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Recombinant fibrinogenase from *Agkistrodon acutus* venom protects against sepsis via direct degradation of fibrin and TNF-α☆

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ABSTRACT

Severe sepsis remains a leading cause of death and disability because of less effective therapy available for this disease. A complex interplay between the inflammatory factors and the coagulation pathways seems to be the fundamental mechanisms for the pathogenesis of sepsis. Here we report that recombinant fibrinogenase II (rF II) from *Agkistrodon acutus* plasmin-independently degraded the thrombi, and inhibited inflammatory responses by direct and specific degradation of tumor necrosis factor alpha (TNF-α) induced by lipopolysaccharide (LPS) without showing proteolytic activities on interleukin-1 (IL-1), cluster of differentiation 68 (CD68) and some other serum proteins. We also report that rF II effectively protected against LPS induced sepsis in a rabbit model. Administration of rF II reduced hepatic and renal damage, decreased the levels of alanine aminotransferase (ALT) and blood urea nitrogen (BUN), and increased survival rate in LPS-induced sepsis rabbits. We further confirmed the rescue effect of rF II on severe sepsis in rat caecal ligation and puncture (CLP) model. Our findings suggest that rF II could effectively protect against sepsis via direct degradation of microthrombi and inflammatory factor TNF-α as well as provide a novel strategy to develop a single proteinase molecule for targeting the main pathological processes of this disease.

1. Introduction

Sepsis is a life-threatening complication of severe infections and the most common cause of death in intensive care units. It has been reported that more than 75,000 cases of sepsis per year in the United States with a mortality of 25–30% [1]. Although much intensive efforts have been made in the past decades to develop treatment of sepsis, currently there is no established therapy for the clinical use yet [2,3].

The basic pathological mechanism of sepsis includes the spread of microvascular thrombosis, which prevents adequate blood supply to organs and leads to multiple organ failure and death [2]. Clinical trials aiming at an interruption of "latent coagulation" in sepsis by administration of coagulation inhibitors have so far failed to demonstrate a statistically significant benefit on survival. Heparin blocks endotoxin initiated clotting but is ineffective in preventing organ failure [4]. It is possible that failure of the anticoagulants to protect against sepsis may not have prevented the decrease in the

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blood supply to organs. Furthermore, the safety of anticoagulants in sepsis has not been established.

Inflammation and coagulation are tightly linked during sepsis [5–7]. Cytokines and inflammatory factors can induce coagulation. Thrombin and other serine proteases interact with protease-activated receptors to promote further activation and additional inflammation [8,9]. TNF-α is one of the most important inflammatory factors. During sepsis, tumor necrosis factor alpha (TNF-α) was elevated during the early period and played a key role in the following pathophysiologic processes [10,11].

Previous use of some proteinases purified from various snake venoms using biochemical methods showed effects on the clinical course of thrombotic disorders [12]. We isolated a novel snake venom fibrinogenase, F IIa, from the *Agkistrodon acutus* snake [13]. It has the ability to directly degrade fibrin *in vitro* and dissolves thrombi effectively *in vivo* [14,15]. In the present study, we cloned a novel gene, expressed, purified and characterized this novel recombinant protease. We found that this recombinant fibrinogenase II (rF II) protected against sepsis through its antithrombotic activity and degradation of TNF-α.

2. Materials and methods

2.1. Materials

All reagents were from Sigma, unless otherwise indicated.

