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Antithrombin III improved neutrophil extracellular traps in lung after the onset of endotoxemia

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ABSTRACT

Background: Coagulation and inflammation are closely linked during acute inflammatory conditions, such as sepsis. Antithrombin (AT) is an anticoagulant that also has anti-inflammatory activities. The effects of therapeutically administering AT III after the onset of endotoxemia or sepsis were not clear. Here, we studied the effects of administering AT III after inducing lethal endotoxemia in mice.

Methods: Mice were injected intraperitoneally with lipopolysaccharide (LPS) to induce endotoxemia. AT III was administered 3 h later. We assessed survival and the severity of endotoxemia and quantified plasma cytokine levels and biochemical markers of liver and kidney function. In the lungs, we examined neutrophil accumulation, neutrophil extracellular traps, alveolar wall thickness, and chemokine (C-X-C motif) *ligand 1 (cxcl-1), cxcl-2*, and high mobility group box 1 expression.

Results: Administering AT III reduced the severity and mortality of LPS-induced endotoxemia as indicated by 24-h survival of 84% of the mice that received LPS + AT III and only 53% of mice given LPS alone ($P < 0.05$). AT III treatment attenuated several changes induced in the lungs by endotoxemia including *cxcl-2* mRNA expression, high mobility group box 1 protein expression, neutrophil accumulation, alveolar septal thickening, and neutrophil extracellular trap formation. AT III did not decrease plasma cytokine levels or plasma urea nitrogen levels that were upregulated as a result of LPS-induced endotoxemia.

Conclusions: Administration of AT III after the onset of endotoxemia improved outcomes in a mouse model. The attenuation of lung inflammation may have a large impact on mortality and morbidity. Because lung inflammation increases the likelihood of mortality from sepsis, AT III could be a useful agent in septic patients.

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Introduction

Antithrombin (AT) III is a glycoprotein that is produced by liver and inactivates target enzymes in both the intrinsic and extrinsic coagulation pathways including thrombin, factor Xa, and factor IXa.[1] Heparin enhances thrombin-AT binding, augmenting the anticoagulatory activity of AT III. AT III also has anti-inflammatory effects,[2] which are mediated by binding to syndecan-4, a heparin sulfate proteoglycan. When heparin binds to AT III, it decreases the affinity of AT III for syndecan-4 and may attenuate the anti-inflammatory effects of AT III.[3,4] In a subgroup analysis of the Kyber-Sept study, a large clinical trial that investigated AT III treatment in patients with sepsis, the mortality of patients with severe sepsis was improved by AT III treatment in patients who did not receive concomitant heparin treatment.[5,6] The anti-inflammatory effects of AT III may play crucially important roles in severely septic patients.

Several animal studies support a potentially beneficial, anti-inflammatory role of AT III during sepsis.[7-12] In septic rat models, AT III inhibited the expression of proinflammatory cytokines and high mobility group box (HMGB)-1 protein, decreased neutrophil recruitment, and attenuated inflammation.[7-9] These models tested the effects of administration of AT III prior to or concomitant with the induction of sepsis. In clinical situations, suppressing inflammation too early may increase the patient's susceptibility to infection.[13] Thus, clarifying the effects of administering AT III after the initiation of sepsis is essential to elucidate the mechanisms underlying the anti-inflammatory effects of AT III and exploit the potential benefits to septic patients. Since coagulation and inflammation are closely connected to each other during sepsis,[14] AT III could be a useful agent to prevent not only coagulation disorders, but also to prevent organ dysfunction due to hyperinflammation. The purpose of this study was to elucidate the effects of administering AT III after the onset of sepsis using a mouse model of endotoxemia.

Material and methods

Animals

This study was approved by our Institutional Animal Care and Use Committee and was carried out according to Kobe University's animal experimentation regulations. Adult (7 to 15 wk old) male C57BL/6J mice (Clea Japan, Tokyo, Japan) were injected intraperitoneally with 20 mg/kg lipopolysaccharide (LPS; *Escherichia coli* O111:B5, Sigma–Aldrich, St Louis, MO) in 1 mL saline and 250 IU/kg AT III (Japan Blood Products Organization, Tokyo, Japan) in 0.5-mL saline under anesthesia. We used the single dose of AT III as much as 250 IU/kg because this dose has been previously shown to be effective to attenuate inflammation and improve survival in acute inflammation model in rats.[7,9,10] The AT III was injected 3 h after LPS injection. Since the peak of plasma levels of inflammatory cytokines such as interleukin (IL)-6 were 3 h after LPS injection in mice,[15] we thought the 3 h after LPS injection was an appropriate time point to administer AT III. Saline was used for vehicle control injections. Mice were randomly divided into four groups: (1) saline control group, 1 mL saline was injected then another 0.5-mL saline was injected 3 h later; (2) AT III group, 1 mL saline was injected, then AT III was injected 3 h later; (3) LPS group, LPS was injected, then saline was injected 3 h later; and (4) LPS + AT III group, LPS was injected first, then AT III was injected 3 h later. After injection, the mice were returned to their cages and allowed access to a standard diet and water ad libitum. Thirty-six mice were utilized to determine animal survival. Mice were killed under anesthesia at 3, 9, and 18 h after LPS injection to collect blood and lung samples.

Mice behavior score

Mouse behavior was observed every 2 h for 24 h after LPS injection with the observers blinded to the treatments received. The severity of endotoxic shock was scored according to the system of Rettew *et al.*[16] with a score of 1 given to mice with ruffled fur but no detectable behavioral differences, a score of 2 given to mice with percolated fur and a huddle reflex but that were still active, a score of 3 for mice that were less active and relatively passive when handled, a score of 4 for inactive mice that exhibited only limited response when handled, a score of 5 for moribund mice, and a score of 6 for dead mice.

FACSArray and ELISA

Tumor necrosis factor (TNF)-α, IL-6, interferon (IFN)-γ, and IL-10 were assayed using Cytometric Bead Array flex sets (BD Pharmingen, Franklin Lakes, NJ). Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ). Data were analyzed by FCAP Array software, version 1.0 (BD Immunocytometry Systems). The limits of detection for TNF-α, IL-6, IFN-γ, and IL-10 were 17.1 pg/mL, 6.5 pg/mL, 5.2 pg/mL, and 9.6 pg/mL, respectively. Plasma HMGB-1 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit specific for HMGB-1 (Shino-Test, Tokyo, Japan), according to the manufacturer's instructions. The ELISA plate was read at 450 nm using a plate reader (Microplate Reader 680, Bio Rad Laboratories).

Biochemical tests

Plasma samples were also sent to a clinical testing laboratory (SRL. Inc.; Tokyo, Japan). Plasma alanine aminotransferase, aspartate aminotransferase, bilirubin, urea nitrogen, and creatinine were quantitated.

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from harvested lungs with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA extracted was reverse-transcribed to yield single stranded cDNA using iScript cDNA Synthesis kits (Biorad, Hercules, CA) according to