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Fibrin(ogen)olytic character of FII$_a$ isolated from *Agkistrodon acutus* venom[1]

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Abstract

Aim: To investigate the fibrin(ogen)olytic character of FII$_a$ isolated from *Agkistrodon acutus* venom *in vitro* and *in vivo*. **Methods:** ^{125}I-labeled human plasma clot lysis was measured *in vitro* and rabbit carotid artery thrombosis was as an *in vivo* model. **Results:** *In vitro*, urokinase (UK) at 25, 35, 40, 45, 60 kU/L and FII$_a$ at 0.08, 0.23, 0.4, 0.5, and 0.7 g/L resulted an equivalent clot lysis (20%, 40%, 50%, 60%, and 80%). UK at 25−60 kU/L induced 27.3%±3.6%, 35.2%±2.3%, 39.3%±2.4%, 44.2%±4.6%, and 51.1%±1.2% fibrinogen degradation. But FII$_a$ at 0.08−0.7 g/L induced 95.4%±0.3%, >95.6%, >95.6%, >95.6%, >95.6% fibrinogen degradation respectively. *In vivo*, UK 40 kU/kg and FII$_a$ 1.0 mg/kg reduced the weight of residual thrombus to 9.0±2.5 mg and 7.8±3.5 mg compared with negative control group (30.0±5.4 mg). But the fibrinogen degradation rate after UK 40 kU/kg and FII$_a$ 1.0 mg/kg treatment was 24.4%±6.2% and 4.1%±7.8%, respectively ($P<$ 0.05, n=6). The order of the lysis speed after UK 125 kU/L treatment was platelet poor plasma (PPP) clots>the whole blood clots>platelet rich plasma (PRP) clots. The sequence for FII$_a$ 0.4 g/L was PRP>PPP>whole blood clots. **Conclusion:** At the same percentage of clot lysis, FII$_a$ degraded more fibrinogen than UK did *in vitro* but less fibrinogen than UK did *in vivo*. The order of the lysis speed was PPP>whole blood clots>PRP clots for UK and PRP>PPP>whole blood clots for FII$_a$.

Introduction

Fibrin-specific clot lysis was initially the major goal to offer the opportunity for effective lysis of pathological thrombus without the risks of a systemic haemorrhagic diathesis[1]. But clinical studies show that even tissue plasminogen activator (t-PA), which exhibits strict selectivity toward fibrin-bound plasminogen, is associated with a significant although variable degree of fibrinogeno-lysis[2–4].

In our previous studies, fibrinolytic enzyme FII$_a$ from *Agkistrodon acutus* venom was shown to dissolve both fibrin and fibrinogen *in vitro*. *In vivo*, FII$_a$ was able to dissolve thrombus without hemorrhage at an effective dose for thrombolysis[5–7]. A few experiments were performed to investigate the fibrin(ogen)olytic character of FII$_a$ such as the specificity to fibrin and fibrinogen and the specificity to different types of clots.

In this study, we compared fibrin(ogen)olytic character of FII$_a$ with urokinase (UK) *in vitro* and *in vivo*.

Materials and methods

Snake venoms *Agkistrodon acutus* venom was collected in Yuanling, Hu-nan Province and lyophilized and stored in desiccator.

Reagents DEAE-Sephadex A-50 and Sephadex G-75 were from Pharmacia (Uppsala, Sweden); human fibrinogen (95% clottable) and bovine fibrinogen (70% clottable) was from Sigma (Saint Louis, USA); aprotinin was from Dadeli Biochemistry and Pharmaceutical Co Ltd (Lanzhou, China); thrombin was from Zhuhai Biochemical Pharmaceutical Factory (Zhuhai, China); UK was from Tianpu Biochemistry and Pharmaceutical Co Ltd (Guangzhou, China); ^{125}I-labeled fibrinogen was from the Department of Experimental Nuclear Medicine of our college by the method of chloramines-T; fibrinogen concentration determination reagent pack (Clauss

method) was from Sun Biotechnology Company (Shanghai, China); human plasma was from Guangzhou Blood Center was centrifugated at $3800 \times g$ for 8 min; whole blood was collected from healthy volunteers ($n=5$ male, age 24.0 ± 4.2 a, weighing 61 ± 2.4 kg). All materials were of analytical grade from commercial sources.

