Hemorrhagic activity and mechanism of FII_a, a fibrinolytic enzyme from *Agkistrodon acutus* venom[1]

Qing-qing WANG[2], Jia-shu CHEN, Xiu-xia LIANG[3], Peng-xin QIU, Yi-wen WANG, Guang-mei YAN

Department of Pharmacology, Zhongshan Medical College, Sun Yat-Sen University, Guangzhou 510080, China

KEY WORDS *Agkistrodon acutus* venom; fibrinolytic enzyme; hemorrhagic protease; endothelial cells

ABSTRACT

AIM: To study the local hemorrhagic activity of a fibrinolytic enzyme (FII_a) from *Agkistrodon acutus* venom and its mechanism. **METHODS:** The local hemorrhagic activity was determined by subcutaneous injection on the back of mouse. The effects of FIIa on factor X, prothrombin, gelatin, and collagen were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Platelet aggregation assays were performed in rat platelet-rich plasma (PRP). Human umbilical vein endothelial cells (HUVEC) were cultured and passaged in complete M199 medium. Cell viability and nuclear morphology change were determined by fluorescein diacetate (FDA) staining and Hoechst 33258 staining, respectively. **RESULTS:** The minimum hemorrhagic dose (MHD) of FIIa was 89 μg. *In vitro*, FII_a (0.25 g/L) degraded factor X, prothrombin, collagen, and gelatin, and dose-dependently (0.25, 0.50, 0.75, and 1.00 g/L) inhibited the platelet aggregation induced by ADP in rat PRP. When HUVEC in culture treated with FII_a, HUVEC showed detachment in a dose-dependent manner, but no apoptosis sign was observed. **CONCLUSION:** FII_a had local hemorrhagic activity, and the mechanism was related to the degradation of factor X, prothrombin, gelatin, and collagen, the inhibition of ADP-induced platelet aggregation, and inducement of HUVEC detachment.

INTRODUCTION

Fibrinolytic enzymes from various snake venoms can degrade fibrin and fibrinogen directly. The fibrinolytic enzyme was firstly isolated from Taiwanese *Agkistrodon acutus* venom[1]. Some fibrinolytic enzymes do not have hemorrhagic activity such as atroxase[2], fibrolase[3], and brevilysin L6[4]. These enzymes without hemorrhagic activity will be prospective in clinical uses, and are widely investigated.

Some fibrinolytic enzymes have hemorrhagic activity such as the fibrinolytic enzyme from Taiwanese *Agkistrodon acutus* venom[1], jararafibrase III/IV from *Bothrops jararaca*[5], ProfibH from *Philodryas olfersii*[6]. The reason for hemorrhage of fibrinolytic enzymes is either they are mixed with hemorrhagic fraction or they have intrinsic hemorrhagic activity. Manning[7] thought that the main mechanism of hemorrhagin was the direct degradation of extracellular matrix or by inducing matrix metalloproteinases (MMP). Inhibition of platelet aggregation[8] and apoptosis of vascular endothelial cell[9] were also responsible for the hemorrhage.

In our previous work, a fibrinolytic enzyme called FII_a has been purified from Anhui *Agkistrodon acutus*

[1] Project supported by the Committee of Science and Technology of Guangdong Province, No 001365.
[2] Now in *Beijing University of Chinese Medicine, Beijing 100029, China.*
[3] Correspondence to Dr Xiu-xia LIANG.
Phn 86-20-8733-0553. Fax 86-20-8733-1577.
E-mail Lxx-yl@163.net
Received 2003-01-06 Accepted 2003-12-24

venom. It consists of a single polypeptide chain with a molecular weight of 26 000. FII_a can degrade fibrin and fibrinogen *in vitro*, and solubilize thrombus *in vivo*[10,11]. However, the local hemorrhagic activity of FII_a has not been elucidated. In this study, we mainly determined the hemorrhagic size of FII_a and investigated the probable mechanism in an attempt to get a better understanding of the local hemorrhage of snake venom and the clinical prospects of FII_a.

MATERIALS AND METHODS

Drugs and reagents *Agkistrodon acutus* venom lyophilized was purchased from Qimen Snake Farm (Anhui, China). DEAE-Sephadex A-50 and Sephadex G-75 were purchased from Pharmacia (Uppsala, Sweden). Bovine fibrinogen, plasmin, factor X, prothrombin, collagen, gelatin, endothelial cell growth factor (ECGF), and heparin were purchased from Sigma (St Louis, USA). Molecular weight protein standards were obtained from NEB (Beveily, USA). Thrombin was obtained from the Biochemical Pharmaceutical Factory (Zhuhai, China). Media 199 and fetal bovine serum (FBS) were obtained from Gibco (NewYork, USA). All other chemicals and solvents used were of analytical grade from commercial sources (Guangzhou, China).

Animals Male and female Kunming strain mice (weighing 20±2 g, Grade II, Certificate No 00A002) and male Sprague-Dawley (SD) rats (weighing 300±12 g, Grade II, Certificate No 00A005) were supplied by the Experimental Animal Center of Zhongshan Medical College, Sun Yat-Sen University.

