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Original Article

Platelet activity and phosphorylation of haematopoietic lineage cell-specific protein 1 in sepsis

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Abstract: Sepsis is a systemic inflammatory response syndrome caused bya severe systemic infection. We aimed at exploring the changes of platelet activation and haematopoietic lineage cell-specific protein 1 (HS1) phosphorylation (p-HS1) in normal and sepsis people. 150 sepsis samples and 50 normal samples were collected from Shanghai pudong public hospital. Platelet parameters, aggregation and p-HS1 expression were measured. In addition, model of LPS-induced platelet was employed for identifying the regulation of p-HS1 expression. In our result, platelet parameters were changed and the aggregation of platelet of sepsis group was increased. p-HS1 of sepsis patients was expressed significantly higher than normal people. Syk and src were the main regulators of p-HS1 expression. In conclusion, platelet activation and HS1 were responsible for the sepsis, and syk and src regulated the phosphorylation of HS1. This study can provide insights for the sepsis treatment.

Keywords: Platelet, phosphorylation, HS1, sepsis

Introduction

Sepsis is interpreted as a systemic inflammatory response syndrome caused by a severe systemic infection, which continues to be the leading cause of morbidity and mortality in severe hemorrhage, burns, and abdominal surgery patients [1]. The incidence of severe sepsis depends on how acute organ dysfunction is defined and on whether that dysfunction is attributed to an underlying infection. Approximately 14000 patients die from sepsis all around the world [2, 3]. Sepsis is associated with bacterial infection, the release of inflammatory cytokineand coagulation abnormalities, but the fundamental mechanism involved in remains unclearly known [4, 5]. Risk factors for severe sepsis are related both to a patient's predisposition for infection and to the likelihood of acute organ dysfunction if infection develops. There are many well-known risk factors for the infections that most commonly precipitate severe sepsis and septic shock, including chronic diseases and the use of immunosuppressive agents [6]. To date, it has also been unclear whether reduced platelet counts lead directly to adverse clinical outcomes in sepsis, or whether they are simply a biomarker for disease severity at presentation.

Increasing evidence has demonstrated that disorders of the circulatory system play a crucial role in the process of sepsis [7, 8]. By adhesion to the subendothelium and aggregation, platelets maintain primary hemostasis by forming hemostatic plugs, occluding sites of vascular damage and allowing the assembly of coagulation complexes responsible for thrombin generation [9]. Studies on platelet function in sepsis have yielded conflicting results. In clinical studies, several investigators have reported decreased platelet aggreg ability during sepsis [10], whereas platelet activation is regarded as an important event inactivation of the coagulation system, and during sepsis, lipopolysaccharides (LPS) and inflammatory cytokines promote platelet activation, which then contributes tomicrothrombi formation in the capillaries [11, 12]. The platelet-neutrophil complex formation was increased during *Streptococcus pyogenes* dissemination and the progression of sepsis, indicating that platelet activation had occurred

Table 1. Comparison of platelet parameters between normal people and sepsis patients

Groups	n	PLT ($\times 10^9$/L)	PDW (%)	MPV (fL)
Normal	50	245±89.3	12.8±5.1	12.4±2.2
Sepsis	150	115±45.1	18.9±7.3	19.5±5.4
P		<0.01	<0.05	<0.01

PLT: platelets; PDW: platelet distribution width; MPV: mean platelet volume.

in vivo, and platelet activation in the circulation precedes accumulation of platelets in damaged organs [13]. Thus, these studies provide novel insights of the potential clinical utility of anti-platelet therapy in the treatment of sepsis.

