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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-

natants were stored at –20 °C until assayed. The concentrations of TNF-α in animal serum and cell culture supernatants were determined using ELISA KIT (R&D Systems, Inc., USA and RapidBio Lab. Calabasas, USA).

2.7. Confocal Immunofluorescence microscopy of TNF-α and cluster of differentiation 68 (CD68) of macrophages

RAW 264.7 macrophages were stimulated with 1 μg/ml LPS in the absence or presence of 1 μg/ml rF II. Cells were then fixed in a 1:1 methanol: acetone solution for 30 min following by blocking in goat serum for 1 h. 1:200 monoclonal anti-TNF-α antibody (Sigma, USA) and 1:100 monoclonal anti-CD68 antibody (Sigma, USA) were added to the cells and incubated at 4 °C overnight. Goat anti-mouse FITC antibody (Jackson Immuno. Research lab., USA) was added for 1 h at room temperature and mounted to glass slides until visualized by confocal immunofluorescence microscopy (Nikon, Japan).

2.8. RT-PCR assay for TNF-α mRNA in macrophages

After incubation LPS and/or rF II for 1 h, macrophages were harvested. Total cellular RNA was extracted using RNA easy kits (Invitrogen, USA). Expression of mRNA was determined by RT-PCR System kit (Promega, Madison, WI) following the manufactures' protocol. The following primers were used: TNF-α sense: 5′-tactgaacttcggggtgattggtcc-3′; antisense: 5′-cagccttgtcccttgaagagaacc-3′. β-actin sense: 5′-ctctgaacccc-taaggccaacc-3′; antisense: 5′-atgccacaggattccataccc-3′. PCR was performed using an initial step of denaturation (1 min at 94 °C), 35 cycles of amplification (94 °C for 1 min, 58 °C for 30 s, and 72 °C for 30 s), and an extension (72 °C for 7 min). The amplifications of TNF-α and β-actin genes are expected to generate 295 bp and 500 bp, respectively. PCR products were analyzed on 2% agarose gels.

2.9. Effects of rF II on interleukin-1 (IL-1) and serum proteins

10 μg the recombinant human IL-1 protein and human serum proteins 10 μg albumin, 10 μg gamma-golubin, 10 μg alpha$_2$-antitrypin, 10 μg haptoglobin were incubated with 10 μg/ml of rF II for 2 h at 37 °C respectively and analyzed the products on 12% SDS-PAGE.

2.10. Animals and drug treatments

All animal experiments were conducted in accordance with the National Guide for the Care and Use of Laboratory Animals and were approved by Sun Yat-sen University Animal Care and Use Committee (Guangzhou, China). Acute pulmonary embolism was performed according to the method of Frank H [17]. An autologous thrombus was produced in a 5-ml tube. 1 ml thrombus fragment was cut according to an established protocol. Blood clots were injected and embolized the pulmonary arteries. Prior research has shown that the emboli typically embolized the descending branch of the right or left pulmonary artery, which measures up to approximately 2 mm in rabbits. One hour after the start of the experiments, they were sacrificed and the tissue of the thromboembolic and non-thromboembolic pulmonary artery was obtained.

Sepsis was induced by infusion with 0.1 mg/kg/h LPS for 6 h in 60 ml (10 ml/h) intravenously through the marginal ear vein. Treatments were started simultaneously with LPS infusion through the contralateral marginal ear vein. Blood samples were taken through a catheter inserted into a femoral artery immediately before LPS infusion, 2 h and 6 h after the start. To determine the extent of organ damage in rabbits we measured plasma levels of blood urea nitrogen (BUN) and alanine aminotransferase (ALT). These were determined by 7170A automatic analyzer (HITACHI, Japan).

Adult male Sprague-Dawley rats weighing 200–250 g were used. Sepsis was induced by the CLP procedure as described previously [18]. In brief, rats were anesthetized with isoflurane. After a midline incision, the cecum was exposed and was ligated ~2/3 of the distance from the distal pole. The ligated cecum was punctured through with an 18-gauge needle and a small portion of feces wad expressed. The abdomen was closed in layers using 4–0 sutures. Where indicated, animals received 1.0 mg/kg rF II, 50 IU/kg heparin and 2 mg/kg xigris for 6 h by intravenous injection 1 h after CLP. Sham animals underwent the same procedure except for ligation and puncture of the cecum.

2.11. Determination of median lethal dose (LD50) of rF II

Male mice of Kunming strain weighing 18–22 g were used. The mice were individually caged, observed for health verification for 7 days prior to testing. According to the method of Bliss [19], based on the results obtained from preliminary toxicity study, the dose of 100% mortality is 80 mg/kg and the dose of 0% mortality is 30 mg/kg. Mice received rF II by intravenous injection in graded doses of 80, 64, 51.2, 40.96 and 32.76 mg/kg (the ratio is 1:0.9) to five different groups of mice. Each animal was observed daily for 7 days following dosing. LD50 were calculated by the Bliss software.

2.12. Data analysis

Differences between data groups were evaluated for significance using Student's *t*-test of unpaired data or one-way analysis of variance. Repeated measures analysis of variance was used for multivariate analysis. All experiments were repeated at least three times and the data were presented as the mean ±S.D. unless otherwise noted. Results of ALT and BUN at 2 h and 6 h were converted to percentages assuming a value of 100% for basal data. Survival curves of CLP were analyzed by the Kaplan–Meyer log-rank test.