Purification of FII_a Isolation procedures were performed by the method previously described[10]. The procedures comprised of three steps including ion-exchange on DEAE-Sephadex A-50 and gel filtration on Sephadex G-75 twice. The final product was homogenous as established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Local hemorrhagic activity of FII_a The method of Kondo[12] was used to determine the local hemorrhagic activity. Test solution (0.1 mL) was injected subcutaneously using the clean shaven back of Kunming mouse. After 6 h the animal was sacrificed and the skin was removed. The diameter of hemorrhagic spot was measured on the underside of the skin. The amount of FII_a that produced a hemorrhagic spot of 5 mm in diameter was defined as the minimum hemorrhagic dose (MHD).

Degradation of factor X, prothrombin, gelatin and collagen FII_a (0.25 g/L, 25 μL) was incubated with 75 μL of blood coagulation factor X (10 kU/L), prothrombin (10 kU/L), gelatin (0.2 %), and collagen (0.1 %) at 37 ºC, respectively. At various time intervals, 0.1 mL of a denaturing solution (urea 10 mol/L, 4 % SDS and 4 % β-mercaptoethanol) was added and the mixture was incubated at 100 ºC for 4 min. Each sample (20 μL) was analyzed by SDS-PAGE[13].

Platelet preparation Platelets were prepared from whole blood collected from a male SD rat, using 3.8 % trisodium citrate (3.8 % trisodium citrate/blood, 1:9, v/v). Platelet-rich plasma (PRP) was obtained after blood was centrifugated at 200×*g* at room temperature for 10 min. After the removal of PRP, the remnant blood was centrifuged at 2000×*g* for the additional 20 min, and the platelet-poor plasma (PPP) was collected. PPP was mixed with PRP to give a platelet count about 3×10^{11}/L. Platelet aggregation was recorded with a Chrono-Log aggregometer (Model 500) at 37 ºC using silicoized glass tubes. Changes in light transmission of the platelet suspensions were recorded under continuous stirring at 900 r/min.

Platelet aggregation After 250 μL of PRP was stirred with variable concentration of FII_a (0.25, 0.5, 0.75, and 1 g/L) in a curvette at 37 ºC, the aggregating agent was added (final concentration: ADP 5 μmol/L). C_{max} and N_{max} were recorded (C_{max} and N_{max} represent the maximal aggregation rates of control and FII_a group, respectively)

Cell cultures Human umbilical vein endothelial cells (HUVEC) were obtained as described by Jaffe *et al*[14]. Cells were cultured on plastic dishes in M199 culture medium that had been supplemented with 20 % FBS, 75 mg/L ECGF, and 0.1 g/L heparin at 37 ºC in a 5 % CO_2/95 % air atmosphere. Cells were stained immunochemically by treatment with antibodies from rabbit against human factor VIII-related antigen (VIII R: Ag), and ultramicroscopy was used to identify that they were endothelial cells[15].

Assay of viability HUVEC were cultured until cultures had reached confluence. Then the medium was replaced with the basal medium (without ECGF and FBS). Cells in test media were kept in an incubator with different concentration of FII_a (0.25, 0.5, 0.75, and 1 g/L). To determine the number of living cells, cells were stained by fluorescein diacetate (FDA)[16].

Nuclear-fragmentation assay HUVEC were cultured on dishes until cultures had reached confluence.

The medium was replaced with the basal medium (without ECGF and FBS). The cells were incubated with different concentrations of FII_a (0.25, 0.5, 0.75, and 1 g/L). Cells were fixed with 4 % glutaraldehyde at room temperature for 10 min before stained with Hoechst 33258[16]. The nuclear fragmentation was examined by fluorescence microscope.

Statistical analysis Data were expressed as mean±SD. Statistical analysis was performed using *t*-test.

RESULTS

FII_a (0.25, 0.5, 0.75, 1, and 1.25 g/L) 0.1 mL was injected subcutaneously, after 6 h the skin was removed. The diameters of hemorrhagic spots measured on the underside of the skin were 0.8±0.5, 2.0±0.4, 4.4±0.4, 5.5±0.5, and 7.5±0.6 mm, respectively. The MHD of FII_a was 89 μg (Fig 1) .

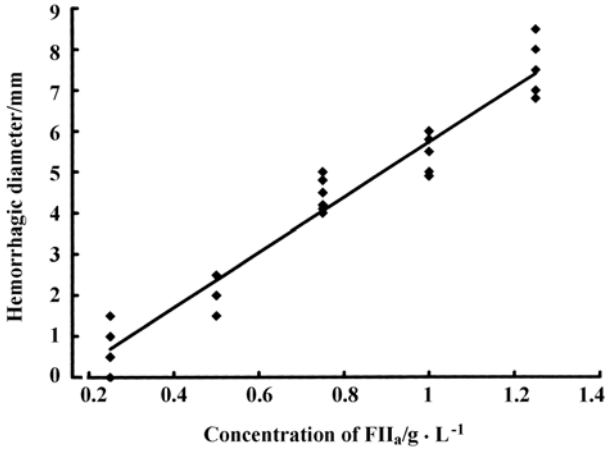

Fig 1. Local hemorrhagic assay of FII_a (n=6).