HS1 is a signaling molecule that functions downstream of glycoprotein activation, may contribute to platelet activation [14, 15]. Furthermore, recent studies have suggested that HS1 is associated with B-Chronic Lymphocytic Leukemia (B-CLL) [16] and systemic lupus erythematosus [17] and also influences numerous functions of natural killer (NK) cells, including lysis of target cells, cell adhesion, chemotaxis, and actin assembly at the lytic synapse [18]. The phosphorylation of HS1 was regulated by syk and src. The Src family member Lyn binds constitutively and steadily to unphosphorylated HS1 through the interaction between Lyn Src homology domain 3 and HS1 proline-rich domain [19]. After Syk-mediated HS1 phosphorylation, Lyn Src homology domain 2 motif can bind transiently to the phosphorylated Tyr-residues [20], causing HS1 hyperphosphorylation. Previous study found that the ratio of phosphorylated HS1 (p-HS1) and total HS1 (t-HS1) was significantly increased in LPS-stimulated platelets, indicating that LPS stimulated platelet activation may be closely related to HS1 phosphorylation [21]. In parallel, the p-HS1/t-HS1 levels in platelets were significantly downregulated in CLP mice treated with carbon monoxidereleasing molecules 2 (CORM2), indicating that CORM released CO downregulates the expression of platelet glucoproteins and inhibits activation of HS1 pathway in sepsis [22].

As a result, we collected 150 cases of patients with sepsis and identify the platelet activation and HS1 expression. In addition, the regulators of HS1 phosphorylation were also studied.

Materials and methods

Ethics statement

The Medical Ethical Committee of Shanghai pudong public hospital approved the study. After written informed consent, blood specimens were obtained from the cubital veins of healthy donors. The Medical Ethical Committee of Shanghai Pudong public hospital gave consent for the useof these samples.

Preparation of washed platelets

Blood was extracted from 50 healthy volunteers and 150 patients with severe sepsis from department of burn, after informed consent, and in accordance with the Medical Ethical Committee of Shanghai Pudong public hospital. Blood was collected into vacuum tubes and anti-coagulated with one-nine of 129 mM trisodiumcitrate. Platelet rich plasma (PRP) was obtained by centrifuging at 120× g for 10 min. Platelets were isolated by centrifuging at 678× g for 10 min and then washed twice with CGS buffer. The platelet poor plasma (PPP) was used to measure platelet aggregation. The platelets were resuspended in Tyrode's buffer for at least 1 h at 37°C before use. For experiments with PRP, the platelet density was maintained at 2×10^8/mL for every experiment.

Platelet adhesion and spreading

Platelet spreading on immobilized fibrinogen was performed as previously described [23, 24]. Fibrinogen was coated on the slides with microtiter wells at 4°C overnight. Platelet adhering and spreading on fibrinogen-coated wells was performed at 37°C for 90 min. Then the cells were washed, fixed, permeabilized, and stained with FITC-labeled phalloidin. Adherent platelets were measured under a fluorescence microscope using a 400× ocular lens.

ATP release

450 μL of Platelet rich plasma (PRP) was added with 50 μL ATP fluorescent agent (JRDUN, Shanghai, China) and incubated for 2 min at 37°C. 5 μM of ADP was added for the induction of ATP release. Platelet functional analyzer was employed for the analysis.