3. Results

3.1. Cloning and preparation of recombinant fibrinogenase II

Based on our cDNA library and database of *A. acutus* venom, which contains over 8000 expressed sequenced tags [16], we screened and cloned a cDNA encoding rF II, which contains 702 base-pairs in its open read frame (Fig. 1A). Its amino acids

(A) cDNA sequence

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   1 aaaagagagg ctgaagctaa tcgtactcct gaacaacaaa tctatgaccc ctacaaatac gttgagactg tctttgttgt
  81 ggacaaagca atggtcacaa aatacaatgg cgatttagat aagataaaaa caagaatgta cgaagctgcc aacaatatga
 161 atgagatgta cagatatatg ttttttcgtg tagtaatggt tggcctaata atttggaccg aagaagataa gattaccgtg
 241 aagccagatg tggattatac tttgaacgca tttgcagaat ggagaaaaac atatttgctg gctgagaaaa aacatgataa
 321 tgctcagtta atcacgggca ttgacttcag aggaagcatt ataggatacg cttacattgg cagcatgtgc cacccgaagc
 401 gttctgtagg aattattcag gattatagcc caataaatct tgtgcttgcc gttataatgg cccatgagat gggtcacaat
 481 ctgggcattc accatgacga cggttactgt tattgcggtg gttacccatg cattatgggt ccctcgataa gccctgaacc
 561 ttccaaattt ttcagcaatt gtagttatat ccaatgttgg gactttatta tgaatcacaa cccagaatgc attgacaatg
 641 aacccttggg aacagatatt atttcacctc cactttgtgg aaatgaactt ttggaggcgt ga
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(B) Amino acid sequence

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   1 KREAEANRTP EQQIYDPYKY VETVFVVDKA MVTKYNGDLD KIKTRMYEAA NNMNEMYRYM FFRVVMVGLI
  71 IWTEEDKITV KPDVDYTLNA FAEWRKTYLL AEKKHDNAQL ITGIDFRGSI IGYAYIGSMC HPKRSVGIIQ
 141 DYSPINLVLA VIMAHEMGHN LGIHHDDGYC YCGGYPCIMG PSISPEPSKF FSNCSYIQCW DFIMNHNPEC
 211 IDNEPLGTDI ISPPLCGNEL LEA
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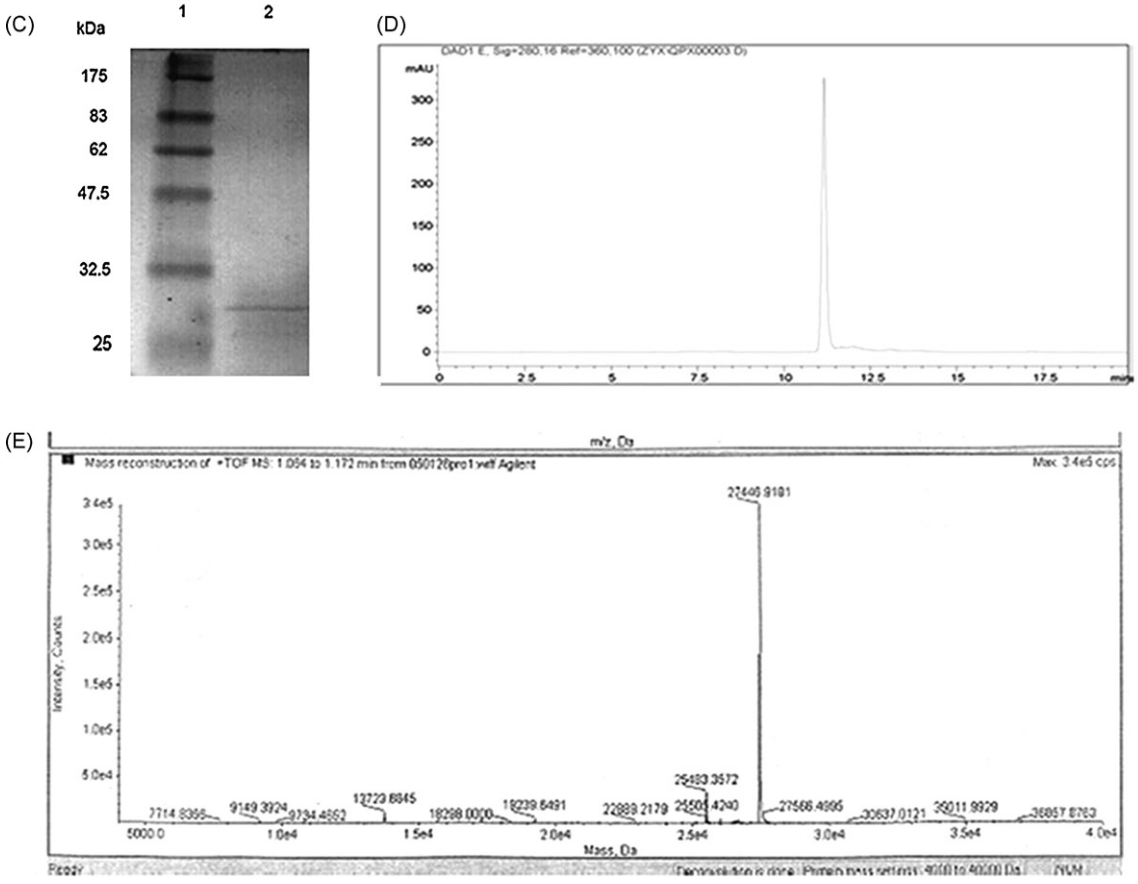

Fig. 1 – Cloning and preparation of rF II. (A) cDNA sequence of rF II. (B) Amino acid sequence of rF II. The active site sequence of metalloproteinase in rF II is boxed. (C) The purity of rF II was examined by SDS-PAGE. Lane 1 is markers; lane 2 is product of gel filtration. (D) The purity of rF II was analyzed by HPLC. (E) The purity of rF II was analyzed by MALDI-TOF MS.