As shown in Fig 2, FII_a, at the concentration of 0.25 g/L, digested factor X, prothrombin, gelatin and collagen in a time-dependent manner. Even after a 6 h-incubation, factor X, prothrombin, gelatin, and collagen appeared unaffected by plasmin.

In rat PRP, FII_a concentration-dependently inhibited platelet aggregation induced by ADP. At the concentration of 1, 0.75, 0.5, and 0.25 g/L, the maximal platelet

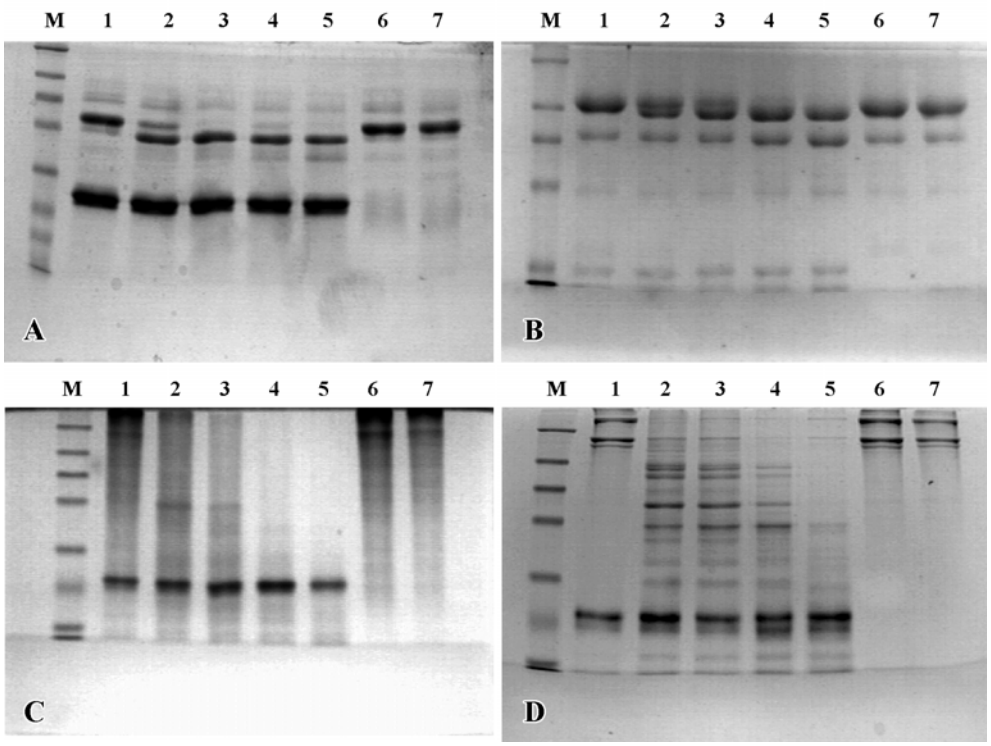

Fig 2. SDS-PAGE analysis of degraded factor X 10 kU/L (A), prothrombin 10 kU/L (B), collagen 0.1 % (C), and gelatin 0.1 % (D) by FII_a (0.25 g/L, 25 μL) in a total volume of 100 μL at 37 ºC. A) Lane 1-5: factor X+FII_a incubated for 0, 5, 15, 30, and 60 min; lane 6: factor X; lane 7: factor X+plasmin incubated for 60 min; B) Lane 1-5: prothrombin+FII_a incubated for 0, 5, 15, 30, and 60 min; lane 6: prothrombin; lane 7: prothrombin+plasmin incubated for 60 min; C) Lane 1-5: collagen+FII_a incubated for 0, 5, 15, 30, and 60 min; lane 6: collagen; lane 7: collagen+plasmin incubated for 60 min; D) Lane 1-5: gelatin+FII_a incubated for 0, 5, 15, 30, and 60 min; lane 6: gelatin; lane 7: gelatin+plasmin incubated for 60 min.

aggregation rate (%) was 18.0±2.3, 24.4±2.6, 34.4±2.7, 50.2±2.9, respectively. The differences were significant when compared with NS group (n=6. ^bP<0.05, ^cP<0.01 *vs* NS group. Tab 1, Fig 3).

Tab 1. Effects of FII_a against ADP- induced platelet aggregation in rat PRP. Mean±SD. n=6. ^bP<0.05, ^cP<0.01 *vs* NS group.

