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Combination of antithrombin and recombinant thrombomodulin attenuates leukocyte–endothelial interaction and suppresses the increase of intrinsic damage–associated molecular patterns in endotoxemic rats

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ABSTRACT

Introduction: Both antithrombin (AT) and thrombomodulin are key players in physiological anticoagulant systems. Because the levels of both factors are known to decrease significantly during severe sepsis, we hypothesized that a combination therapy would be effective.

Methods: A sepsis model was established using the intravenous infusion of lipopolysaccharide (LPS). A dose of 125 IU/kg of AT, 0.25 mg/kg of recombinant thrombomodulin, or a combination of both agents was injected immediately after LPS infusion ($n = 7$, each). Intravital observation of the mesenteric microcirculation was performed, and leukocyte adhesion and blood flow were calculated at 3 h after LPS infusion. Immediately after the observation, blood samples were obtained and coagulation markers, organ damage markers, the circulating levels of nucleosome and high-mobility group box 1 were measured.

Results: Microscopic findings revealed the suppression of leukocyte adhesion and thrombus formation in the combination group. The number of adhesive leukocytes on the endothelium was significantly suppressed ($P < 0.01$), and the blood flow in venules was better maintained in the combination group compared with the placebo control ($P < 0.01$). The blood samples showed the suppressed activation in coagulation, no significant changes were observed in the organ damage markers in the treatment groups. The circulating levels of nucleosome and high-mobility group box 1 were both decreased significantly in the combination group compared with the placebo control ($P < 0.01$).

Conclusions: The coadministration of AT and recombinant thrombomodulin is effective for the suppression of leukocyte activation and cell death during sepsis.

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1. Introduction

The tight cross talk between the coagulation system and inflammatory reactions during sepsis has attracted attention, and anticoagulant therapies have been expected to be beneficial not only for septic coagulopathy but also for severe sepsis [1–3]. Two major physiological anticoagulant systems, the thrombomodulin–protein C system and the antithrombin (AT)–heparan sulfate system, have been intensively investigated. AT administration has been recommended in the Japanese Guidelines for disseminated intravascular coagulation (DIC) [4], and a recent Japanese clinical study demonstrated the possible efficacy of AT for septic DIC [5]. As for thrombomodulin, recombinant thrombomodulin (rTM) was approved in Japan for the treatment of DIC in 2008 [6]. Subsequently, a phase 2B clinical trial involving severe sepsis with suspected DIC was conducted in countries including North America, Europe, and Asia, and the possible efficacy of this treatment was announced [7]. Following this study, an international phase 3 trial is currently underway [8]. Because both AT and thrombomodulin activities are known to decrease significantly and have independent effects on the coagulation system, a combination therapy could potentially be beneficial. On the basis of this hypothesis, we examined the combination effects in the lipopolysaccharide (LPS)-challenged model and reported that the combination therapy attenuates organ damage, histologic changes, and leads to an improvement in survival [9].

With regard to the pathophysiology of severe sepsis, the role of damage-associated molecular patterns (DAMPs) has attracted attention [10]. DAMPs including nucleosome and high-mobility group box 1 (HMGB1) are released from the damaged host cells and are known to induce further cellular damage. In the present study, we hypothesized that the anticoagulant therapy might modulate the leukocyte activation, which leads to the decreased levels of circulating DAMPs, and planned to reveal the mechanism of action of anticoagulant therapy.

2. Material and methods

2.1. Sepsis model and treatment groups

A total of 28 ten-week-old Wistar rats (Japan Clea Co, Tokyo, Japan) were used. All the experimental procedures were conducted after obtaining the approval of the Ethical Committee for Animal Experiments of Juntendo University. All the rats were provided with standard rat chow and water *ad libitum*. Rats were anesthetized with intraperitoneal sodium thiopental (100 mg/kg; Pentothal; Sigma Chemical Co, St Louis). For continuous anesthesia, propofol (Sigma Chemical Co) infusion and fluids (10 mL/kg/h sodium chloride) were administered *via* the caudal vein; then 8.0 mg/kg of LPS (*Escherichia coli* O55-B5; Difco Laboratories, Detroit) diluted with 0.15 mL of sterile physiological saline was infused intravenously. The animals were divided into four groups. A dose of 125 IU/kg of AT (CSL Behring Japan Co, Tokyo, Japan) (AT group), 0.25 mg/kg of rTM (ART-123; Asahi Kasei Pharma Co, Tokyo, Japan) (rTM group), or 125 IU/kg of AT and 0.25 mg/kg of rTM (AT/rTM group) were administered intravenously immediately after LPS infusion ($n = 7$, each). In the control group ($n = 7$), animals were given LPS and saline.

2.2. Intravital microscopy

The mesenteric microcirculation was examined using intravital microscopy, as previously described [11,12]. Briefly, the abdomen was opened with a median incision and the mesentery was displayed and immobilized on a special stand. The microcirculation was then observed using the microscopic system (Nikon Microphot-FX Microscope; Nikon Co, Ltd, Tokyo, Japan). At 3 h after LPS infusion, each field was recorded for 3 min at 30 frames/s using a video system (Ikegami CA-300; Ikegami Tsushinki Co, Ltd, Tokyo, Japan). In each animal, six fields in succession were randomly selected. The images obtained were used to document and analyze the number of adherent leukocytes at the postcapillary venules. A leukocyte was defined as adherent to a venule if it remained stationary for 30 s. The number of leukocytes that had adhered to the venules at 3 h after LPS infusion was counted by two independent persons, and the mean value was calculated. To analyze the red blood cell (RBC) velocity, a venule with a diameter of approximately 50 μm was examined. The venule was observed, and the images were recorded using a high-speed camera (Memrecam GX-1; Nac Image Technology Inc, Tokyo, Japan) at 3 h after LPS infusion. Then, the RBC velocity was calculated using particle image velocimetry (Digimo Co, Ltd, Tokyo, Japan).

2.3. Blood sampling and measurement

Immediately after the microscopic observations, the rats were sacrificed under deep anesthesia in an ether chamber. Blood samples were obtained from the inferior vena cava, and citrated plasma samples were obtained by whole blood centrifugation and were stored at −80°C until assay. Fibrin and fibrinogen degradation products (FDP), and fibrinogen levels and the levels of the organ damage markers, such as alanine aminotransferase (ALT), total bilirubin, creatinine, and blood urea nitrogen, were measured in the samples. Determinations of FDP and fibrinogen were performed by an enzyme-linked immunosorbent assay (ELISA) kit (Teikoku Laboratories, Tokyo, Japan). The extracellular nucleosome levels were measured using a sandwich ELISA kit (Cell Death Detection ELISAplus Kit; Roche Diagnostics, Indianapolis, IN). A total of 20 μL of citrated plasma was diluted 1:4 in 1% bovine serum albumin, 0.5% Tween, and 1 mmol/L of ethylenediaminetetraacetic acid in phosphate-buffered saline and added to streptavidin-coated microtiter plates containing biotinylated mouse anti-histone antibody and peroxidase-conjugated anti-DNA antibodies. After standard washing steps, the peroxidase activity of the retained immunocomplexes was developed by incubation with 2,2′-azino-di(3-ethylbenzthiazoline-sulfonate) and read in a spectrophotometer at 405 nm. In the same samples, the HMGB1 level was measured using a commercially available sandwich ELISA kit (Shino-test, Tokyo, Japan) in a manner similar to that used to measure the nucleosomes.