2.2. Cloning and preparation of rF II

In a typical preparation, the salivary glands from *A. acutus* venom were homogenized and centrifuged at 10,000 × *g* for 10 min. A cDNA library was constructed [16]. Amplification of fibriongenase II DNA coding the mature peptide by PCR. Primer 1 is 5′-gtctcgagaaaagagaagctgaa-3′; Primer 2 is 5′-gagcggccgctcacgcctccaa-3′. After restriction digestion and sequencing, recombinant transfer plasmid pPIC 9K (Invitrogen, USA) was constructed successfully. Recombinant plasmid was digested by Sac I, and transformed into *Pichia pastoris* KM71 cells by electroporation. Transformants were selected by their ability to grow on minimal media MM/MD and then by their resistance to G418. Selected transformants were grown at 30 °C in 1 L baffled shake flasks containing 0.3 L rich medium. After induction for 48 h, the culture was collected and then centrifuged to remove cells and precipitates at 15,000 × *g* at 4 °C. The supernatant was ultrafiltered and then applied to a column of Unosphere S previously equilibrated with 0.01 M Tris–HCl (pH 8.0). The fraction displaying fibrinogenolytic activity was then mixed with 2.5 M NaCl and applied to another column of butyl sepharose 4FF previously equilibrated with 2.5 M NaCl buffer. The fraction was eluted with 1.8 M NaCl buffer at a flow rate of 100 ml/h at 25 °C. A UV detector was set at 280 nm to monitor the absorbance. The fraction was separated from salts by dialysis and lyophilized.

The purity of rF II was quantified by high performance liquid chromatography (HPLC). rF II was dissolved and applied to a gel-filtration column of G2000-SW_{XL} (7.8 mm × 30 cm; Tosoh Co., Japan) to perform an analysis of purity. Protein concentrations in column effluents were quantified by measuring absorbance at 280 nm. The fraction was analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using an Agilent-equipment under the following conditions: accelerated voltage 25 kV, in the linear analysis mode.

2.3. Fibrinolytic activity assays

The fibrin plate lysis assays were conducted in 110-mm plates as follows: the mixture consisting of 25 ml rabbit plasma and 10 U thrombin. After 30 min at room temperature, different concentrations of rF II and tissue-type plasminogen activator (t-PA) (Boehringer Ingelheim, Germany) (0, 0.25, 0.5, 1, 2, 4, 8 μM) were placed on the surface and incubated for 30 min at 37 °C. 8 μM rF II and t-PA were incubated for different times (0, 5, 15, 30 min) at 37 °C. The lysis areas were determined.

For the inactivation of intrinsic plasminogen, fibrin plates were placed at 82 °C for 1 h. Different concentrations of rF II and t-PA (0, 0.25, 0.5, 1, 2, 4, 8 μM) were placed on the surfaces and incubated for 180 min at 37 °C. 8 μM rF II and t-PA were incubated for different times (0, 30, 60, 120, 180 min) at 37 °C. The lysis areas were determined.

Fibrin degradation was also shown on 12% SDS-PAGE. Fibrin was formed from fibrinogen as follows: 1.2 μM human fibrinogen was clotted with 5 μl of thrombin (100 NIH units/ml), both in 50 mM Tris–HCl buffer. The fibrin clots were allowed to form for 1 h at room temperature. 0.04 μM rF II was added to every clot and incubated at 37 °C for 0, 0.5, 1, 2, 4, 8, 12, 24 h. Then 0.3 ml of denaturing solution was added and the mixture was incubated over night before electrophoresis.

2.4. Plasminogen activation assays

Human plasminogen was incubated at a final concentration of 10 U/ml in 50 mM Tris–HCl buffer. The reaction was initiated by the addition of rF II to the final concentrations of 0.5–2 μg/ml. Aliquots were taken at various time intervals and assayed for plasmin activity. They were introduced into plastic cuvettes which contained 0.5 μl of D-Pro-Phe-Arg-*p*NA (P7959, 0.2 mM). The mixture was incubated for 30 min at 37 °C. The reaction was stopped and the formation of *p*-nitroanilide was monitored at 405 nm. 0.01 U/ml Plasmin (with no plasminogen) and 10 μg/ml t-PA was used as positive control.

2.5. Direct degradation effect of rF II on TNF-α protein

10 μg recombinant rabbit TNF-α protein and mouse TNF-α protein (Protech Company, USA) were incubated with 10 μg/ml rF II at 37 °C for 2 h. Each sample was analyzed by 12% SDS-PAGE.

2.6. Measurement of TNF-α concentration

The plasma of rabbits were collected in tube and stored at −20 °C until assayed. RAW 264.7 macrophages were placed in 96 well plates. The cells were stimulated with 1 μg/ml lipopolysaccharide (LPS) for 1, 2, 3 and 4 h respectively, in the absence or presence of 1 μg/ml rF II. Cell culture super-