Group	Dose/ g·L^{-1}	Platelet aggregation against ADP/%	Inhibition/ %	IC_{50}/ g·L^{-1}
NS	-	56.6±4.8		
	0.25	50.2±2.9^b	10.3±13.9	0.66
FII_a	0.5	34.4±2.7^c	38.5±10.2	
	0.75	24.4±2.6^c	56.9±3.5	
	1	18.0±2.3^c	67.8±6.7	

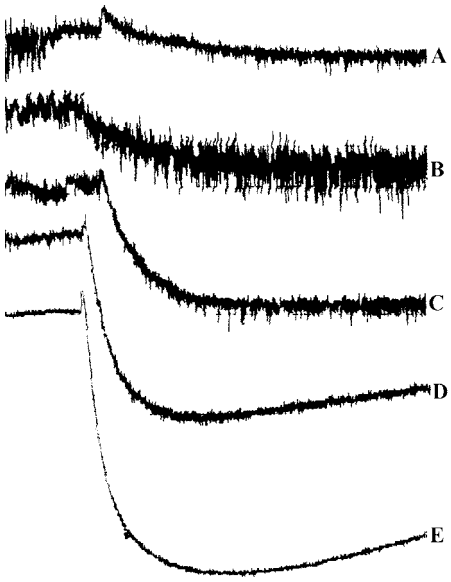

Fig 3. Inhibitory effect of FII_a on platelet aggregation induced by ADP in rat PRP. Platelet aggregation induced by ADP was considered 100 % aggregation. A, B, C, D) FII_a 1, 0.75, 0.5, and 0.25 g/L; E) normal saline.

HUVEC were isolated from human umbilical cord vein by trypsin digestion and grew on dishes in M199 supplemented with 20 % (v/v) FBS, heparin 5 kU/L, ECGF 150 mg/L, penicillin 500 U/L, and streptomycin 0.5 mg/L at 37 ºC in a 5% CO_2/95 % air atmosphere. It was identified by the "cobblestone" on morphology exhibited by confluent monolayer and positive staining for factor VIII-related antigen (VIII R:Ag) and Weibel-Palade body (Fig 4).

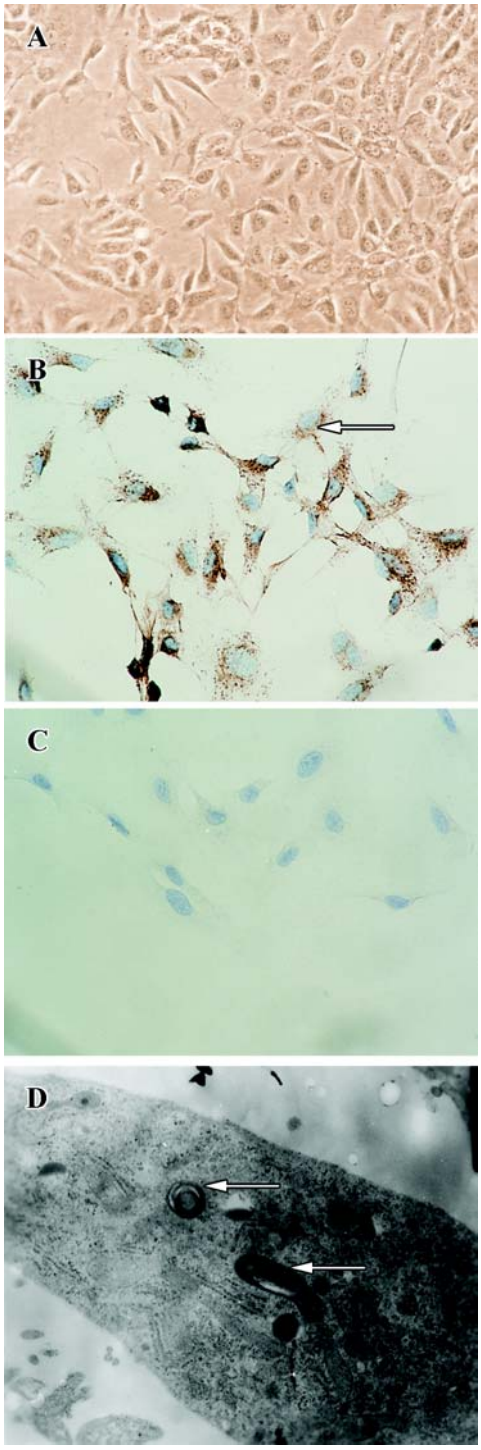

Fig 4. A) The morphology of normal HUVEC (magnification, ×400). B) Photomicrograph of immunocytochemical staining of VIII R: Ag. White arrow indicates brown reaction product which shows at the location of antigen (magnification, ×400). C) Photomicrograph of immunocytochemical staining of VIII R: Ag (negative control). D) Electromicrograph of HUVEC. White arrow indicates weibel-palade body.