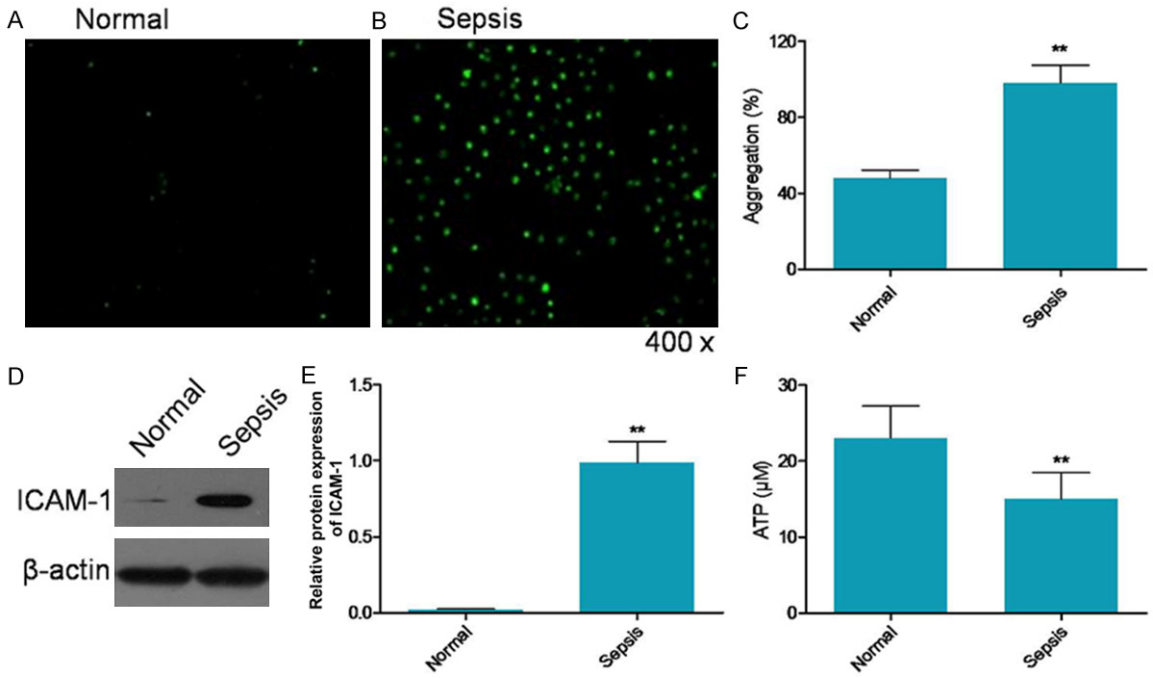

Figure 1. Increased adhesion of platelet in sepsis patients. A-C. Spreading of platelets in normal and sepsis patients. D, E. ICAM-1 expression was measured by western blot, and β-actin was also detected as the control of sample loading. F. ATP release of normal and sepsis groups. Data was presented as mean ± SD, n = 6, $^{**}P<0.01$ *versus* sepsis group.

Platelets stimulation model

LPS (10 mg/mL) was used to stimulate PRP to imitate the condition of coagulation under sepsis [25]. The PRP was assigned to 4 groups randomly. The model group did not undergo any treatment, whereas the LPS group received LPS simulation for 30 min, the PP2 group, piceatannol group and PP2 + piceatannol group underwent the same simulation and immediate administration of PP2, piceatannol and PP2 + piceatannol with dose of 10 mM. Samples were incubated in a CO_2-incubator at 37°C, 95% humidity, and 5% CO_2. After the intervention, the correlation indices were detected.

Western blot

Platelets were harvested and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a poly vinylidene fluoride membrane (Millipore, Bredford, USA). The blots were blocked with 5% skim milk, followed by incubation with primary antibodies. Antibodies against ICAM-1 and β-actin were purchased from Abcam. Antibodies against p-HS1 and total HS1 (t-HS1) were purchased from Santa. Blots were then incubated with goat anti-mouse secondary antibody (Beyotime, Shanghai, China) or goat anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Millipore).

Statistical analysis

Data was presented as the mean ± SD. Statistical analysis was performed by one-way ANOVA and the significant differences between two groups were analyzed by posthoc test (SNK). An alpha value of $P<0.05$ was considered statistically significant.

Results

Characters of blood platelet parameter in sepsis patients

Numbers (PLT), platelet distribution width (PDW) and mean platelet volume (MPV) of

