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The effect of plasma-derived activated protein C on leukocyte cell-death and vascular endothelial damage

Toshiaki Iba *, Kunihiko Nagakari

Department of Emergency and Disaster Medicine, Juntendo University Graduate School of MedicineJapan

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ABSTRACT

Introduction: The role of leukocyte and its death in the progression in inflammation attracts attention nowadays. The purpose of this study is to examine the effects of activated protein C (APC) on leucocyte cell death and vascular endothelial damage in sepsis.
Methods: Wistar rats were infused with lipopolysaccharide (8.0 mg/kg) concomitantly with either a low dose (0.5 mg/kg), a high dose (5.0 mg/kg) of plasma-derived APC or albumin. One and 3 hours after the injections, the mesenteric microcirculation was observed by intravital microscopy. The serum levels of nucleosome and High Mobility Group Box 1 (HMGB1) were measured in each group. In another series, cultured leukocyte cell-death in the medium supplemented with serum obtained from each group was examined *in vitro*.
Results: Microcirculatory disturbance was significantly suppressed in both the high-dose and low-dose groups compared to the control group ($P < 0.01$, 0.05, respectively). The bleeding area was significantly increased in the control and high-dose groups ($P < 0.05$, 0.01, respectively). Serum levels of cell death markers such as nucleosome and HMGB1 were significantly decreased in the treatment groups ($P < 0.01$), and the protective effect was more pronounced in high-dose group. Cell death suppression was most prominent in high-dose group and the formation of neutrophil extracellular traps (NETs) was significantly suppressed in the treatment groups.
Conclusion: Low-dose plasma-derived APC exerted protective effects on the microcirculation without increasing the risk of bleeding. The protective effect against leukocyte cell death and the suppressive effect on NETs formation of APC might be related to its beneficial effects.

Introduction

It has been clarified that positive feedback between inflammation and coagulation leads to reciprocal activation of both pathways. Activated protein C (APC), a major natural inhibitor of the coagulation system, suppresses coagulation by inactivating factors VIIIa and Va, and exerts profibrinolytic activities through inhibition of plasminogen activator inhibitor-1 (PAI-1), and decreases the production of thrombin-activatable fibrinolysis inhibitor (TAFI) [1,2]. In addition, its anti-inflammatory effects exerted through the mediation of endothelial protein C receptor (EPCR) and protease-activated receptor (PAR)-1 [3,4], and its protective effects on the endothelium [5] have been reported.

Recombinant APC (drotrecogin alfa) had been recommended for the treatment of severe sepsis from 2001 through 2011 [6]. However, drotrecogin alfa was taken off from the market since the last clinical trial targeting septic shock failed to demonstrate the survival benefit [7]. Although the debate on the efficacy of APC is still ongoing [8–10], drotrecogin alfa is no longer available. Instead, a plasma-derived APC (pd-APC, CTC-111) is still available in Japan, and a randomized double-blind trial comparing the efficacy of CTC-111 with that of unfractionated heparin (UFH) was performed [11] in DIC patients. Though this is a small study, the results demonstrated a significant decrease of the 28-day mortality in the CTC-111group (20.4% vs. 40%, $p < 0.05$). However, the clinical as well as basic evidences that support the efficacy of CTC-111 are quite limited.

The recent topic in this field is the tight link between coagulation and inflammation, and the fact that activated neutrophils play major roles in both phenomena is widely accepted [12]. Since anticoagulants are strong candidates for the management of a disrupted coagulation/inflammation system and also for the regulation of the activated neutrophils, we attempted to evaluate the effect of pd-APC in an animal model of sepsis as well as *in vitro* study.

Materials and Methods

Animal Model

All experimental procedures were conducted according to guidelines approved by the Ethical Committee for Animal Experiments of

* Corresponding author at: Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113–8421, Japan. Tel.: +81 3 3813 3111x5818; fax: +81 3 3813 5431.
E-mail addresses: toshiiba@cf6.so-net.ne.jp (T. Iba), kun@juntendo-urayasu.jp (K. Nagakari).

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Juntendo University. Wistar rats, 12- to 15-weeks old were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). The rats were anesthetized by intraperitoneal injection of 40 mg/kg of sodium pentobarbital (Sumitomo Dainippon Pharma, Tokyo, Japan), followed by administration of 8.0 mg/kg of lipopolysaccharide (LPS) (*E. coli* O55-B5, Difco Laboratories, Detroit, USA) via the tail vein. Then, immediately after the LPS injection, either 0.5 mg/kg (low-dose) or 5.0 mg/kg (high-dose) of pd-APC (CTC-111, Kaketsuken, Kumamoto, Japan) was administered (n = 10 in each group). A third group of 10 animals was given 5% human albumin (CSL Behring, Marburg, Germany) intravenously immediately after the injection of LPS, at a volume equal to that of pd-APC administered in the treatment groups. These animals were served as the controls.

