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A novel fibrinogenase from *Agkistrodon acutus* venom protects against DIC via direct degradation of thrombosis and activation of protein C

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ABSTRACT

The incidence of disseminated intravascular coagulation (DIC), which leads to multiple organ dysfunction and high mortality, has remained constant in recent years. At present, treatments of DIC have focused on preventing cytokine induction, inhibiting coagulation processes and promoting fibrinolysis. Recent clinical trials have supported the use of antithrombin and activated protein C supplementation in DIC. To better understand the mechanism of treatment on DIC, we here report a novel fibrinogenase from *Agkistrodon acutus* (FIIa) that effectively protected against LPS-induced DIC in a rabbit model, and detected the tissue factors expression in HUVE cells after using FIIa. In vivo, administration of FIIa reduced hepatic and renal damage, increased the concentration of fibrinogen, the activities of protein C, the platelet count, APTT, PT, FDP, the level of AT-III and t-PA, decreased the level of PAI-1, and increased survival rate in LPS-induced DIC rabbits. In vitro experiments, we further confirmed that FIIa up-regulated the expression of t-PA and u-PA, down-regulated the expression of PAI-1, and directly activated protein C. Our findings suggest that FIIa could effectively protect against DIC via direct degradation of microthrombi and activation of protein C as well as provide a novel strategy to develop a single proteinase molecule for targeting the main pathological processes of this disease.

1. Introduction

Disseminated intravascular coagulation (DIC) is an acquired syndrome characterized by the activation of intravascular coagulation and subsequent intravascular fibrin formation. This process may be accompanied by secondary fibrinolysis, which often leads to a bleeding tendency, or deficient fibrinolysis [1,2], which can cause diffuse microthrombi formation in the organs, severe cases can lead to multiple organ function failure and finally death [3]. DIC is a life threatening syndrome arising from various causes including disseminated sepsis. It is generally associated with an adverse outcome [4,5]. If severe or life-threatening hemorrhage occurs, replacement with platelets, fresh plasma, and possibly cryoprecipitate, is indicated [6], but the intravascular fibrin formation and organ dysfunction are often irreversible by the fact that the timing of therapy is crucial. The standard anticoagulant therapy such as heparin and low molecular weight heparin is thought to enhance the effects of thrombolysis by preventing formation of new fibrin in organs. Thus far, however, there is no indication from clinical studies that anticoagulation offers any survival benefit in patients with DIC. Another therapeutical choice in treating DIC is to administer plaminogen activators(t-PA, u-PA) to induce fibrinolysis of existing and developing clots. Experimental studies with u-PA have shown modest effects [7]. Plasminogen activator inhibitor(PAI)-1 is the principal inhibitor of plasminogen activation and appears to be the most involved in DIC [8,9]. Increased PAI-1 has been associated with a predisposition to thrombosis, which is a specific inhibitor of t-PA released from endothelial cells. These conditions may "overflow" the systemic circulation that leads to systemic fibrinolysis and degradation of other clotting proteins [10], increases the bleeding tendency. Combination therapy with multiple anticoagulatory agents may ultimately prove to be the best approach for treating DIC. One recent study found that combining ATIII and protein C-supplement therapy reduced TNF-α levels and hypotension associated with

Abbreviations: APC, activated protein C; APTT, activated partial thromboplastin time; AT-III, antithrombin III; DIC, disseminated intravascular coagulation; EGF, endothelial growth factor; FDP, fibrin(-ogen) degradation products; FIIa, fibrinogenase II from *Agkistrodon acutus*; HUVECs, human umbilical vein endothelial cells; LPS, Lipopolysaccharide; PAI-1, plasminogen activator inhibitor-1; PT, plasma prothrombin time; t-PA, tissue-type plasminogen activators; TNF-α, tumor necrosis factor-α; u-PA, urokinase-like plasminogen activators; VEGF, vascular endothelial growth factor.

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endotoxin-induced experimental sepsis, though no effect on coagulation parameters was evident [11]. Recombinant human activated protein C has shown efficacy in decreasing mortality in patients with sepsis accompanied with DIC, but it is not clear to which extent this benefit is caused by amelioration of DIC. Many therapeutic avenues are being developed based on recent findings into the causes and progression of DIC, but really no effective therapy available [12–14].

FIIa is a noval fibrinolytic enzyme purified from *Agkistrodon acutus* venom, which is belonged to the snake vemon metalloproteinase family. The crystal structure of FIIa features that it has a Zn^{2+} ion in the active site which is essential for hydrolytic activity [15,16]. In vitro FIIa directly degrades α-chains and β-chains of fibrin/fibrinogen, whereas in vivo studies show it dissolves thrombus without activating plasminogen or influencing the activities of t-PA, urokinase, and PAI-1 [17]. This shows that FIIa has a different mechanism of action from t-PA and urokinase. Additionally, in the examination of tissue sections from kidney, liver, heart, and lung [17], the thrombolytic activities of FIIa lacked hemorrhaging. These results suggest that FIIa should be a safe and attractive agent for treating DIC.