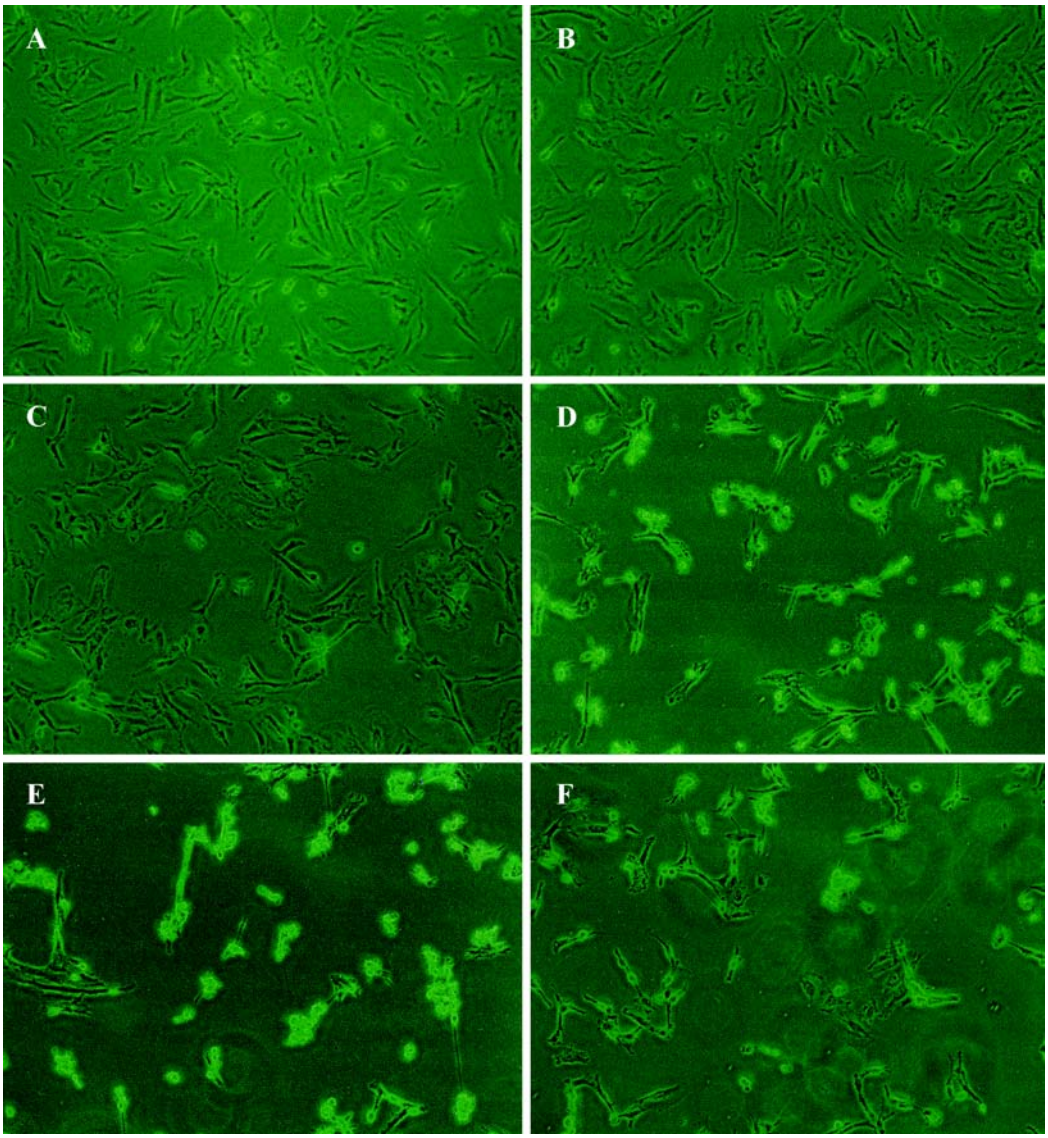

Fig 5. The morphology change of HUVEC treated with FII_a (magnification, ×100). A) normal saline; B) plasmin 0.05 kU/L; C, D, E, F) FII_a 0.25, 0.5, 0.75, and 1 g/L.

After exposure to FII_a for 20 min, HUVEC in culture showed distinct morphologic changes. The cell was detached, round and the distance between two cells became wider. The morphologic changes were in a dose- dependent manner (Fig 5). As shown in Fig 6, HUVEC could be stained by FDA after incubated with FII_a. FII_a-induced nuclear changes were evaluated under a fluorescence microscope using Hoechst 33258 staining. In cells treated with FII_a, the nuclei showed no condensation when compared with those of NS group (Fig 7). The results of FDA and Hoechst 33258 staining showed that HUVEC treated with FII_a were still alive.

DISCUSSION

In this paper, the local hemorrhagic activity of FII_a was studied. The result shows FII_a has local hemorrhagic activity. Its MHD is 89 μg/0.1 mL when injected subcutaneously. Compared with the hemorrhagins, the MHD of DaHT-1, DaHT-2 and DaHT-3 from Hunan *Agkistrodon acutus* snake venom are 0.5, 0.8, and 2.6 μg, respectively. The MHD of the crude venom from Anhui *Agkistrodon acutus* snake was 5 μg[17] . The MHD of Elegatoxin from *Trimeresurus elegans*[18] is 0.8 μg , and Mutalysin I from *Bushmaster*[19] is 0.5 μg. The hemorrhagic activity of FII_a is much lower than those of hemorrhagins.

