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Citrullinated Histone H3: Early Biomarker of Neutrophil Extracellular Traps in Septic Liver Damage

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ARTICLE INFO

Article history:
Received 3 February 2018
Received in revised form
7 July 2018
Accepted 3 August 2018
Available online xxx

Keywords:
Citrullinated histone H3
Sepsis
Neutrophil extracellular traps
Septic liver damage
Phosphodiesterase 3 inhibitor

ABSTRACT

Background: Neutrophil extracellular traps (NETs) play a crucial role in host defense, but excess and prolonged interaction of NETs with platelets can cause severe inflammation and host organ damage. Modification of histone H3 by citrullination is involved in *in vitro* NET formation. The phosphodiesterase III inhibitor, cilostazol (Ciz), which has a protective effect on liver sinusoidal endothelial cells and inhibits platelet aggregation, may prevent organ damage caused by excess NETosis. In this study, we investigated whether citrullinated histone H3 (H3Cit) could serve as a biomarker for the detection of critical liver damage in sepsis and the efficacy of phosphodiesterase-III inhibition for preventing the liver dysfunction induced by NETosis.

Materials and methods: Mice injected with lipopolysaccharide (LPS; 1 mg/kg) were used as a sepsis model with or without treatment with Ciz (200 mg/kg). H3Cit, myeloperoxidase, and neutrophil elastase levels were measured by immunohistochemistry. We evaluated H3Cit-positive neutrophils in the peripheral blood by flow cytometry.

Results: Immunohistochemistry revealed that H3Cit-, neutrophil elastase-, and myeloperoxidase-positive cell numbers in the livers peaked at 12 h after LPS administration. However, flow cytometry showed a significant increase in H3Cit-positive neutrophils in the peripheral blood only 4 h after LPS injection. Treatment with Ciz significantly ameliorated all parameters.

Conclusions: H3Cit is a useful biomarker for early detection of NETosis or liver dysfunction, and Ciz may be an effective treatment for septic liver damage.

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0022-4804/$ – see front matter © 2018 Published by Elsevier Inc.
https://doi.org/10.1016/j.jss.2018.08.014

Introduction

Sepsis is a state of systemic inflammatory response to infection that can be fatal unless sufficiently treated. Alterations in organ function vary widely in sepsis, from mild changes to irreversible organ failure.[1] Liver dysfunction is a component of multiple-organ dysfunction syndrome in sepsis and is associated with poor prognoses.[2,3] Useful early biomarkers are thus required to predict and prevent severe organ failure during sepsis. The ability to evaluate and predict the degree of organ dysfunction would have a major clinical impact and would influence treatment strategies for sepsis patients.

It was recently reported that during sepsis, neutrophils secrete extracellular net-like structures called neutrophil extracellular traps (NETs), which respond to damage-associated molecular patterns and pattern-associated molecular patterns, including bacteria.[4,5] Bacterial lipopolysaccharide (LPS) can activate neutrophils and subsequently catalyze intracellular histone arginine citrullination by peptidyl arginine deiminase 4.[6] Citrullinated histone, particularly histone H3 (H3Cit), was identified in the formation of NETs during chromatin decondensation.[7,8] H3Cit may thus be considered a biomarker for NETs in sepsis and could be a novel therapeutic target.[9]

NETs have been reported to play a crucial role in the inflammatory environment during infection, but excess and prolonged exposure to NETs results in endothelial damage.[10] Interactions between neutrophils and platelets accelerate NET formation, as well as thrombosis due to aggregation of intravasated platelets. Excessive microvascular thrombosis can then cause disorders of the microcirculation, leading to organ dysfunction.[11,12] We have previously reported that endothelial damage and detachment after NET formation followed by extravasated platelet aggregation (EPA) are the root causes of organ dysfunction such as liver damage during sepsis.[13,14] Therefore, we hypothesized that an antiplatelet drug, the phosphodiesterase III (PDE-III) inhibitor cilostazol (Ciz), may aid in the prevention and treatment of organ damage in sepsis by inhibiting platelet aggregation, as well as increasing cyclic AMP levels in endothelial cells.[15-18]

In this study, we examined whether H3Cit is a useful biomarker for early detection of septic organ damage, especially liver injury, and whether Ciz could be a useful treatment for the disease.