To better understand the effect of FIIa, which may be a potential clinical use in DIC, here we report the activity of FIIa on a lipopolysaccharide (LPS)-induced model of DIC and investigated the mechanism of its action in laboratory.

2. Materials and methods

2.1. Reagents

LPS, heparin, and human fibrinogen (95% clottable) were purchased from Sigma (St. Louis, Mo). The fibrinogen concentration determination reagent pack (Clauss method) and the reagent packs for the activity assays of antithrombin III (ATIII), protein C, plasminogen, PAI-1, and t-PA were obtained from Sun Biotechnology Company (Shanghai, China); The human t-PA ELISA kit and the human u-PA ELISA kit were purchased from ASSAYPRO (USA). The human PAI-1 ELISA kit, the human recombined TNF-α and its antibody, the VEGF, the t-PA antibody and the u-PA antibody were purchased from R&D systems, Inc (USA). The protein C and EGF were purchased from abcam (USA), and the human APC ELISA kit was from USCN LIFE (USA); all other reagents were analytical grade from commercial sources.

2.2. Animals

Adult male New Zealand white rabbits (weight 2–3 kg) were supplied by the Experimental Animal Center of Guangdong Province.

2.3. Purification of the enzyme

The fibrinogenase II(FIIa) isolated from *Agkistrodon acutus* vemon, was prepared according to the method previously described [18].

2.4. Experiments in vivo

2.4.1. Experimental animal models

All procedures were conducted according to the ethical guidelines of the Animal Care and Use Committee at SUN Yat-Sen University. DIC experimental models were performed by the method of Jose Hermida [19], which were induced by infusing LPS in 60 ml of saline solution at a rate of 100 μg/kg/h (10 ml/h) through the marginal ear vein of rabbits over a period of 6 h. Animals were anesthetized by an intramuscular injection of 30 mg/kg ketamine hydrochloride, followed by intramuscular supplements of ketamine hydrochloride given throughout the experiment.

Treatments, which were according to the method of Lin [20], started simultaneously with LPS infusion through the contralateral marginal ear vein. Six different groups were established, one of which contains 10 animals: treatment groups (low-, medium-, and high-dose FIIa) were given 0.1, 0.3 and 0.6 mg/kg/h in 60 ml of saline solution over a period of 6 h (10 ml/h). The LPS control group was infused with saline solution, which was at a rate of 10 ml/h, over a period of 6 h. The heparin control group was infused with heparin at a rate of 100 IU/kg/h (10 ml/h) over a period of 6 h. The normal control group, which was given neither LPS nor FIIa, was infused with saline solution through both marginal ear veins of the animals.

2.4.2. Sample collection and handling

Blood samples were taken through a catheter inserted into a femoral artery immediately before LPS infusion and at 2 and 6 h postinfusion. Blood samples were collected in 3.8% sodium citrate (1:10 vol/vol citrate/blood). The blood was centrifuged at 2000 × *g* for 15 min at 4 °C. Blood for the measurement of t-PA was collected in Stabilyte tubes (Biopool, Umea, Sweden) in order to avoid the interference with its inhibitors. All samples were stored at −70 °C until assayed.

2.4.3. Laboratory methods

Fibrinogen consentration was measured according to the method of Clauss. ATIII, protein C, plasminogen, PAI-1, and t-PA activity were measured according to the reagent pack instruction based on chromogenic substrates.

2.4.4. Tissue preparation for histologic examination

Kidney, liver tissue specimens were fixed in formalin, embedded in paraffin, stained with phosphotungstic acid-hematoxylin stain, and examined for the presence of fibrin microthrombi by a pathologist.

2.5. Experiments in vitro

2.5.1. Human umbilical vein endothelial cells (HUVEC) cultures

Primary HUVEC were isolated from normal umbilical cords as described by Jaffe EA [21]. HUVEC were cultured in Medium 199 (Gibco BRL, Grand Island, NY, USA) containing 20% fetal bovine calf serum (Hyclone, Logan, UT, USA), endothelial growth supplement (Sigma, St. Louis, MO, USA), 5% penicillin/streptomycin (Gibco BRL), and 25 ug/ml heparin (Sigma), and 50 ng/ml ECGs (abcam). For assays, HUVEC were plated at a concentration of 1×10^5 cell/ml and grown for 24 h with a humidified atmosphere of 5% CO_2 at 37 °C prior to experimentation. All experiments utilized cells grown within five passages.

In the following experiments, the concentration of FIIa is 2.5 μg/ml and the density of HUVEC is 1×10^5/well.

2.5.2. The action of FIIa on TNF-α-induced PAI-1 cell model

2.5.2.1. Observation of PAI-1 protein by immunofluorescence. Four different groups were established, control group was given M199 only. The positive group was given TNF-α (100 ng/ml), meanwhile the treatment group was given TNF-α (100 ng/ml) and FIIa. The fourth group was only given FIIa. The protocol of immunofluorescence is the same as the above describe, except that the primer antibody is PAI-1 antibody.

2.5.2.2. Measurement of PAI-1 concentration by ELISA assays. The concentration of PAI-1 in the supernatant was determined using Human Serpin E1/PAI-1 ELISA kit (R & D systems, USA), according to the assay procedures.