sequence was deduced from the cDNA sequence (Fig. 1B). We then successfully expressed the gene in *P. pastoris* and characterized a recombinant fibrinogenase II. rF II shared significant degrees of identity with known proteases [20]. The catalytic triad, H155, E156, H159, G162 and H164, which is a conserved sequence in zinc-dependent metalloproteinases, was also found in rF II (Fig. 1B). The purity of rF II was examined by SDS-PAGE and HPLC. The results showed that there was only one fragment on SDS-PAGE and only one peak was eluted on HPLC (Fig. 1C lane 2; Fig. 1D). We further

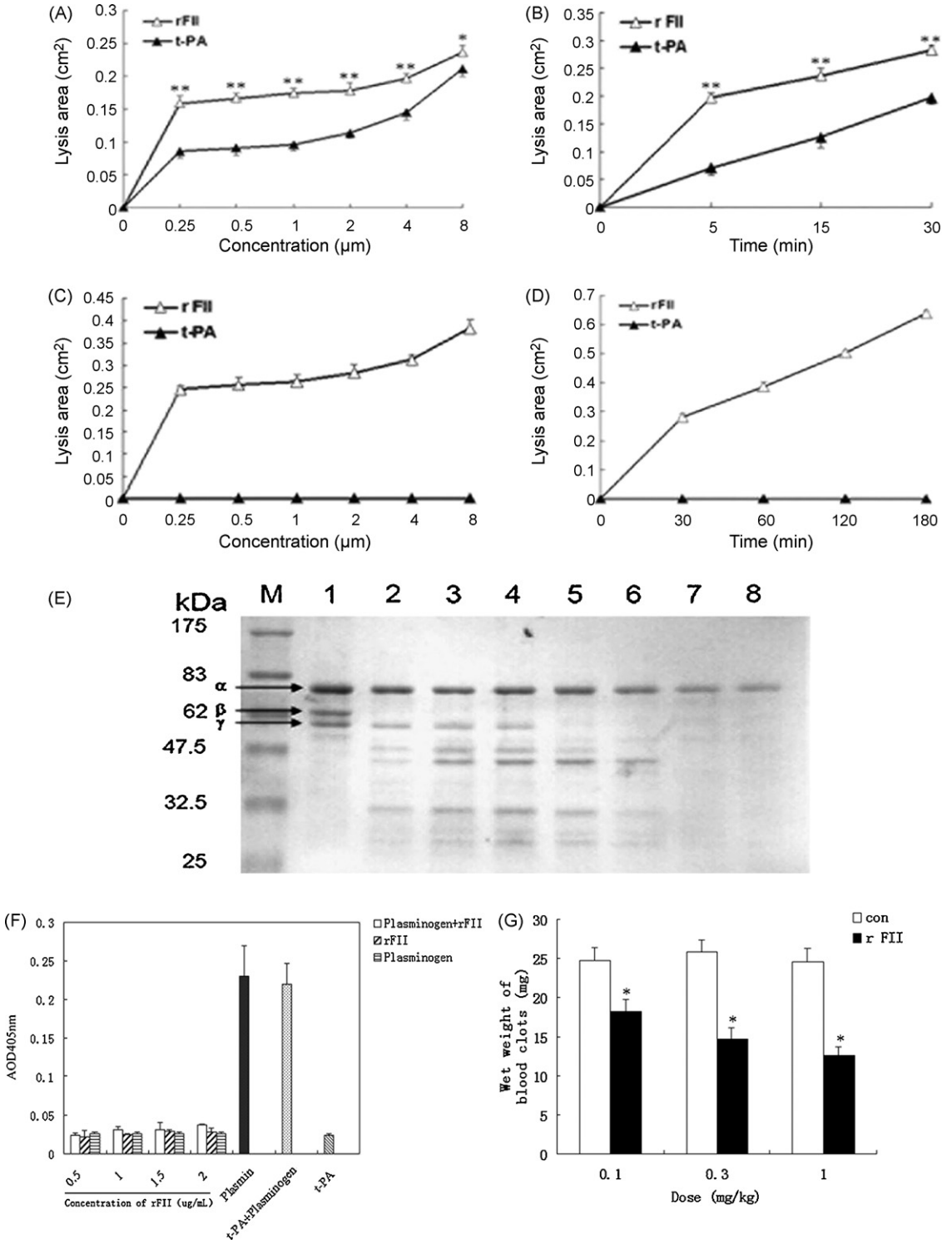

Fig. 2 – Fibrinolytic activity of rF II *in vitro* and *in vivo*. (A and B) Analysis of fibrinolytic activity of rF II on rabbit plasma plates in different concentrations and different times. (C and D) Analysis of fibrinolytic activity of rF II on inactive rabbit plasma plates in different concentrations and different times. Plasma plates were prepared as described in Section 2. *$P < 0.05$, **$P < 0.01$. Data represent mean ± S.D. of three independent experiments. (△) rF II; (▲) t-PA. (E) Fibrin degradation by rF II. The fibrin clot was prepared as described in Section 2. Markers were shown on the extremely left. Lanes 1–8 represent the following time-points sequentially: 0, 0.5, 1, 2, 4, 8, 12, 24 h. (F) Effect of rF II on plasminogen activation. Plasminogen was incubated with rF II at 37 °C for 1 h. (G) Thrombolysis effect of rF II on blood clots in pulmonary artery of rabbits ($n = 10$). Data are presented as the mean ± S.D. *$P < 0.05$.

determined rF II's actual molecular weight by MALDI-TOF MS. As shown in Fig. 1E, rF II's molecular weight was 27.4 kDa, which was consistent with the values calculated from its amino acid sequence and the result of SDS-PAGE.