Materials and methods

Experimental animals

Male BALB/c mice ($n = 72$; 6 wk old, 20–30 g; Charles River Laboratories, Wilmington, MA, USA) were used in this study. To induce sepsis, 1 mg/kg *Escherichia coli* 0111:B4 LPS (L4130; Sigma–Aldrich, St. Louis, MO, USA) was intraperitoneally administered per our previous report.[14] A schematic depiction of the experimental timeline for this study is shown in Figure 1. To dynamically monitor liver damage in combination with NET formation in septic mice, we employed three animal groups: (1) the control group, without LPS administration or pretreatment with Ciz; (2) the LPS group, with the injection of LPS (1 mg/kg)

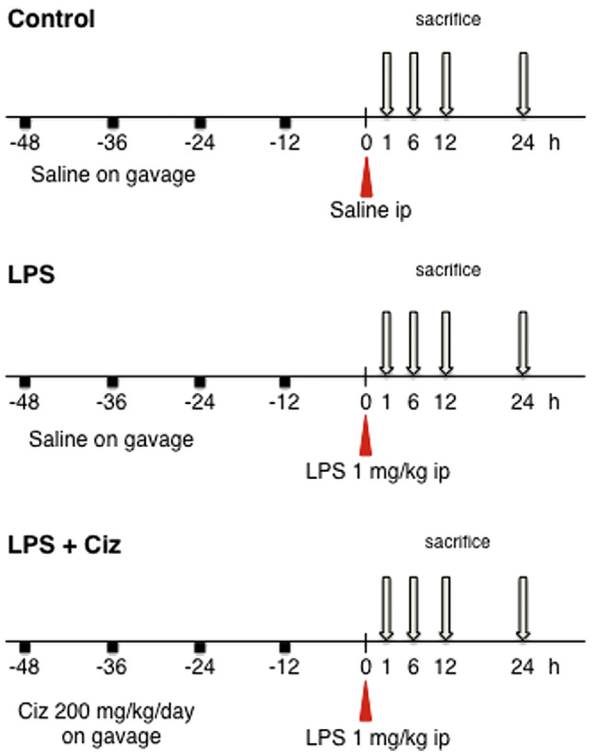

Fig. 1 – Experimental timeline. Mice were sacrificed for subsequent analyses 1, 6, 12, or 24 h after intraperitoneal (i.p.) injection of saline or lipopolysaccharide (LPS). Number of animals in each group: Control, $n = 24$; LPS, $n = 24$; and LPS + Ciz (cilostazol), $n = 24$. (Color version of figure is available online.)

without pretreatment with Ciz; and (3) the LPS + Ciz group, with both LPS injection and Ciz pretreatment. To examine its preventive effects on septic liver damage in mice, the PDE-III inhibitor Ciz (200 mg/kg; Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan), was administered as the pretreatment by gavage twice a day starting 48 h before LPS injection and continuing thereafter. All mice were housed in cages and given free access to chow and water throughout the experiment. The animals were treated in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The Committee on Animal Experimentation of Kanazawa University approved the animal experiments (AP-111868).

Specimen extraction

At 1, 6, 12, and 24 h following intraperitoneal injection of LPS, mice were administered isoflurane anesthesia and exsanguinated. Blood samples were collected, and liver tissues were removed after euthanasia.

Histological and immunohistochemical identification of NETs

Isolated livers were fixed by immersion in 10% formaldehyde at 4°C overnight. The paraffin-embedded liver sections were stained with hematoxylin and eosin. Immunohistochemical