Cellular Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

LPS enhances platelets aggregation via TLR4, which is related to mitochondria damage caused by intracellular ROS, but not extracellular ROS

Guo Feng, Xinyu Yang, Yanqiu Li, Xiang Wang, Shiming Tan, Fangping Chen*

Department of Hematology, The Third Xiangya Hospital, Central South University, No. 138 Tongzi Road, Changsha, Hunan, PR China

ARTICLE INFO

Keywords:
Lipopolysaccharide
TLR4
Platelet aggregation
Intracellular ROS

ABSTRACT

Platelet is an important cell contributing to hemostasis and immunity. Bacterial lipopolysaccharide (LPS), mainly functioning by stimulating toll-like receptor 4 (TLR4), mediates platelet activation and sepsis. However, the inter-relationship between these players in sepsis remains unknown. We found that the aggregation of platelets was enhanced in complete blood of sepsis patients than that of healthy donors. PRP isolated from complete blood of healthy donors was used in the following study to filter out the interference of irrelevant cells. The results shown that the maximum aggregation rate (MAR) was significantly higher in LPS-challenged PRP model than that of controls, and administration of the specific TLR4 inhibitor, TAK242, reduced the MAR in this model. LPS promoted P-selectin expression and intracellular ROS production, and both TAK242 and N-acetyl-L-cysteine (NAC) could depressed the LPS-induced increase of P-selectin and intracellular ROS. H_2O_2 administration increased P-selectin expression partially but had little effect on intracellular ROS, thought it increased mitochondrial damage. In vivo, LPS increased both intracellular ROS and CD62P comparing with that of controls, effects that were prevented by TAK242. Furthermore, platelet aggregation through LPS-TLR4 pathway was involved in AKT, PKC and p38 phosphorylation but not cGMP/cAMP pathway. In conclusion, this study shows that intracellular ROS, not extracellular ROS such as H_2O_2, plays a crucial role in facilitating platelet aggregation via LPS/TLR4 pathway, and this process was involved in AKT, PKC and p38 phosphorylation but not cGMP/cAMP pathway. The results would helpful for understanding the role of intracellular ROS and LPS-TLR4 pathway in platelet aggregation.

1. Introduction

It is wildly known that there is an intricate relationship among inflammation, thrombosis and sepsis. Inflammation and thrombosis jointly contribute to the pathophysiological process of sepsis, inflammation could result in blood hypercoagulability and thrombosis, and thrombosis-inducing cytokines release would promote inflammation [1]. The disbalance between inflammation and thrombosis is one of the major public health issues, and even results in high mortality worldwide. Platelet is the major component in primary hemostasis, a defense mechanism to prevent bleeding, and plays a crucial role in innate immunity and adaptive immunity [2]. The activation of platelets is closely related to inflammatory storm, microthrombus, disseminated intravascular coagulation and multiple organ failure [3,4]. It is reported that sepsis could lead to thrombocytopenia, adhesion decreasing, and haemal bacterial load increasing, and finally result in inefficient of hemostasis [5,6]. However, some reports show that sepsis is associated with enhanced platelets adhesion, P-selectin expression and platelet factor 4 concentrations [4,7–10].

Toll-like receptors (TLRs) are a group of conservative pattern recognition receptors that could activate innate and adaptive immunity, and TLR4 is well known for the recognition of lipopolysaccharide (LPS), a pathogenic element located on Gram-negative bacteria. By combining with TLR4 of the platelet, LPS has multiple effects on thrombocytic function and immunity. Some studies demonstrated that LPS could decrease platelets and led to disability of platelets [11]. Another report shows that LPS failed to active platelet in the MyD88-/- mice model, indicating that LPS aggregates platelets via MyD88 pathway [12]. In addition, intracellular oxygen free radicals (ROS) generated from NADPH in platelets contributes to platelets aggregation. Thrombin, thromboxane and collagen promote nitrogen oxides generation, resulting in intracellular ROS increase, platelets activation, and finally

* Corresponding author.
E-mail addresses: fgblood@csu.edu.cn (G. Feng), 29374794@qq.com (X. Yang), 1097583986@qq.com (Y. Li), 1085172502@qq.com (X. Wang), tanshiming009@163.com (S. Tan), xychenfp@qq.com (F. Chen).

https://doi.org/10.1016/j.cellimm.2018.04.002
Received 10 January 2018; Received in revised form 16 March 2018; Accepted 2 April 2018

thrombogenesis [13].

It remains unclear that whether LPS enhances platelet aggregation via TLR4. Here we show that LPS enhances platelets aggregation via TLR4, which is related to intracellular ROS, protein kinase B (AKT), protein kinase C (PKC) and p38.

2. Materials and methods

2.1. Ethics statement

Appropriate blood samples of clinical sepsis patients were collected according to International Guidelines for management of Sepsis and Septic Shock: 2016. Informed written consent was obtained from each sepsis patient/family before collection of samples. We recruited 14 healthy volunteers, without a history of hematological diseases and without taking any drug that might affect the function of platelets, from The third Xiangya Hospital of Central South University. And all donors gave their informed consent according to the declaration of Helsinki. Male BALB/c mice (20–25 g) were purchased from SLAC Laboratory Animal Central (Changsha, China). Animals were housed in an air conditioned room (temperature, 25 ± 5 °C; relative humidity, 55 ± 5%) with regular light and dark cycle and allowed to acclimate for 7 days. All the procedures were under the guideline of the Third Xiangya Hospital of Central South University Human Research Committee.