3.2. *Fibrinolytic activity of rF II in vitro*

To determine the bioactivity of rF II, we firstly investigated its fibrinolytic effect *in vitro*. Fibrinolytic activity of rF II was measured on fibrin plates formed from rabbit plasma, and compared with t-PA, a standard agent clinically used in antithrombi therapy. The fibrinolytic activity of rF II was higher than t-PA at the same concentrations (Fig. 2A). We further analyzed the effect of rF II on fibrin plates at different reactive times (0–30 min). The fibrinolytic activity of rF II was also higher than t-PA at the same reactive times (Fig. 2B). As shown in Fig. 2E, SDS-PAGE analysis showed that the β-chain of fibrin was degraded mainly within 30 min. Meanwhile, the γ-chain was also being degraded completely 2 h after incubation. The intensity of α-chain changed slowly over time.

3.3. *Effect of rF II on fibrin is independent of activation of plasminogen*

Given that most of the fibrinolytic drugs, such as t-PA, exert their actions via the conversion of endogenous plasminogen to plasmin, we further examined whether rF II degrades fibrin through activation of plasminogen or through direct cleavage of thrombi. We measured fibrinolytic activity of rF II on inactive fibrin plates formed from rabbit plasma. After inactivation of plasminogen on fibrin plates at 82 °C, the fibrin clots were still hydrolyzed by rF II in a dose- and time-dependent manner. However, t-PA had no effect on the inactivated fibrin plates at the same concentrations and times (Fig. 2C and D).

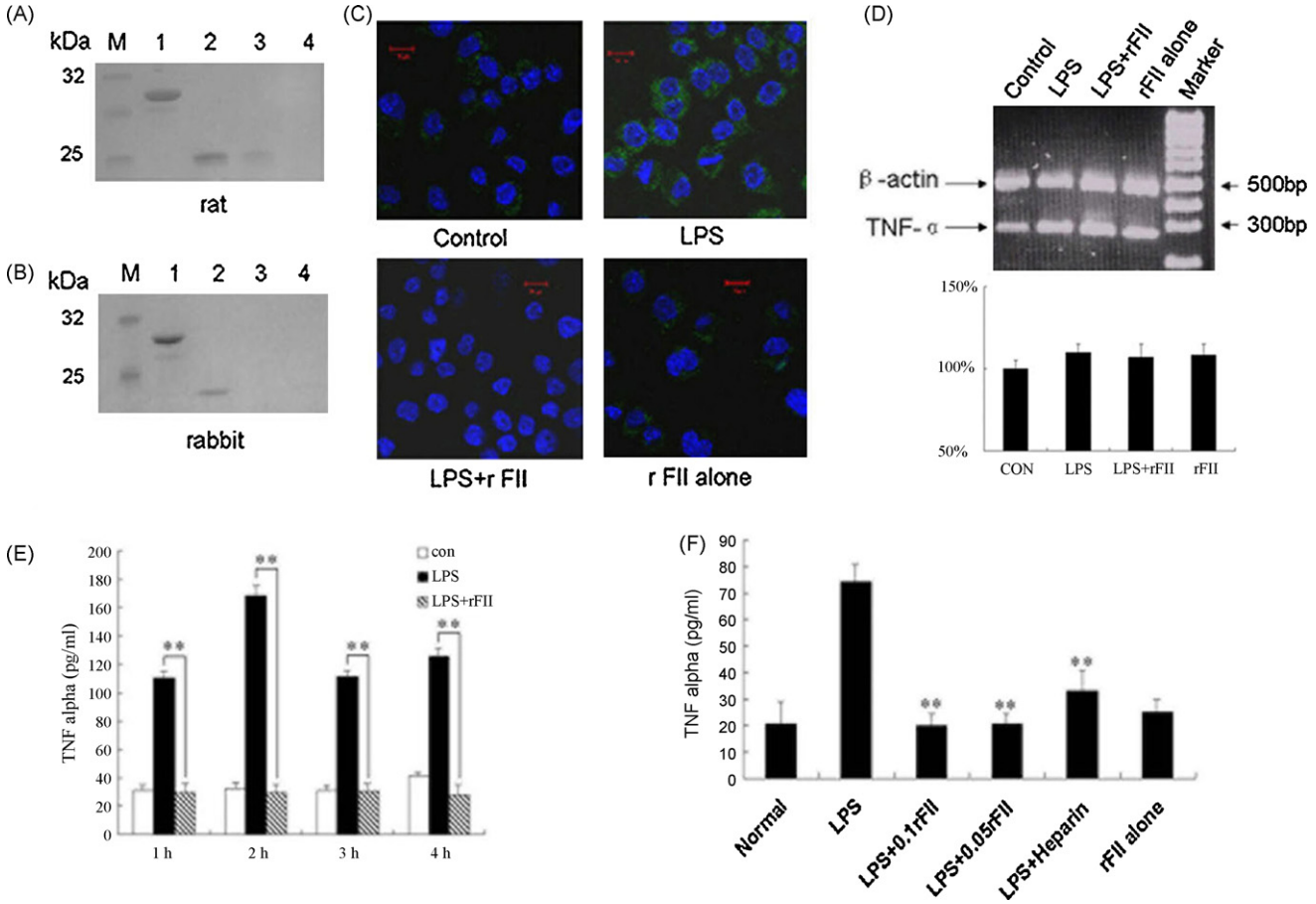

Fig. 3 – Degradation of rF II on TNF-α *in vitro* and *in vivo*. (A) Directly proteolytic effect of rF II on rat TNF-α protein. Markers were shown on the extremely left. Lane 1: rF II protein; lane 2: rat TNF-α protein; lane 3: rat TNF-α protein plus rF II; lane 4: work concentration of rF II. (B) Directly proteolytic effect of rF II on rabbit TNF-α protein. Markers were shown on the extremely left. Lane 1: rF II protein; lane 2: rabbit TNF-α protein; lane 3: rabbit TNF-α protein plus rF II; lane 4: work concentration of rF II. (C) Immunofluorescence assay analyzing the proteolytic effect of rF II on TNF-α on macrophages. Blue: hochest 33258 staining; Green: TNF-α. Scale bars represent 10 μm. (D) Effect of rF II on LPS induced TNF-α mRNA expression in macrophages. (E) Proteolytic effect of rF II on macrophages releasing TNF-α in supernatants. Data represent mean ± S.D. of three independent experiments. $^{**}P < 0.01$ as compared to LPS group. (F) Effect of rF II on plasma levels of TNF-α in rabbits at 2 h. $n = 6$, $^{**}P < 0.01$ as compared to LPS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

