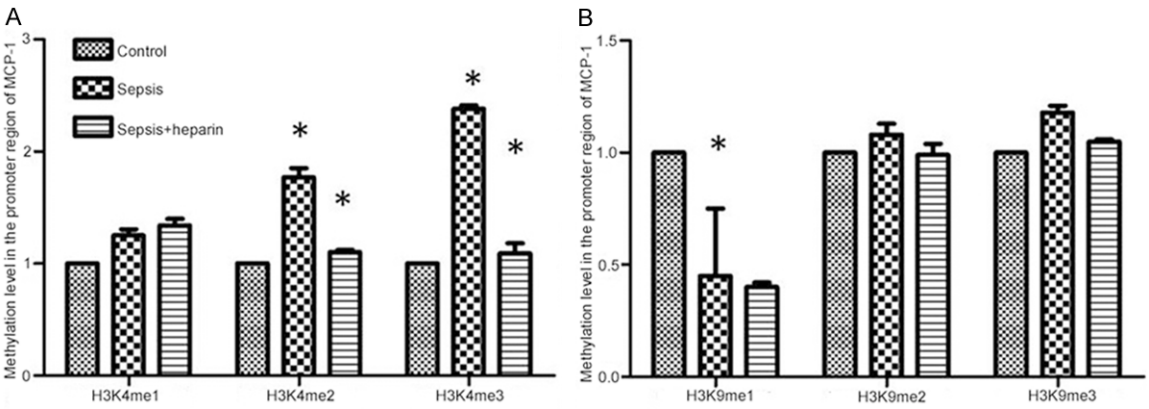

Figure 3. The effects of heparin on the expression of NF-kB, P38 and MAPK 1: Control, 2: Low dose group, 3: High dose group. *Compared with control group, P<0.05.

lar endothelial cell injury was dose dependent, the protein levels of serum sTM decreased with the increased concentration of heparin. The most obvious inhibition effects were in 4 μg/ml and 2 μg/ml heparin intervention group (P<0.05). There was no difference among 0.25 μg/ml and 0.125 μg/ml heparin intervention group and DMSO group (P>0.05).

The effects of heparin on the APC protein

The results of ELISA indicated that heparin could inhibit the expression of APC gene and the effects of heparin on the APC protein was dose dependent, the protein levels of APC decreased with the increased concentration of heparin. The most obvious inhibition effects were in 4 μg/ml and 2 μg/ml heparin intervention group (P<0.05). There was no difference among 0.25 μg/ml and 0.125 μg/ml heparin intervention group and DMSO group (P>0.05).

The effects of heparin on the expression of NF-kB, P38-MAPK

The results of Western Blotting showed that heparin could inhibit the expression of NF-kB, P38 and MAPK which involved in NF-κB signal pathway. The protective effects of heparin on the vascular endothelial cell injury were related with its on inhibition on MAPK and NF-kB signal pathway (**Figure 2**).

The effects of heparin on the methylation of histone in the promoter region of MCP-1

Compared with normal group, two methylation and trimethylation of H3K4 were higher in sepsis group (P<0.05), there was no difference between the two groups in one methylation of H3K4 (P>0.05). The intervention of heparin can reverse the changes of methylation (P<0.05, **Figure 3A**). One methylation of H3K9 decreased in sepsis group (P<0.05), there was no difference between the two groups in two methylation and trimethylation of H3K9, and the intervention of heparin cannot reverse the changes of one methylation of H3K9 (P<0.05, **Figure 3B**). The H3K4 methyltransferase SET7/9, MLL and menin which may be involved in the process of H3K4 methylation increased in sepsis group (P<0.05) and the intervention of heparin can decrease these (P<0.05, **Figure 4A-4C**). The H3K4 demethylase LSDl decreased in sepsis group (P<0.05) and the intervention of heparin can increase it (P<0.05, **Figure 4D**).

Discussion

Vascular endothelial cells (VEC) are a layer of flat cells covering the inner surface of blood vessels, which locate between the vessel lumen and vascular smooth muscle cells as a barrier of the blood and the vessel wall. VEC has many important biological functions. It is not only the regulation barrier of vascular permeability, but also is the largest and active endocrine and paracrine organ in the body [6-10]. When the inflammatory signals, blood pressure and hormone levels in the circulation changed, VEC could synthesize and secret many vaso-active substances such as nitric oxide (NO), prostacyclin, endothelin (ET), angiotensin-II, Plasminogen activator inhibitor (PAI), Fibroblast growth factor (FGF), Transforming