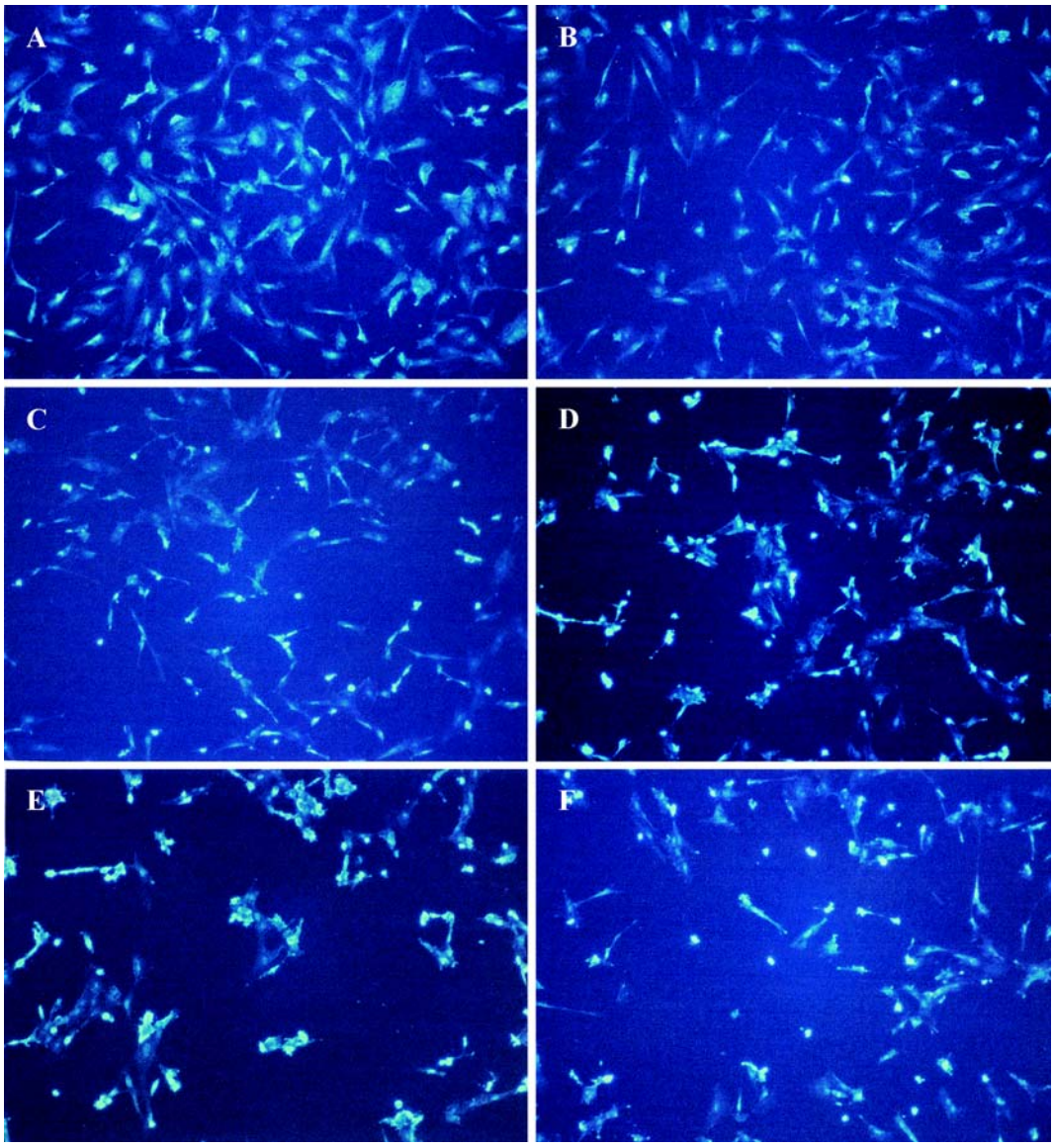

Fig 6. FDA staining of HUVEC treated with FII_a (magnification, ×100). A) normal saline; B) plasmin 0.05 kU/L; C, D, E, F) FII_a 0.25, 0.5, 0.75, and 1 g/L.

The main reasons for hemorrhage were that dysfunction of blood coagulation and platelet, hyperfibrinolysis or secondary hyperfibrinolysis, damage of vascular endothelial cells, and degradation of basement membrane. α-Fibrinolytic enzyme from snake venom may inhibit platelet aggregation by hydrolyzing α-fibrinogen to prevent fibrinogen from combining with fibrinogen receptor (GPIIb-IIIa) on platelet membrane such as α-fibrinolytic enzyme from *Agkistrodon contortrix contortrix*[20], *A rhodostoma*[21] and *T mucrosquamatus*[22]. Some fibrionolytic enzymes may also influence platelet membrane protein GPIb-IX-V complex, GPIa-IIa, GPVI, GPIIb-IIIa, and von Willebrand factor (or collagen). In the present study, some fibrinolytic enzymes with KGD (Lys-Gly-Asp) sequence such as Barbourin[23] has high specificity with platelet membrane GPIIb-IIIa. The data of our previous study show FII_a degrades α and β chains of fibrinogen[10]. Thus, FII_a inhibits the platelet aggregation is probably dependent on this effect.