To further confirm the direct effect of rF II on the fibrin clots, activation of human plasminogen by rF II was determined by measuring the amidolytic activity of active plasminogen on P7959. After pre-incubating rF II with plasminogen for 1 h at 37 °C, the amidolytic activity of the activated plasmin was detected. There was no amidolytic activity detected after pre-incubation of different concentrations of rF II in 1 h (Fig. 2F). Meanwhile, the absorbance of plasmin was 0.17, with about 4 fold higher than that of rF II. It demonstrated that rF II would not activate plasminogen. There was no amidolytic activity detected after pre-incubation of t-PA. However, the absorbance of t-PA and plasminogen was still high. These results further demonstrated that t-PA exerts its action via the conversion of endogenous plasminogen to plasmin but rF II can directly hydrolyze fibrin without activating intrinsic plasminogen. Taken together, these results suggested that rF II is a plasmin-independent fibrinolytic enzyme.

3.4. *Thrombolysis effect of rF II in vivo*

The *in vivo* thrombolysis effect of rF II was studied in a rabbit pulmonary artery thrombotic model. rF II was delivered at doses of 0.1, 0.3, 1 mg/kg and the thrombolysis effect on pulmonary artery were tested 1 h after injection. rF II significantly decreased the thrombi weight in pulmonary artery in dose-dependent manner (Fig. 2G). These results were consistent with a fibrinolytic effect of rF II on fibrin *in vitro*.

3.5. *Direct degradation effect of rF II on TNF-α in vitro*

It has been shown that the overproduction of cytokines is associated with septic shock and organ failure during sepsis. TNF-α is an important inflammatory marker and generally increases significantly during the early period of sepsis [10,11]. Therefore, we tested whether or not rF II had proteolytic activity on TNF-α protein. We incubated the recombinant rabbit TNF-α protein and rat TNF-α protein with 10 μg/ml of rF II for 2 h at 37 °C and analyzed the products on SDS-PAGE. We found that the recombinant rabbit and rat TNF-α proteins were mostly degraded within 2 h by rF II (Fig. 3A and B).

We then evaluated effects of rF II degradation of TNF-α in a cellular model. We observed that 1 μg/ml of LPS stimulated expression of TNF-α on the cell surface and in the supernatants of cultured macrophages. TNF-α was significantly reduced when incubated with rF II at a concentration of 1 μg/ml in the simultaneous presence of 1 μg/ml of LPS at 2 h (Fig. 3C). Moreover, levels of LPS-induced TNF-α in supernatants were also reduced by co-treatment with rF II in 1, 2, 3, 4 h (Fig. 3E).

In order to eliminate the possibility that down-regulation of LPS-induced TNF-α by rF II was due to inhibition of synthesis, we further examined the effect of rF II on LPS-induced TNF-α