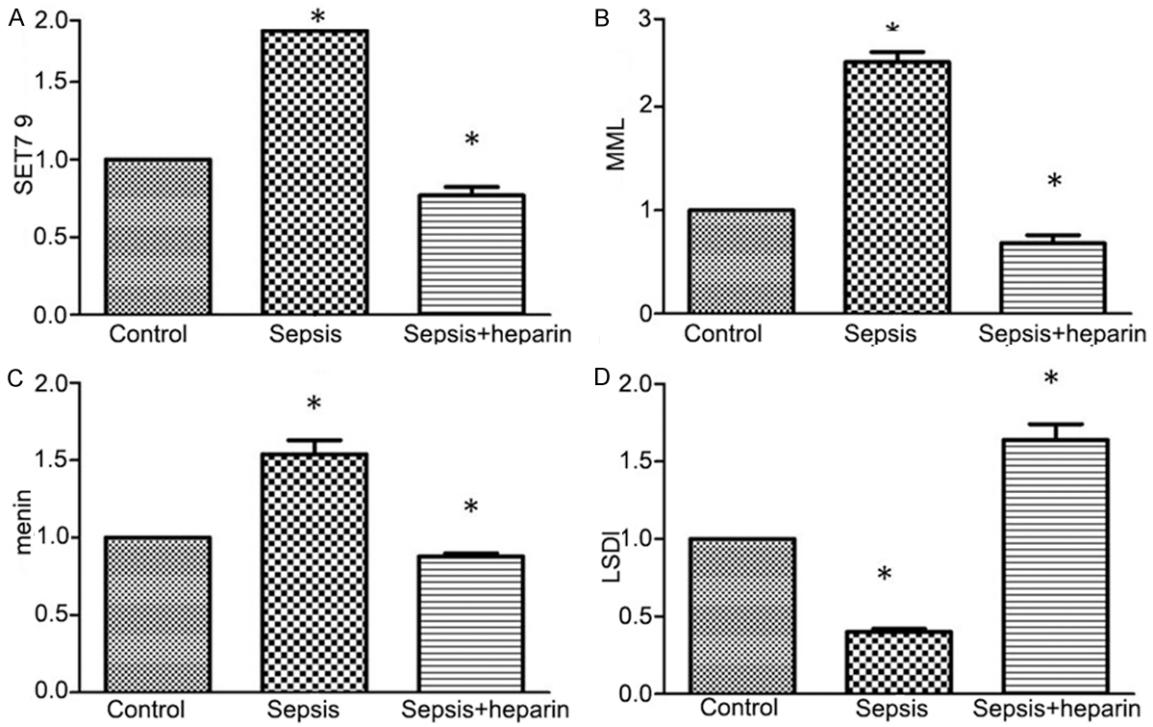

Figure 4. The effects of heparin on the methylation of histone in the promoter region of MCP-1. *Compared with control group, P<0.05.

growth factor (TGF), Platelet-derived growth factor (PDGF) and a variety of adhesion molecules to regulate vascular equilibrium. The interaction among these factors could maintain vascular relaxation and contraction state, regulate vascular tension, inhibit aggregation of platelets, maintain the balance of body coagulation and fibrinolysis system and blood flow to prevent thrombosis, regulate the expression of adhesion molecules and the growth, proliferation and migration of vascular smooth muscle cells, prevent the infiltration of inflammation and harmful substances [11-16].

Endothelin (ET) is an important vascular constriction factor, it is a polypeptide composed of 21 amino acids and its production is regulated by many agonists, it is mainly secreted in vascular endothelium. ET has three isomers: ET-1, ET-2 and ET-3, its receptor has two subtypes: ETα and ETβ. ET-1 could promote the proliferation and migration of vascular smooth muscle cells, increase vascular permeability and stimulates the production of monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and other inflammatory factors. NO and ET maintain the normal vascular diastolic function. Adhesion molecules produced by vascular endothelial cells such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin and MCP-1 regulate the adhesion and aggregation of blood cells in the blood vessel walls [17, 18].

Vascular endothelial was susceptible to damage induced by various factors, mechanical damage of hemodynamics, chemical factors such as tobacco, drugs, pathogens, immune complex deposition and lipid deposition all can lead to endothelial cell dysfunction. Endothelial cells synthesized and secreted a variety of active substances under the influences of these risk factors, the balance among the cytokines was destroyed which causing a series of pathological changes such as vasomotor abnormalities, adhesion and infiltration of leukocytes, thrombosis and proliferation of vascular smooth muscle cells. Many diseases especially the initiation and occurrence of atherosclerosis had important relationship with the endothelial dysfunction [19-21].

Endothelial cells regulate the function of blood vessels through secreting a large number of active molecules, such as NO, eNOS, ET-1,

VCAM, ICAM, MCP-1, vWF, PAI-1 and CRP. Therefore, these molecules could be bio-markers to detect endothelial function damage.

Endothelial cells dysfunction had two characteristics: One was the changes of vasoconstrictor activity, which was related with the reducing effect of NO partly; the other was the increased secretion of adhesion molecules. Hyperglycemia, advanced glycation end products of protein, lipoprotein and inflammatory factors can induce the expression of adhesion molecules. Thus it could be a marker for evaluating the activity of endothelial cells by determining the concentration of adhesion molecules in blood at the early stage of atherosclerosis.

Many studies have shown that adhesion and chemokine factors such as ICAM-1, VCAM-1, E-selectin and MCP-1 were associated with the atherosclerosis and cardiovascular disease [22].

We found that heparin could protect vascular endothelial cell injury and the mechanism was related with the effect of heparin on histone methylation in gene promoter region. Heparin can regulate gene expression in vascular endothelial cells by influence histone methylation of H3K4me2 and H3K4me3 in gene promoter region which were related with endothelial function and endothelial inflammation, and further revealed the key role of epigenetic regulation in the occurrence of vascular diseases. Heparin also influenced the regulation of MAPK and NF-κB signaling pathway. These results could provide new ideas and methods for the application of heparin in the prevention and treatment of vascular disease.

Disclosure of conflict of interest

None.

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