Observation of the Microcirculation

In 5 animals each of the low-dose, high-dose and control groups, the mesenteric microcirculation was examined by intravital microscopy. The method for the intravital microscopic examination is described elsewhere [13]; in brief, the abdomen was opened under anesthesia by a median incision and the mesentery was exposed and immobilized on a stand. The microcirculation was observed using the Eclipse Pol microscopic system (Nikon Co., Ltd., Tokyo, Japan) at one and three hours after the LPS injection. In each animal, six successive fields were selected and each field was recorded for 5 minutes at the speed of 30 frames/second by a high-vision recording system (EOS 5D Mark III, Canon Co., Ltd., Tokyo, Japan). The images obtained were used to document and analyze the bleeding area. The bleeding area at one hour in the control group was expressed as 1.0, and the ratios of the area at one and three hours to this area were plotted in each group. To analyze the RBC velocity, a venule with a diameter of approximately 20–30 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 one and three hours after the LPS injection. Then, the RBC velocity was calculated by particle image velocimetry (Digimo Co., Ltd., Tokyo, Japan). The baseline RBC velocity was deemed as 1.0 and the decrease in the ratio was monitored. Finally, the mesenteric venule was stained with 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI, 0.01 mg/ml in Tris-EDTA buffer solution with 10 mM 2-mercaptoethylamine, pH 7.4) to visualize the damaged cells.

Measurement of Cell Death Markers

Five animals in each group that were not used for the intravital microscopy were used for this study. Blood samples were obtained from the inferior vena cava at three hours after the LPS injection under anesthesia. The sampling was also performed in healthy untreated rats as the normal group. Serum samples were immediately isolated by centrifugation at 4 °C at 1400 × g for 15 min, and stored at −20 °C. The extracellular nucleosome levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) assay (Cell Death Detection ELISAplus Kit; Roche Diagnostics, Indianapolis, IN). A total of 20 μL of citrated serum was diluted 1:4 in 1% bovine serum albumin, 0.5% Tween, and 1 mmol/L ethylenediamine tetraacetic 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 the standard washing steps, the peroxidase activity of the retained immunocomplexes was developed by incubation with ABTS (2,2'-azino-di[3-ethylbenzthiazoline-sulfonate]) and read in a spectrophotometer at 405 nm. In the same samples, the High Mobility Group Box 1 (HMGB1) level was measured using a commercially available sandwich ELISA kit (Shinotest, Tokyo, Japan) in a manner similar to that used to measure the nucleosomes.

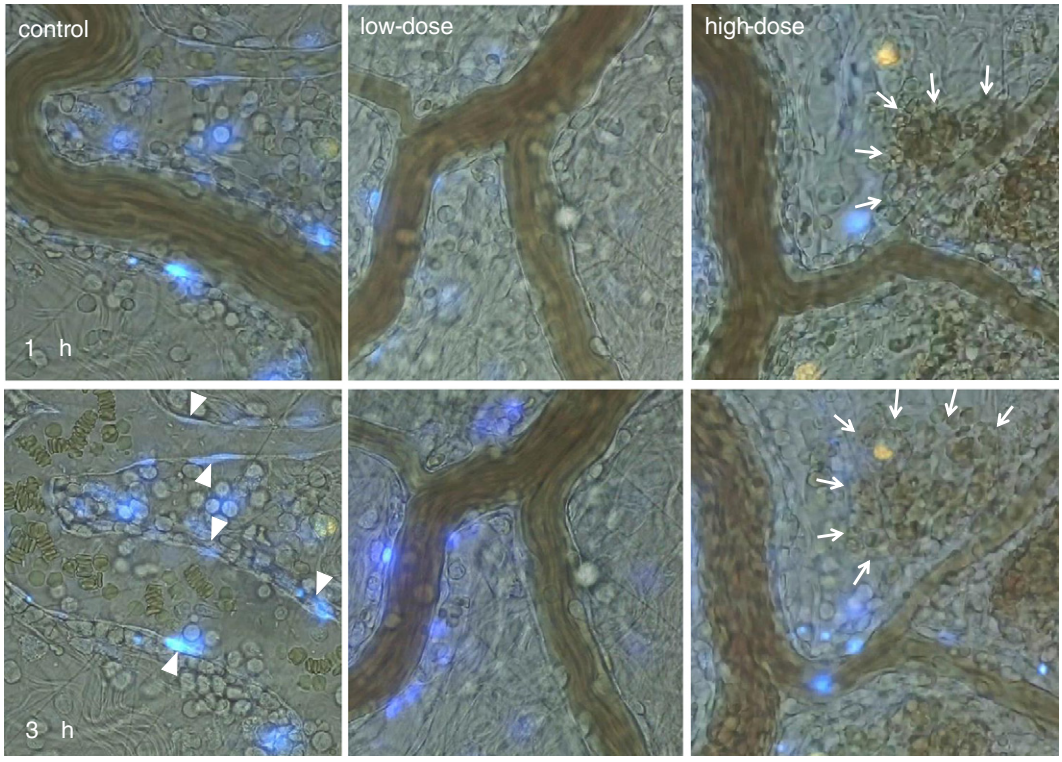

Fig. 1. Effect of activated protein C on the microcirculation and endothelial damage in endotoxemia. Intravital microscopy demonstrates the effect of plasma-derived activated protein C (pd-APC) on the microcirculatory disturbance in endotoxemic rat. High-dose pd-APC caused bleeding (arrows, right column). The endothelial damage depicted by fluorescence live imaging using DAPI (4′,6-diamidino-2-phenylindole) (arrow heads) was attenuated by pd-APC treatment. The photographs are overlays of the immunofluorescent and bright images. (objective lens x20).