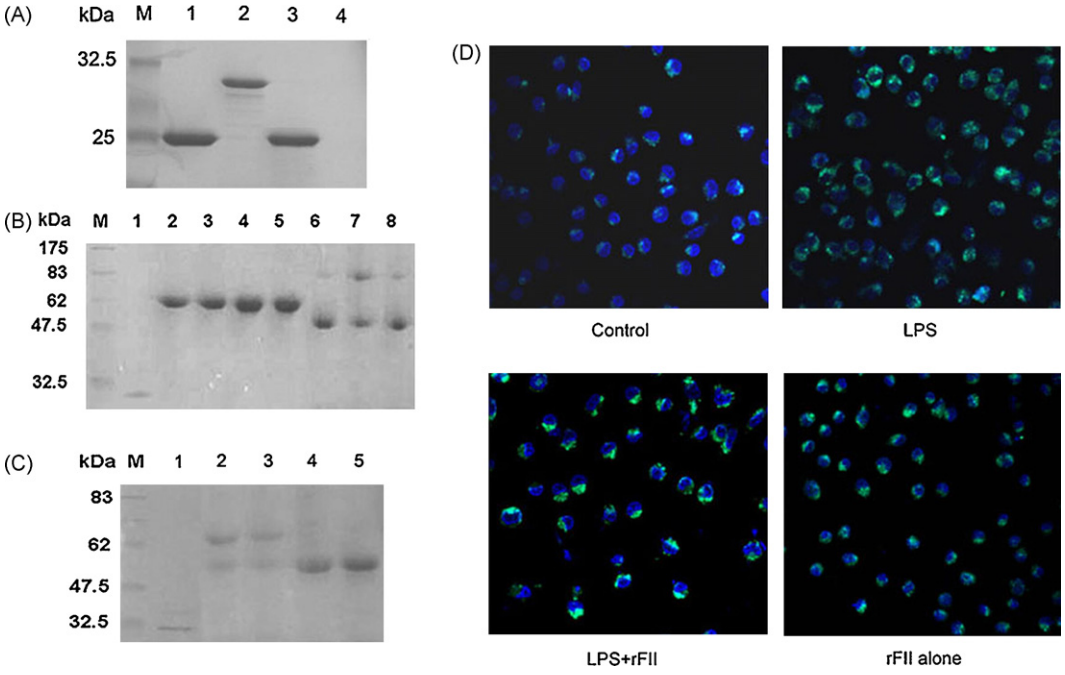

Fig. 4 – Effect of rF II on IL-1, CD68 and serum proteins. (A) Effect of rF II on human IL-1 protein. Markers were shown on the extremely left. Lane 1: human IL-1 protein; lane 2: rF II protein; lane 3: IL-1 protein plus rF II; lane 4: work concentration of rF II. (B) Effects of rF II on human albumin and γ-globulin. Markers were shown on the extremely left. Lane 1: rF II; lanes 2–5: 0, 2.5, 5, 10 μg/ml rF II incubated with human albumin; lanes 6–8: 0, 5, 10 μg/ml rF II incubated with human γ-globulin. (C) Effects of rF II on human α_2-antitrypin and haptoglobin. Markers were shown on the extremely left. Lane 1: rF II; lane 2: human α_2-antitrypin; lane 3: human α_2-antitrypin + rF II; lane 4: human haptoglobin; lane 5: human haptoglobin + rF II. (D) Immunofluorescence assay analyzing the effect of rF II on CD68 in macrophages. Blue: hochest 33258 staining; Green: CD68. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

mRNA expression in macrophages. As can be seen in Fig. 3D, the levels of mRNA of TNF-α was not altered by co-stimulation with rF II, showing that down-regulation of TNF-α by rF II was not related to inhibition of mRNA synthesis.

3.6. Degradation effect of rF II on TNF-α in vivo

To demonstrate effects of rF II cleavage of TNF-α *in vivo*, we determined the plasma levels of TNF-α in LPS treatment rabbits. Rabbits were injected with 0.1 mg/kg/h of LPS and levels of TNF-α were dramatically increased at 2 h (Fig. 3F). However, infusion of 0.05 mg/kg and 0.1 mg/kg of rF II significantly reduced the increased plasma levels of TNF-α at 2 h (Fig. 3F).

3.7. Specificity of rF II activity

It is known that the haemorrhagic toxins present in the snake venoms act directly on the capillary basement membrane and serum proteins. This damage can induce to cause internal haemorrhage, by which their clinical use has been limited [21]. Therefore, we observed whether or not rF II had specificity of its proteolytic activities upon some important proteins. We first incubated some recombinant human serum proteins with 10 μg/ml of rF II for 2 h at 37 °C. Human albumin, gamma-golubin, alpha$_2$-antitrypin and haptoglobin were not degraded by rF II (Fig. 4B and C). We further examined whether or not rF II had proteolytic activities on IL-1, another important inflammatory molecule, and CD68, an important surface protein of macrophages. As can be seen in Fig. 4A, rF II had no proteolytic activity on IL-1. Similarly, CD68 was also not reduced by rF II in the simultaneous presence of LPS (Fig. 4D). These series of results suggest that rF II possesses relatively high specificity compared with its proteolytic activities of fibrin and TNF-α.

3.8. The protective effect of rF II on LPS induced sepsis

Given the findings noted above, we further investigated the effects of rF II on LPS-induced sepsis in a rabbit model. 34.8% (8/23) of rabbits infused with 0.1 mg/kg/h LPS survived by the first 24 h after the start of the experiment, whereas all of the saline control group animals survived (Fig. 5A). The survival rates of the rF II treatment were significantly increased: seven of eleven rabbits (63.6%) survived after a low dose of rF II and fourteen of eighteen rabbits (78%) survived after a high dose of rF II respectively. The survival rate of heparin treatment group was 72.7% (8/11). The rabbits infused with only rF II all survived.

3.9. The effects of rF II on liver and renal functions in rabbits in sepsis induced by LPS

We further investigated the effects of rF II on liver and renal functions in rabbits. Plasma levels of ALT, an indicator of liver injury, were increased by LPS infusion. However, the levels of ALT were significantly lower in rF II treatment animals as well as in heparin treatment rabbits (Fig. 5B). A similar finding was observed with regard to plasma levels of BUN, which is an indicator of renal injury. An increase in plasma BUN levels was observed in the LPS group at 6 h. The levels of BUN were significantly suppressed by rF II as well as by heparin (Fig. 5C).

3.10. rF II rescues sepsis in rat caecal ligation and puncture (CLP) model

We investigated the potential therapeutic effect of rF II in the rat model of caecal ligation and puncture, a clinically relevant model for human sepsis because it causes lethal peritonitis produced by polymicrobial infection [22]. rF II treatment was started 1 h after the induction of sepsis. The results showed that rF II at dose of 1 mg/kg significantly improved survival (Fig. 6). The survival rate of CLP-induced sepsis was increased by rF II from 18.2% to 72.7% ($p = 0.001$, Fig. 6). Xigris, the only agent clinically used in sepsis therapy [23], increased survival rate from 18.2% to 50% ($p = 0.004$, Fig. 6). However, the survival rate of heparin treatment group is 30%, without statistical significance compared with control group ($p = 0.385$, Fig. 6). All of animals in the sham group survived (data not shown).

3.11. Determination of LD50 of rF II

We examined the safety profile of rF II in further detail. The LD_{50} of rF II was measured with Kun-ming mice. The LD_{50} value of rF II was determined as 53.5 mg/kg, which was much higher than the doses that we used in the present study, suggesting a relatively big safety window for use. However, even though there is no any bleeding in the animals when the dose of rF II up to 32.8 mg/kg, we do find haemorrhage in some animals when the dose reaching or beyond 53.5 mg/kg.

4. Discussion

In this study, we report for the first time that rF II cloned and prepared from *A. acutus* venom fraction had significantly protective effect against the lethal effects of sepsis in rabbits. Reduction of LPS induced biochemical damage was observed. As a snake venom fibrinolytic enzyme, rF II had directly proteolytic effect on fibrin clots. In addition, rF II may involve in inhibition of the inflammatory responses by direct degradation of TNF-α induced by LPS. Both activities of rF II may be necessary to protect against sepsis induced lethality. rF II had also been demonstrated to be able to significantly rescue rats of caecal ligation and puncture.