Animals Male New Zealand white rabbits (3–4 months old, weighing 2.2 ± 0.1 kg, Grade II, certificate No 2001A033) were provided by the Experimental Animal Center of Zhongshan Medical College.

Purification of the enzyme FII_a, the fibrinolytic enzyme from *Agkistrodon acutus* venom was prepared according to the method described by Chen *et al*[7].

Lysis of ^{125}I-labeled human plasma clots *In vitro*, ^{125}I-labeled clots were prepared by the modified method of Gurewich *et al*[8]. Human plasma 5.5 mL were mixed with ^{125}I-labeled fibrinogen (6.105×10^4 Bq) plus 55 µL $CaCl_2$ (1 mol/L). This solution (0.5 mL) was added to 5-mm (ID) glass tube in the presence of 5 µL thrombin (100 kU/L). The clots were incubated at 37 ℃ for 30 min and kept overnight at room temperature. All the clots were washed three times with 0.9% saline and the total radioactivity was measured before being transferred to 10-mm (ID) test tubes.

Plasma (4 mL) were incubated (37 ℃, 6 h) with radiolabeled clots in the presence of UK 20, 30, 40, 60, and 80 kU/L or FII_a 0.05, 0.1, 0.2, 0.4, and 0.8 g/L, respectively. The reaction was terminated by adding aprotinin (1×10^6 kU/L final concentration) into test tubes containing different concentrations of UK or edetic acid (5 µmol/L final concentration) into test tubes containing different concentrations of FII_a. Aliquots (1 mL) were removed for the measurement of radioactivity.

Clot lysis was calculated according to the formula: Clot lysis=Radioactivity in aliquot (modified volume)/total radioactivity×100%. The concentrations of UK or FII_a which could induce 20%, 40%, 50%, 60%, and 80% clot lysis were determined based on the concentration-response curve.

The same experiments were repeated with UK and FII_a at the concentrations that could induce 20%, 40%, 50%, 60%, and 80% clot lysis. Aliquots were removed for the measurement of fibrinogen concentration to get the fibrinogen degradation at the same percent of clot lysis.

Each experiment was performed three times.

Fibrinogen degradation Fibrinogen degradation was performed according to the reagent pack instruction by the Clauss method.

Carotid artery thrombosis The rabbit carotid artery thrombosis model was established according to the method of Wang *et al*[9]. Forty-eight rabbits were randomly divided into 8 groups ($n=6$): 0.9% saline 1 mL/kg, as the negative control group; UK 5, 10, 20, and 40 kU/kg group; FII_a 0.5, 1.0, and 2.0 mg/kg group.

Two hours after the initiation of the carotid thrombosis, UK and FII_a were administered via ear-edge vein of rabbit. One hour later, the residual thrombus within the carotid artery was excised and blotted. The wet weight was measured. Blood was drawn from the right femoral artery cannula before and 1 h after administration for the measurement of plasma fibrinogen concentration. The blood sample was drawn into a plastic syringe containing 3.8% sodium citrate as the anticoagulant (1: 9 *v/v*, citrate/blood) and was centrifuged at $1500 \times g$ 4 ℃ for 15 min. The platelet-free plasma was stored at -70 ℃ until assayed.

Lysis of platelet-rich plasma (PRP) clots, platelet-poor plasma (PPP) clots, and whole-blood clots Whole blood from healthy volunteers was mixed with 3.8% sodium citrate (1:9 *v/v*, citrate/blood) and immediately centrifuged at room temperature at $250 \times g$ for 10 min to obtain PRP. PPP was obtained by centrifuging the original blood sample at $1500 \times g$ for 10 min[10]. ^{125}I-labeled clots made from PRP, PPP, and whole blood were prepared as described above.