2.2. Materials

LPS and 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemicals (St. Louis, MO, USA). TAK242 (TLR4 receptor inhibitor) was purchased from MedChem Express (New Jersey, USA). H_2O_2 was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). N-acetyl-L-cysteine (NAC) and JC-1 were purchased from Byotime (Shanghai, China). Adenosine diphosphate (ADP) were purchased from PLR-06 Sinowa (Jiangsu, China). PE-CD62P (P-selectin) was purchased from BD Biosciences (San Jose, CA, USA). Antibodies against total-protein kinase B (Akt), phospho-Akt (Ser473, p-Akt), total-P38 mitogen-activated protein kinases (p38), phospho-p38 (Thr180/Tyr182, p-p38), phospho-protein kinase C substrates (Ser, PKC), GAPDH, RIPA buffer, PMSF, protease and phosphatase inhibitor cock-tails were obtained from Cell Signaling Technology (Beverly, MA, USA). Cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) were purchased from Enzo Life Sciences (NYC, NY, USA).

2.3. Preparation of human platelets

Peripheral venous blood of sepsis patients, healthy volunteers and mice was collected and treated with anti-coagulant. The whole anti-coagulant blood was centrifuged for 10 min at 150*g* for platelet-rich plasma (PRP) collection. PRP were washed with Ca^{2+} free-Tyrodes-HEPES buffer containing 50 ng/mL PGE1 and then centrifuged for 10 min at 850 g. Washed platelets was suspended in Ca^{2+} Free-Tyrodes-HEPES buffer after the supernatant was discarded.

2.4. Blood count

Complete blood cell counter was used for blood count of the whole blood, collected from sepsis patients, healthy donor and mice.

2.5. Measurement of platelet aggregation

Platelet aggregation was measured by Platelet Function Analyzer PL-12 (Sinowa, China). Human complete blood, including healthy samples and sepsis samples, were pretreated with ADP (15 mM) or vehicle at 37 °C in an aggregometry sample tube, and PRP (30 × 108/ml) were pretreated with ADP (15 mM, 5 min) or vehicle, with LPS (10 ug/ml, 30 min) and/or TAK242 (132 nM, 30 min) at 37 °C in an aggregometry sample tube, and then the aggregation trace and Maximum Aggregation Rate (MAR%) were recorded.

2.6. Flow cytometric analysis

PRP (2 × 107/mL) were separately incubated with LPS (10 ug/ml), LPS (10 ug/ml) and TAK242 (132 nM), TAK242 (132 nM), NAC (5 mM,), H_2O_2 (100 uM), vehicle at 37 °C for 30 min. Then, platelets were incubated with FITC-CD62P antibody, DCFH-DA, JC-1 in dark at 37°C with for 15 min. Analysis was processed by a flow cytometer (BD Calibur, USA).

2.7. Biochemical analysis

Washed platelets (3×10^8/mL) were treated with pharmaceutical measurement as above. After incubation, processed samples were lysed and equal-amounts of protein were subjected to 12% SDS-PAGE and transferred onto PVDF for subsequent probing.

2.8. cGMP/cAMP analysis

Washed platelets (3×10^8/mL) were treated with pharmaceutical measurement as above. Via ELISA method, processed samples were analyzed by Multiskan Ascant (Bio-Tec instrument, USA) to test cGMP/cAMP concentrations.

2.9. LPS-induced sepsis and prevention by TLR4 inhibitor in vivo

Male BALB/c mice (20–25 g) received dorsal subcutaneous injection of LPS (0.18 ug/ml) on day 2 and day 3, the TLR4 inhibited group mice received dorsal subcutaneous injection of TAK242 (5 mg/kg) on Days 1–3 and LPS (0.18 ug/ml) on Days 2–3, the vehicle group received subcutaneous injection of saline solution with equivalent volume from Days 1 to 3, then PRP separated from heart blood was analyzed by flow cytometric analysis.

2.10. Statistical analysis

GraphPad Prism 7 (GraphPad Software Inc, La Jolla, CA, USA) was used for statistical analyzation of data and generation of figures. The results were presented as the mean ± the standard error of the mean (SEM). P value < 0.05 was considered as statistically significant.

3. Results

3.1. Platelet aggregation was enhanced in sepsis patients, and TAK242 inhibited platelets aggregation induced by LPS

We analyzed the MAR of both sepsis patients and healthy donors. The results shown that the MAR of sepsis patients was significantly higher than that of healthy donors. And ADP activated platelet of both sepsis patients and healthy donors (Fig. 1A). Aiming to eliminate the interference of other blood cells of complete blood, we conducted our further study on PRP from healthy donors. PRP was pretreated with LPS, TAK242, LPS and ADP, LPS and TAK242. The results demonstrated that LPS activated platelet, interestingly, TAK242, a specific TLR4 inhibitor, reversed platelets aggregation induced by LPS (Fig. 1B).

The results illustrated that platelet aggregation was enhanced in sepsis patients, and the specific TLR4 inhibitor, TAK242, could alleviate platelets aggregation induced by LPS, indicating that LPS stimulates platelet aggregation may via TLR4.