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Full Length Article

Protection of the endothelial glycocalyx by antithrombin in an endotoxin-induced rat model of sepsis

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ABSTRACT

Introduction: Injury and loss of the endothelial glycocalyx occur during the early phase of sepsis. We previously showed that antithrombin has a protective effect on this structure *in vitro*. Here, we investigated the possible protective effects of antithrombin in an animal model of sepsis.
Methods: Wistar rats were injected with endotoxin, and circulating levels of syndecan-1, hyaluronan, albumin, lactate and other biomarkers were measured in an antithrombin-treated group and an untreated control group (n = 6 in each group). Intravital microscopy was used to observe leukocyte adhesion, microcirculation, and syndecan-1 staining.
Results: The circulating levels of syndecan-1 and hyaluronan were significantly reduced in the antithrombin-treated group, compared with the untreated controls. Lactate levels and albumin reduction were significantly attenuated in the antithrombin-treated group. Intravital microscopic observation revealed that both leukocyte adhesion and blood flow were better maintained in the treatment group. The syndecan-1 lining was disrupted after endotoxin treatment, and this derangement was attenuated by treatment with antithrombin.
Conclusion: Antithrombin effectively maintained microcirculation and vascular integrity by protecting the glycocalyx in a rat sepsis model.

1. Introduction

The endothelial glycocalyx is a critical component of the vascular wall that regulates selective permeability, blood cell interactions, and maintains blood flow [1–4]. Following injury due to hypoxia [5], trauma [6], and sepsis [7] major constituents of the glycocalyx, including syndecans and hyaluronan, are shed from the endothelial surface. In septic patients, the circulating levels of glycocalyx components are higher in the presence of critical illness complicated by acute respiratory distress syndrome [8] or disseminated intravascular coagulation [9]. Studying the endothelial glycocalyx is important because it represents one of the earliest sites of injury during acute inflammation and its structural and functional distortion can lead to organ dysfunction and death [10]. Developing new therapeutic approaches aimed at protecting the endothelial glycocalyx from inflammatory insults represents an important therapeutic approach. Although preclinical research has identified different agents capable of protecting the glycocalyx, none of them are available for clinical use at present [11]. Previous investigations suggest that plasma might convey protective substances to the glycocalyx that included antithrombin and albumin as promising candidates [12,13]. Chappell et al. [14] reported that antithrombin could reduce the shedding of the endothelial glycocalyx in an animal model of ischemia/reperfusion. In addition, antithrombin's anticoagulatory and anti-inflammatory functions may help protect this important bioactive layer. We previously reported that antithrombin can bind to heparans in the glycocalyx preserving vascular integrity *in vitro* [15,16]. In the present study, we investigated the influence of antithrombin on the glycocalyx in an animal model of sepsis.

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2. Materials and methods

2.1. Sepsis model and treatment group

Ten-week-old Wistar rats (purchased from Sankyo Laboratory Service Co., Tokyo Japan) were used for the experiment. 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, USA). Then, 8.0 mg/kg of lipopolysaccharide (LPS, *E. coli* O55-B5; Difco Laboratories, Detroit, USA) diluted with 0.15 mL of sterile physiological saline was infused intravenously. In the treatment group, 500 IU/kg of antithrombin (Nihon Pharmaceutical Co., Tokyo, Japan) was administered intravenously immediately after LPS infusion (n = 12, each). In the control (vehicle) group (n = 12), animals were given LPS and saline.

2.2. Blood sampling and measurement

At 3 and 6 h after the LPS infusion, rats were sacrificed under anesthesia in an ether chamber (n = 6 for each group at each timing). Blood samples were obtained from the inferior vena cava. The citrated plasma samples were obtained by whole blood centrifugation and were stored at −80 °C until assay. An additional three rats without any treatment were used as a normal group for blood sampling. Standard analytic techniques were used to measure alanine aminotransferase (ALT), blood urea nitrogen (BUN), and albumin. The lactate was determined using a blood gas analyzer (ABL715; Radiometer, København, Denmark). Syndecan-1 was measurement using an enzyme-linked immunosorbent assay (ELISA) kit for syndecan-1 (Cloud-Clone Corp., Katy, TX, USA), and hyaluronan was measured using a hyaluronan Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