Plasma (4 mL) were incubated with these three types of radiolabeled clots in the presence of FII_a 0.4 g/L or UK 125 kU/L (final concentration) at 37 ℃. Aliquots were removed for the measurement of radioactivity in plasma at subsequent times during a course of 12 h. The time-response relationship of FII_a and UK for these three types of clots were compared. Each experiment was perfomed three times[11].

Data analysis Values were expressed as mean±SD. Analysis of variance and *t*-test was used for comparisons of the mean values. $P<0.05$ was considered significant.

Results

Fibrin(ogen)olytic character of FII_a *in vitro* *In vitro* both UK and FII_a dose-dependently dissolved clots. Based on the dose-effect relationship of UK and FII_a, UK at 25, 35, 40, 45, 60 kU/L and FII_a at 0.08, 0.23, 0.4, 0.5, and 0.7 g/L caused an equivalent clot lysis (20%, 40%, 50%, 60%, and 80%, Figure 1, 2).

At the calculated concentration, which could induce the same clot lysis, the fibrinogen degradation induced by FII_a was much higher than that induced by UK. In the negative control group, the fibrinogen degradation was 0.01%±0.02% (0.02±0.05 g/L).

Fibrin(ogen)olytic character of FII_a in rabbit carotid artery thrombosis UK or FII_a reduced the weight of residual thrombus and the level of fibrinogen in plasma in a
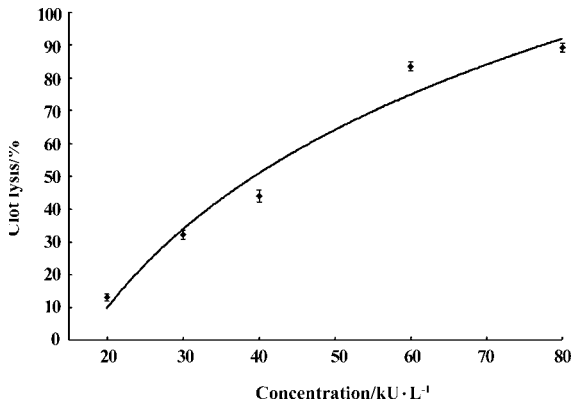
Figure 1. UK dose-dependently dissolved ^{125}I-labeled human plasma clots. In the negative control group, the clot lysis was 11.0%±1.0%.
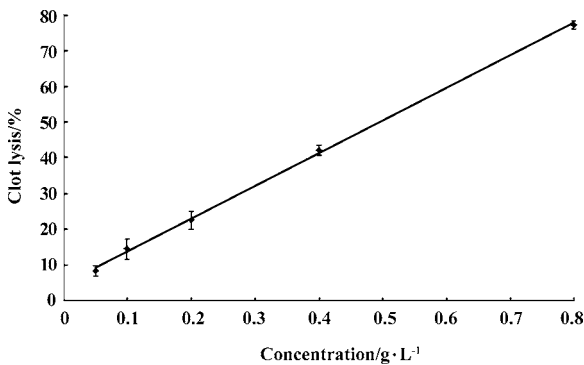
Figure 2. FII$_a$ dose-dependently dissolved ^{125}I-labeled human plasma clots. In the negative control group, the clot lysis was 11.0%±1.0%.

dose-dependent manner. In the group treated with UK 40 kU/kg, the weight of residual thrombus was 9.0±2.5 mg. In the group treated with FII$_a$ 1.0 mg/kg, the weight of residual thrombus was 7.8±3.5 mg. The fibrinolytic effects of these two groups were approximately the same, but the fibrinogen degradation of these two groups were 24.4%±6.2% and 4.1%±7.8%, respectively (*P*<0.05, *n*=6). In the negative control group, the weight of residual thrombus was 30.0±5.4 mg, and the fibrinogen degradation was 2.7%±2.7%. The difference between UK and control or FIIa and control was statistically significant (*P*<0.01, *n*=6, Table 3, 4).