The hallmark of the coagulation disorder in sepsis constitutes the imbalance between intravascular fibrin formation and its removal. Severely reduced anticoagulant capacity and inhibited fibrinolysis are opposed to a massive activation of coagulation, finally leading to overwhelming fibrin formation and consumption of clotting factors and inhibitors. Abundant intravascular fibrin formation leads to microvascular thrombosis, which contributes to the development of multiple organ failure that is often life threatening or fatal [24]. Recent results suggested that promoting fibrinolysis might be a promising target for therapy strategies during sepsis [25]. In this study, we observed that fibrin clots were hydrolyzed by rF II more quickly and efficiently than by t-PA *in vitro*. Most importantly, unlike urokinase and t-PA, rF II lysed

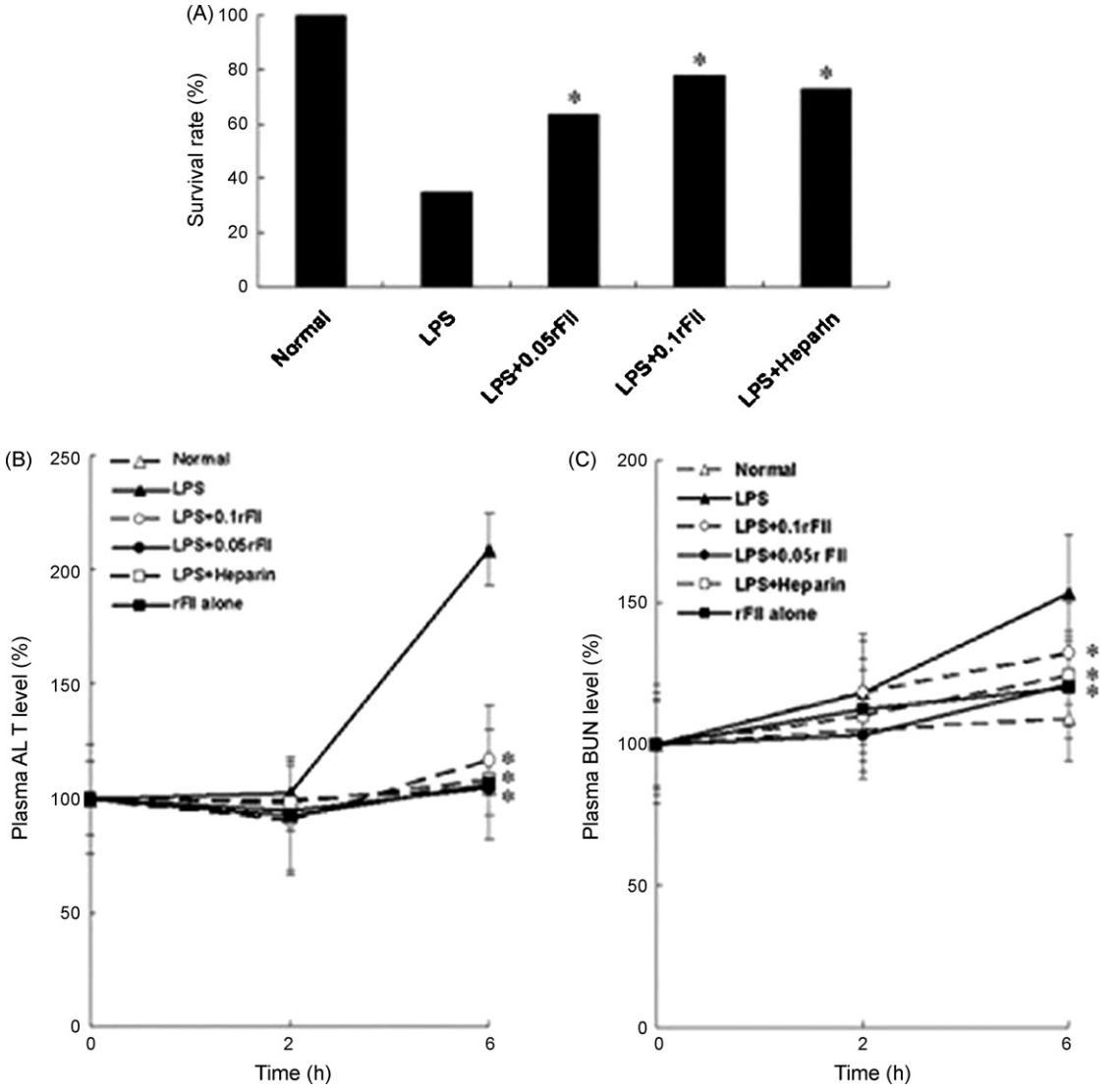

Fig. 5 – Effects of rF II on survival rates and the plasma levels of ALT and BUN in LPS-induced sepsis rabbits. (A) Survival rates were calculated at 24 h after the start of the experiment. *P < 0.05 as compared to the LPS group. (B) Effect of rF II on the plasma levels of ALT. (C) Effect of rF II on the plasma levels of BUN. Results of ALT and BUN at 2 h and 6 h were converted to percentages assuming a value of 100% for basal data. Values are expressed as the mean ± S.D. percent of the initial value before LPS infusion. *P < 0.05, **P < 0.01 as compared to LPS group.

fibrin by direct proteolysis without activating intrinsic plasminogen. Consistently, rF II effectively decreased the thrombi weight in a rabbit pulmonary artery thrombotic model. Since rF II does not activate plasmin, many complex secondary effects such as platelet activation would be avoided. The advantages of the plasmin-independent fibrinolysis of rF II may further demonstrate by the evidence that no bleeding in any of the rF II treatment rabbits in our study, and a much bigger safety window compared with that of the current standard anticoagulation agent heparin or thrombolytic agent t-PA. However, one important fact we need to emphasize here is that rF II at its extreme high dose may result in internal haemorrhage in some animals.