In vitro, because FII_a degrades factor X and prothrombin, the blood coagulation can be influenced. The degradation of collagen and gelatin may destroy vascular basement membrane. FII_a also induced HUVEC detachment in a dose-dependent manner in short time, but not induced apoptosis of HUVEC. This effect suggests that FII_a may enhance vascular permeability, and the effect is different from *Graminelysin I*[24] which induced apoptosis of HUVEC. It seems possible that all these effects are responsible in part for the local hem-

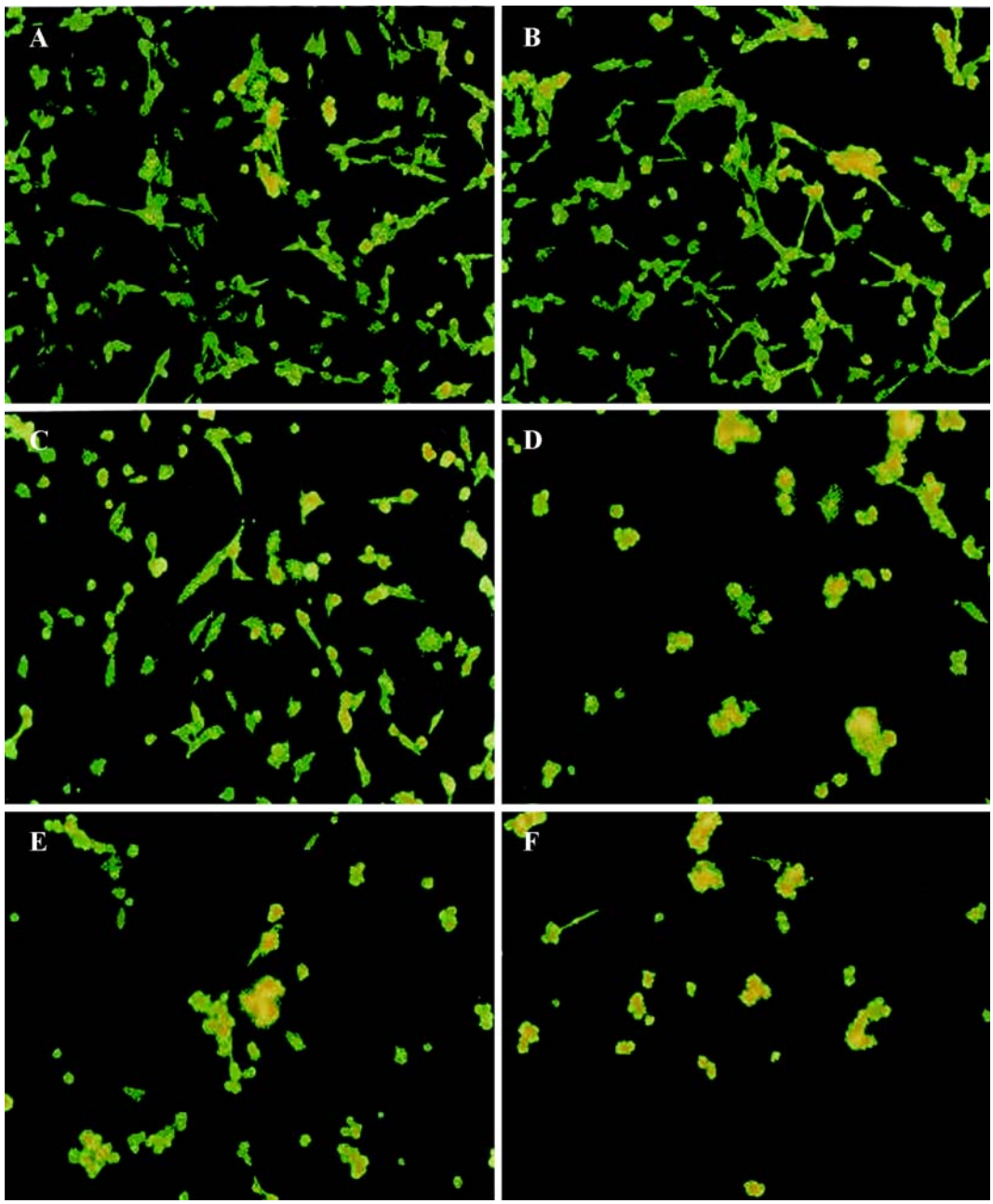

Fig 7. Nuclear fragmentation in HUVEC induced by FII_a (magnification, ×100). A) normal saline; B) plasmin 0.05 kU/L; C, D, E, F) FII_a 0.25, 0.5, 0.75, and 1 g/L.

orrhagic activity of FII_a.

This is the first study reporting the local hemorrhagic activity and its mechanism of FII_a. When the thrombolytic activities of FII_a were determined *in vivo*, hemorrhage was not observed by microscopic examination of tissue sections from kidney, liver, heart, and lung[11]. It seems there is some range between MHD and the effective dose for thrombolysis. In the other hand, the hemorrhagic process may be different between subcutaneous injection and intravenous injection.

REFERENCES

1 Ouyang C, Huang TF. Purification and characterization of the fibrinolytic principle of *Agkistrodon acutus* venom. Biochim Biophys Acta 1976; 439: 146-53.