Fibrin(ogen)olytic character of FII$_a$ in lysis of platelet-rich clots, platelet-poor clots and whole-blood clots The kinetics of FII$_a$-induced lysis were characterized by an initial lag phase followed by an accelerated lysis, whereas lysis by UK lacked the lag phase. The order of the lysis speed after UK 125 kU/L treatment PPP clots>the whole blood clots> PRP clots. The sequence for FII$_a$ 0.4 g/L was PRP clots>PPP clots>whole blood clots. The clot lysis of these three types

Table 1. Fibrin(ogen)olytic effects of UK on ^{125}I-labeled human plasma clots *in vitro*. *n*=3. Mean±SD. [c]*P*<0.01 *vs* control.

Concentration/ kU·L^{-1}	Fibrinogen degradation/%	Fibrinogen degradation/g·L^{-1}
25	27.3±3.6[c]	0.88±0.12[c]
35	35.2±2.3[c]	1.10±0.07[c]
40	39.3±2.4[c]	1.26±0.08[c]
45	44.2±4.6[c]	1.42±0.15[c]
60	51.1±1.2[c]	1.64±0.04[c]

Table 2. Fibrin(ogen)olytic effects of FII$_a$ on ^{125}I-labeled human plasma clots *in vitro*. *n*=3. Mean±SD. [c]*P*<0.01 *vs* control.

| Concentration/ g·L^{-1}	Fibrinogen degradation/%	Fibrinogen degradation/g·L^{-1}
0.08	95.44±0.28[c]	3.07±0.01[c]
0.23	>95.62	>3.07
0.4	>95.62	>3.07
0.5	>95.62	>3.07
0.7	>95.62	>3.07

Table 3. The thrombolysis and fibrinogen degradation by FII$_a$ in rabbit carotid thrombosis model. *n*=6. Mean±SD. [c]*P*<0.01 *vs* control.

Dosage/ mg·kg^{-1}	Residual thrombus/mg	Fibrinogen degradation/%
0.5	12.5±3.9[c]	1.2±9.9[c]
1.0	7.8±3.5[c]	4.1±7.8[c]
2.0	3.4±2.2[c]	6.8±5.2[c]

Table 4. The thrombolysis and fibrinogen degradation by UK in rabbit carotid thrombosis model. *n*=6. Mean±SD. [c]*P*<0.01 *vs* control.

Dosage/ kU·kg^{-1}	Residual thrombus/mg	Fibrinogen degradation/%
5	21.1±3.8[c]	10.3±9.9[c]
10	17.2±2.5[c]	15.2±6.3[c]
20	12.2±2.8[c]	19.9±7.0[c]
40	9.0±2.5[c]	24.4±6.2[c]

of clots with saline was below 10% during the course of 12 h (Figure 3 and 4).
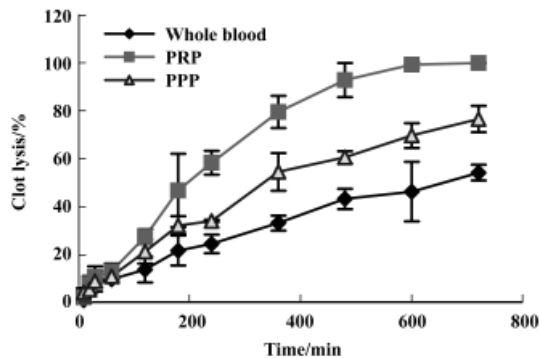
Figure 3. The clot lysis of human whole blood, platelet rich plasma (PRP), and platelet poor plasma (PPP) by FII_a 0.4 g/L. $n=6$. Mean±SD.
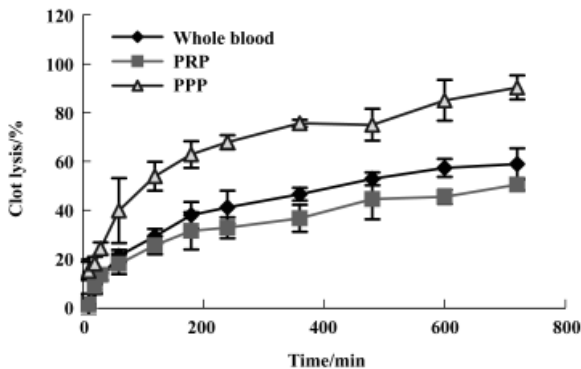
Figure 4. The clot lysis of human whole blood, platelet rich plasma (PRP), and platelet poor plasma (PPP) by UK 125 kU/L. $n=6$. Mean±SD.