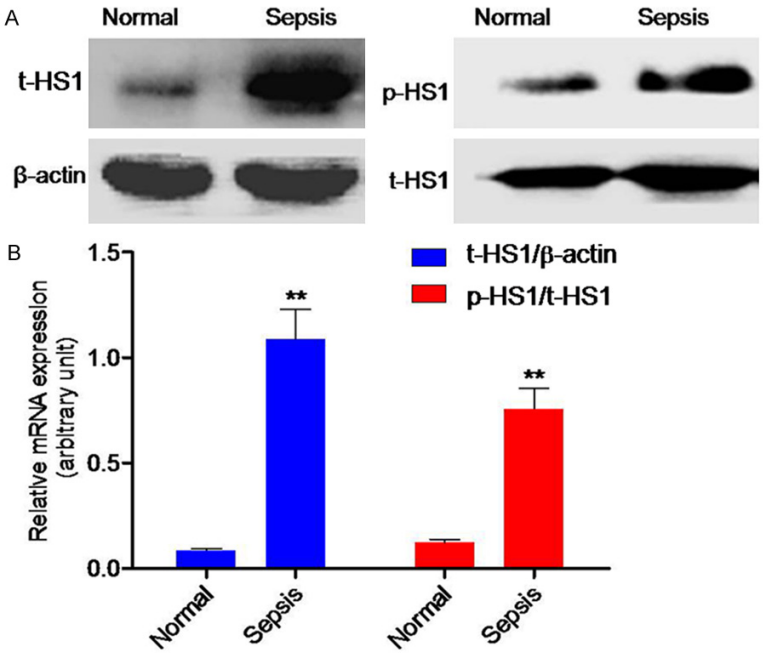

Figure 2. Phosphorylation of HS1 in sepsis patients. A, B. p-HS1 and t-HS1 expression were measured by western blot, and β-actin was also detected as the control of sample loading. Data was presented as mean ± SD, n = 6, $^{**}P<0.01$ versus sepsis group.

platelet were identified as previously described. As shown in **Table 1**, a significant decrease was seen in PLT of sepsis patient compared with the normal people. Increased PDW was normally observed in acute myeloid leukemia, giant platelet syndrome, and thrombotic diseases [26, 27]. Abnormal increase of MPV can result in the formation of thrombus [28]. In the present study, PDW and MPV both dramatically raised up compared with the normal person (**Table 1**).

Increased adhesion of platelet in sepsis patients

Platelet adhesion and spreading on fibrinogen are important indicators of thrombotic diseases [21]. We found tha tplatelet numbers in sepsis patients was increased compared to normal groups, and adhesion was promoted significantly (**Figure 1A-C**). Expression of ICAM-1 adhesion molecule was closely related to cell adhesion. From the western blot analysis, ICAM-1 expression in platelet of sepsis patients was notably higher than that of normal people (**Figure 1D** and **1E**).

Release of ATP from platelet to peripheral blood reveals the platelet activation. As a result, we measured the concentration of ATP in the peripheral blood. **Figure 1F** showed that ATP concentration in sepsis patients was reduced significantly compared with the normal people.

Phosphorylation of HS1 in sepsis patients

HS1 plays a key role in platelet functional responses, and t-HS1 expression and p-HS1 were measured by western blot [21, 29]. As shown in **Figure 2A** and **2B**, t-HS1 and p-HS1 expression in platelet of sepsis patients were both increased compared with that of the normal people.

Effects of syk and src on phosphorylation of HS1

Syk and src play important role in the phosphorylation of HS1. We use the inhibitors of syk and src to explore the subsequent effect on the phosphorylation of HS1. As shown in **Figure 3A** and **3B**, LPS dramatically induced the phosphorylation of HS1. PP2 as the inhibitor of sykeffectively reduced the p-HS1 level compared with the LPS-induced group. Piceatannol is the inhibitor of src which also decreased the p-HS1/t-HS1 level. In addition, combination of PP2 and piceatannol play more effective role in the reduction of p-HS1/t-HS1 expression.