As the onset of multiorgan dysfunction syndrome has been shown to forecast mortality in sepsis, protection of organs particularly liver and kidney function is important in treatment of sepsis. It has been demonstrated that the presence of the intravascular thrombi appears to be related to the clinical dysfunction of the organs [26]. As the fibrinolytic drug, rF II could prevent the fibrin deposits induced by LPS to ameliorate the reduced blood supply to organs. We observed plasma levels of ALT and BUN, which were increased by LPS, were significantly lower by rF II. Moreover, directly proteolytic effect on TNF-α of rF II was also benefit as TNF-α is the critical mediator of LPS-induced organ failure [27].

It is known that, during sepsis, inflammation plays an important role. The level of TNF-α increases significantly during the early period of sepsis and initiates production of the cascade of inflammatory mediators including other cytokines and adhesion molecules [28]. Consequently, abnormalities of hepatic and renal function are seen. These changes ultimately lead to death. Recently studies reported

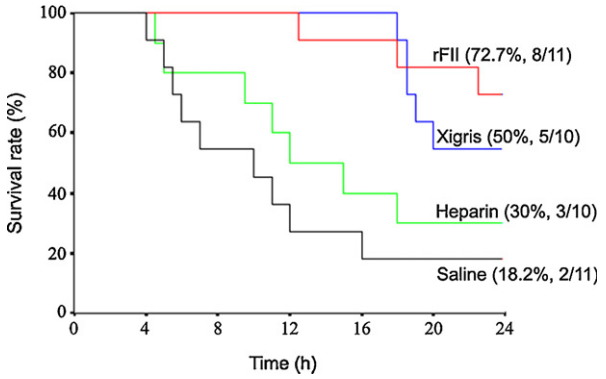

Fig. 6 – The protective effect of rF II on sepsis in rat caecal ligation and puncture. Sepsis was induced in rats after CLP. 1 mg/kg rF II, 50 IU/kg heparin and 2 mg/kg xigris was administered for 6 h by intravenous injection 1 h after CLP procedure. Survival was monitored over 24 h.

that anti-TNF antibody had reduced mortality and attenuated the severity of organ dysfunction in sepsis patients [29,30]. Those reports suggest that reduction of plasma levels of TNF-α may have benefit effect on sepsis. In the present study, we found that rF II inhibited the inflammatory responses by reduction of the increased plasma levels of TNF-α. It has been reported that the PIII class of snake venom metalloproteinases (SVMPs) and A Disintegrin And Metalloproteases (ADAMs) share significant structural similarities [31]. TNF-α converting enzyme (TACE or ADAM 17) is a membrane-bound enzyme responsible for the processing of TNF-α. rF II is a snake venom metalloproteinase. It is possible that rF II could degrade TNF-α by its proteolytic activity. In accordance with this hypothesis, we observed the effects of rF II on TNF-α *in vitro* and *in vivo*. We found that rF II significantly reduced the increased plasma levels of TNF-α in rabbits and directly degraded TNF-α protein in molecular, cellular models. It has been reported that many natural metalloproteinases contribute to inflammatory response and cytokines produce [32,33]. Other snake venoms could degrade cytokines by its proteolytic activity or reduce the inflammatory response [34,35]. Our findings are consistent with recent report that a metalloproteinase from *Bothrops jararaca* venom could degrade TNF-α by its proteolytic activity [35].

It has been well demonstrated that the haemorrhagic toxin elements existed in the snake venoms may digest directly on the capillary basement membrane and serum proteins, because of their nonspecific proteolytic activities, by which a series of disadvantages, including internal haemorrhage and inflammation, were induced. Our and other previous studies have also demonstrated that there are hundreds of proteinases in the snake venom and it is quite difficult to get a real single pure proteinase by biochemical methods because the structural differences between some different proteinases in the snake venom are very little. However, these very little structural changes may result in different biological activities, in some cases, from thrombolytic to haemorrhagin [36,37]. This may be one of the main reasons for limitation of snake proteinases in clinic use. Therefore, a reasonable strategy to solve this problem is to clone a single specific gene and make a recombinant proteinase. It may possess higher specificity for its substrate and higher activity for its catalysis. As a result, it could increase the safety for future clinical use. Our results from reaction of rF II with different kinds of serum proteins and IL-1 as well as macrophage surface protein CD68 suggested that rF II possess relatively high specificity compared with its proteolytic activity to fibrin and TNF-α. It means rF II may have potential advantages in its safety for use. This point of view was further supported by the determination of LD_{50} of rF II. The LD_{50} value of rF II was as higher as 53.5 mg/kg, compared with its concentration used *in vivo* as 1 mg/kg. It provides convincing evidence that the safety of rF II is well. However, as mentioned before, at high concentration of 53.5 mg/kg, rF II does induce bleeding, suggesting that bleeding remains a potential disadvantage for its overdose use in future clinical trials.

The rat "caecal ligation and puncture" model, which is considered by researchers in the field as a standard for sepsis research, because of its similarity with human diseases of ruptured appendicitis or perforated diverticulitis, as well as with the hemodynamic and metabolic phases of human sepsis [18,22]. In this rescue experiment, we found that rF II has significant therapeutic effects on sepsis as well as xigris, the only approved drug that reduces mortality of adult severe sepsis patients [23]. However, heparin, administered after onset of sepsis, just showed a minor therapeutic effect in this model even though it significantly prevents the animal death in the LPS-induced sepsis model when simultaneously used.

In conclusion, rF II may have protective effect on sepsis through direct degradation of fibrin clots and TNF-α. These therapeutic effects support the hypothesis that fibrinogenase may be used for reducing microthrombi and anti-inflammation of sepsis. However, we emphasize that the true utility of rF II in combating sepsis will require further direct testing in clinical trials.

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The authors declare that they have no competing financial interests.

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