Discussion

In this paper, the fibrin(ogen)olytic character of FII_a was studied and compared with UK. The *in vitro* results showed that both FII_a and UK degraded clots and fibrinogen in a dose-dependent manner. FII_a degraded 95.4%±0.3% fibrinogen when the clot lysis was 20% and UK degraded 51.1%± 1.2% fibrinogen when the clot lysis was 80%. *In vitro*, FII_a degraded more fibrinogen than UK did at the same percentage of clot lysis.

The *in vivo* results showed that both FII_a and UK could dose-dependently dissolve clots and fibrinogen. The fibrinolytic effects of UK 40 kU/kg or FII_a 1.0 mg/kg were approximately the same, but the fibrinogen degradation effects were 24.4%±6.2% and 4.1%±7.8%, respectively ($P<0.05, n=6$). *In vivo*, FII_a degraded less fibrinogen than UK did at the same percentage of clot lysis. The *in vivo* and *in vitro* results of the experiments are in accordance with the data of our previous studies[5,6,12].

UK and FII_a have different sensitivity and kinetics to different types of clots. UK dissolved most effectively the PPP clots, then the whole blood clots and PRP clots. The sequence for FII_a was PRP>PPP>whole blood clots.

Attention should be paid as to why the conclusion *in vitro* did not accord with the conclusion *in vivo*. It could be explained that the dosage of FII_a was much higher *in vitro* than that used *in vivo*, while the dosage of UK was opposite. The dosage was at least one of the causes of the inconsistency.

The main component of thrombus in rabbit carotid artery thrombosis model was platelet[9] and the main component in *in vitro* clots was fibrin. FII_a dissolves most effectively the platelet rich clots compared with UK that dissolves most effectively fibrin rich clots (Figure 3 and 4), which could explain why FII_a dissolved clot at less dosage *in vivo* than *in vitro* but the clot lysis effect of UK was just the opposite.

Our previous study showed that FII_a influenced blood coagulation by inhibiting platelet aggregation induced by ADP in rat PRP and degrading factor X and prothrombin[12]. It is also one of the reasons in the dosage inconsistency.

There are two opinions about the mechanism of the fibrinolytic enzyme from snake venom inhibiting platelet aggregation. (1) Fibrinolytic enzymes inhibit platelet aggregation by hydrolyzing α-fibrinogen to prevent fibrinogen from combining with fibrinogen receptor (GPIIb-IIIa), such as α-fibrinolytic enzyme from *Agkistrodon contortrix contortrix*[13], *A rhodostoma*[14], and *T mucrosquamatus*[15]. (2) The disintegrin-like domain may target the fibrinolytic enzyme to a particular site such as platelets where the metalloproteinase domain may cleave relevant substrates including integrins, coagulant proteins, matrix, or other latent proteins[16]: such as collagen receptor antagonist *Jararhagin* from *Bothrops jararaca*[17], *Mutalysin* I from *Lachesis muta muta*[16], *Crovidisin* from *C viridis*[18]; such as GPIIb-IIIa antagonist *Barbourin*[19] with KGD sequence. Our previous study showed FII_a degraded α and β chains of fibrinogen[5]. Is there any relationshiop between the inhibitory activity of FII_a on platelet aggregation and the proteolytic activity of FII_a on fibrinogen or membrane protein? This topic will be further explored in the near future.

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