2.3. Observation of the microcirculation

In a different series of animals (n = 6 in each group), the mesenteric microcirculation was examined using intravital microscopy. The intravital microscopic examination was performed as follows. First, the abdomen was opened under anesthesia by a median incision, and the mesentery was exposed and immobilized on a stand. The microcirculation was then observed using the Eclipse Pol microscopic system (Nikon Co., Tokyo, Japan) at 3 and 6 h after the LPS injection. In each animal, 6 successive fields were selected, and each field was recorded for 5 min at a speed of 30 frames/s using a high-vision recording system (α7 III; SONY Co., Tokyo, Japan). To count the adherent leukocytes on the endothelium, images were obtained and used by two independent examiners working in a blinded manner to count the number of adherent leukocytes in each field at 3 and 6 h. A leukocyte was defined as having adhered to a venule if it remained stationary for 30 s. To analyze the red blood cell (RBC) velocity, a venule with a diameter of approximately 20–50 μm was examined and images were recorded using a high-speed camera (Memrecam GX-1; Nac Image Technology Inc., Tokyo, Japan) at 3 and 6 h after the LPS injection. Then, the RBC velocity was calculated using particle image velocimetry (Digimo Co., Tokyo, Japan). The baseline RBC velocity was deemed as 1.0, and the decrease in the ratio was calculated.

2.4. Immunofluorescent staining for syndecan-1

After the microcirculation observation, the endothelium was stained using anti-syndecan-1 antibody (B-A38; Abcam PLC, Cambridge, UK). The target vessels were infused with anti-syndecan-1antibody diluted to 1:500 with physiological saline and then examined using immunofluorescent microscopy.

2.5. Statistical analysis

The statistical analysis was performed using a one-way ANOVA with the Dunnett post-hoc test using statistical software (StatView II ™). Data were presented as the mean ± standard error (SE). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Laboratory data

The elevation in ALT was not remarkable at 3 h after LPS administration, and no significant difference was seen between the treatment group and the control group. In contrast, the elevation was significantly suppressed in the treatment group at 6 h after LPS administration ($P < 0.05$). The BUN level was lower in the treatment group than in the control group, but the difference was not significant. The decrease in the albumin level was significantly attenuated at both 3 and 6 h in the treatment group ($P < 0.05$). The lactate level was lower in the treatment group at 6 h ($P < 0.05$) (Table 1).

3.2. Syndecan-1 and hyaluronan measurements

Fig. 1 (left panel) shows the changes in the plasma levels of syndecan-1. The syndecan-1 level in normal rats was below 5.00 ng/mL. The syndecan-1 levels increased over time from 3 to 6 h and reached 42.62 ± 3.10 ng/mL at 6 h in the control group. The syndecan-1 levels were significantly decreased by the antithrombin treatment (31.75 ± 3.22 ng/mL; $P = 0.036$). The plasma level of hyaluronan in the normal rats was below 50.0 ng/mL, and it increased at 3 h after LPS administration (256.2 ± 18.4 ng/mL) and then decreased at 6 h. The plasma hyaluronan level was significantly suppressed by the antithrombin treatment at 3 h (155.6 ± 18.7 ng/mL, $P = 0.032$) (Fig. 1, right).

3.3. Intravital microscopy findings

Leukocyte adhesion on the endothelial surface had already started at 1 h after LPS administration. The intact endothelium was observed as a smooth, thin monolayer of endothelial cells, and the glycocalyx was

Table 1
Laboratory findings of the endotoxemic rats treated with or without antithrombin.

Parameters		ALT (IU/L)	BUN (mg/dL)	Albumin (mg/dL)	Lactate (mmol/L)
Normal group		15–32	12.7–14.5	4.7–5.1	0.8–0.9
3 h	Control group	71.33 ± 7.49	35.17 ± 2.65	2.68 ± 0.22	3.42 ± 0.25
	Treatment group	59.17 ± 4.50	30.67 ± 1.71	3.48 ± 0.21[a]	2.95 ± 0.20
6 h	Control group	118.33 ± 12.43	42.83 ± 3.41	2.43 ± 0.16	4.52 ± 0.29
	Treatment group	76.50 ± 8.53[a]	39.83 ± 2.99	3.13 ± 0.21[a]	3.50 ± 0.35[a]

ALT alanine aminotransferase, BUN, blood urea nitrogen.
[a] Significant difference from the control group at $P < 0.05$.