2 Tu AT, Baker B, Wongvibulsin S, Willis T. Biochemical characterization of atroxase and nucleotide sequence encoding the fibrinolytic enzyme. Toxicon 1996; 34: 1295-300.

3 Pretzer D, Schulteis BS, Smith CD, Vandervelde DG. Fibrolase, a fibrinolytic protein from snake venom. Pharm Biotechnol 1993; 5: 287-314.

4 Terada S, Hori J, Fujimura S, Kimoto E. Purification and amino acid sequence of brevilysin L6, a non-hemorrhagic metalloprotease from *Agkistrodon halys brevicaudus* venom. J Biochem (Tokyo) 1999; 125: 64-9.
5 Maruyama M, Tanigawa M, Sugiki M. Purification and characterization of low molecular weight fibrinolytic/hemorrhagic enzymes from snake (*Bothrops jararaca*) venom. Enzyme protein 1993; 47: 124-35.
6 Assakura MT, Reichl AP, Mandelbaum FR. Isolation and characterization of five fibrin(ogen)olytic enzymes from the venom of *philodryas olfersii* (green snake). Toxicon 1994; 32: 819-31.
7 Manning MC. Sequence analysis of fibrolase, a fibrinolytic metalloproteinase from *Agkistroden contortrix contortrix*. Toxicon 1995; 33: 1189-200.
8 Kamiguti AS, Mourada Silva AM, Laing GD, Knapp T, Zuzel M. Collagen-induced secretion-dependent phase of platelet aggregation is inhibited by the snake venom metalloproteinase jararhagin. Biochim Biophys Acta 1997; 1335: 209-17.
9 Masuda S, Hayashi H, Arakis T. Two vascular apoptosis inducing proteins from snake venom are members of the metalloprotease/disintegrin family. Eur J Biochem 1998; 253: 36-41.
10 Liang XX, Chen JS, Zhou YN, Qiu PX, Yan GM. Purification and biochemical characterization of FIIa, a fibrinolytic enzyme from *Agkistrodon acutus* venom. Toxicon 2001; 39: 1133-9.
11 Chen JS, Liang XX, Qiu PX, Yan GM. Thrombolysis effect with FIIa from *Agkistrodon acutus* venom in different thrombosis model. Acta Pharmacol Sin 2001; 22: 420-2.
12 Kondo H, Kondo SI, Kezawa H. Studies on the quantitative method for determination of hemorrhagic activity of *Habu* snake venom. Jpn J Med Sci Biol 1960; 13: 43.
13 Gasmi A, Chabchoub A, Guermazi S, Karoui H, Elayeb M, Dellagi K. Further characterization and thrombolytic activity in a rat model of fibrinogenase from *Vipera Lebetina* venom. Thromb Res 1997; 86: 233-42.
14 Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antibemophilic factor antigen by cultured human endothelial cells. J Clin Invest 1973; 52: 2757-64.
15 Bouonassisi J, Venter JC. Hormone and neutrotransmitter receptors in an established endothelial cell line. Proc Natl Acad Sci USA 1976; 73: 1612-6.
16 Yan GM, Lin SZ, Irwin RP, Paul SM. Activation of proteins bidirectionally affects apoptosis of cultured cerebellar granule neurons. J Neurochem 1995; 65: 2425-31.
17 Xu X, Wang C, Liu J, Lu Z. Purification and characterization of hemorrhagic components from *Agkistrodon Acutus* Venom. Toxicon 1981; 19: 663-4.
18 Nikai T, Suzuki Y, Komori Y, Sugihara H, Fox JW, Nonogaki T, *et al*. Partial amino acid sequence and biological characterization of elegatoxin, hemorrhagic toxin from *Trimeresurus elegans* (*Sakishimahahu*) venom. J Nat Toxins 1999; 8: 189-202.
19 Estevao-Costa MI, Diniz CR, Magalhaes A, Markland FS. Action of metalloproteinases mutalysin I and II on several components of the hemostatic and fibrinolytic systems. Thromb Res 2000; 99: 363-76.
20 Manning MC. Sequence analysis of fibrolase, a fibrinolytic metalloproteinase from *Agkistrodon contortrix contortrix*. Toxicon 1995; 33: 1189-200.
21 Huang TF, Chang MC, Peng HC, Teng CM. A novel alpha-type fibrinogenase from *Agkistrodon rhodostoma* snake venom. Biochim Biophys Acta 1992; 1160: 262-8.
22 Sugihara H, Mori N, Nikai T, Kishida M, Akagi M. Comparative study of three proteinases from the venom of the Chinese hahu snake (*Trimeresurus mucrosquamatus*). Comp Biochem Physiol B 1985; 82: 29-35.
23 Perutelli P. Disintegrins: potent inhibitors of platelet aggregation. Recenti Prog Med 1995; 86: 168-74.
24 Wu WB, Chang SC, Liau MY, Huang TF. Purification a molecular cloning and mechanism of action of graminelysin I, a snake venom-derived metalloproteinase that induces apoptosis of human endothelial cells. Biochem J 2001; 357: 719-28.