Discussion

Sepsis with its host of associated complications remains a major clinical challenge with persistently high mortality of 9-49% [30, 31]. Sepsis plays crucial role in stimulation of inflammation and blood coagulation activation. Serious cross reaction often happens between blood coagulation activation and inflammatory reaction. Clotting system disorder caused by sepsis is described as abnormal platelet activation, which associated with platelet membrane glycoproteins and downstream signaling. HS1 plays a key role in the process of platelet activation [21, 29]. Accordingly, focusing on the function of platelet membrane glycoproteins

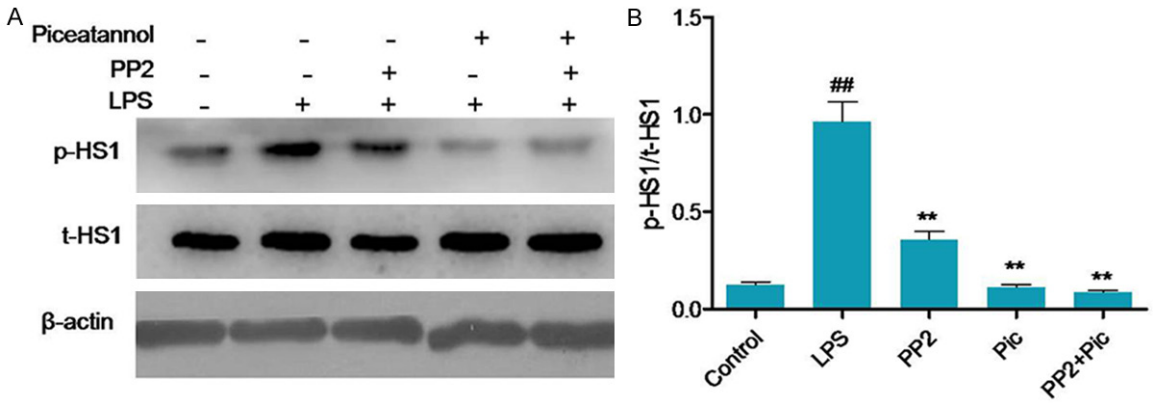

Figure 3. p-HS1 expression was regulated by syk and src in LPS-induced platelet. A, B. Platelets were induced by LPS, inhibitors of syk and src regulated the p-HS1 expression. p-HS1 and t-HS1 expression were measured by western blot, and β-actin was also detected as the control of sample loading. Data was presented as mean ± SD, n = 6, $^{\#\#}P<0.01$ *versus* control group; $^{**}P<0.01$ *versus* LPS group.

and its regulators can provide insights and new treatment for sepsis.

The present study found that platelet amount sepsis patients was significantly lower than that of normal people, and the significant difference was observed in platelet distribution width and mean platelet volume between sepsis patients and normal people. Increased PDW was normally observed in acute myeloid leukemia, giant platelet syndrome, and thrombotic diseases. Abnormal increase of MPV can result in the formation of thrombus. The findings suggested changes of platelets amount and function were associated with sepsis, which can resulted in platelet injury [32]. ICAM-1 plays an important role in adhesion of platelet, and the expression of ICAM-1 was increased in sepsis patients. Release of ATP from platelet to peripheral blood reveals the platelet activation, we concentration of ATP in the peripheral blood was decreased in sepsis group. We further found that adhesion, aggregation and ATP release of platelet in sepsis patients were significantly promoted that the normal people which demonstrated that the consumption of platelets was promoted. This phenomenon possibly resulted the happening of disseminated intravascular coagulation.

HS1 is considered as an important protein in the activation of platelet, and HS1 level can be up regulated by LPS treatment [21]. HS1 is involved in the cytoplasm of platelet. The phosphorylation of HS1 is regulated by syk and src, which correlates closely with the activation of platelet. In our results, expression of p-HS1 was up regulated in sepsis patients. p-HS1 was translated from cytoplasm to cytomembrane, which consequently makes impact on the function of platelet.

In conclusion, this research demonstrated that parameters, adhesion ability and ATP release of platelet in sepsis patients was significantly changed compared with the normal people. HS1 in sepsis patients was phosphorylated which regulated by syk and src. The above work could provide insights for the treatment of sepsis.

Disclosure of conflict of interest

None.

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