生物工程学报
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Chin J Biotech 2008, November 25; 24(11): 1955-1961
Chinese Journal of Biotechnology ISSN 1000-3061

研究报告

重组葡激酶和水蛭素融合蛋白的血栓靶向性机制

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摘　要: 为解释以凝血因子 Xa（FXa）的识别序列为连接肽的葡激酶和水蛭素的融合蛋白（命名为 SFH）在体内的强溶栓和低出血的特征，研究分析了 SFH 的两个血栓靶向性作用机理。首先采用 ELISA 和免疫组化的方法在体外分析了由水蛭素游离的 C 末端赋予的 SFH 对血栓的靶向性，结果显示 SFH 对凝血酶和富含凝血酶的血栓具有更高的亲和力。为阐明 SFH 抗凝活性在血栓部位的靶向性释放，构建表达了仅在水蛭素 N 末端连接 FXa 识别序列的水蛭素衍生物（命名为 FH）。体外试验结果表明完整的 FH 无抗凝活性，在体内 FH 可以发挥抗栓作用，且出血副作用较低，这些结果说明 FXa 的识别序列可以封闭水蛭素的抗凝活性，在体内 FH 可以由于 FXa 的成功裂解而释放其抗凝活性，且其抗凝活性可能仅局限于血栓局部。这就间接说明了 SFH 的抗凝活性可以在血栓局部进行靶向性释放。以上两个血栓靶向性作用机理是 SFH 在体内发挥更高溶栓效率和降低出血副作用的重要机制。

关键词: 葡激酶, 水蛭素, 血栓靶向性, 溶栓, 抗凝

Two Characteristics of a Recombinant Fusion Protein Composed of Staphylokinase and Hirudin: High Thrombus Affinity and Thrombus-targeting Release of Anticoagulant Activity

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Abstract: To improve thrombolytic effect, a fusion protein SFH composed of staphylokinase (SAK) and hirudin (HV) with blood coagulation factor Xa (FXa) recognition peptide as a linker, was designed. SFH showed improved thrombolytic effect and low bleeding *in vivo*. Two thrombus-targeting mechanisms might account for the above features of SFH. This study was designed to study the two thrombus-targeting mechanisms of SFH. ELISA and immunohistochemistry assay were used to study the improved thrombus selectivity of SFH and the results showed that SFH, compared with SAK, displayed higher affinity for thrombin and thrombin-rich thrombus. To verify the thrombus-targeting release of anticoagulant activity of SFH, FH-a derivative of HV with only FXa recognition sequence at N terminus of HV was designed and used in animal tests. In inferior vena cava thrombosis model, FH showed equal antithrombotic effect as HV, indicating that HV could be successfully released from FH by FXa cleavage *in vivo*. More importantly, no prolongation of plasma TT, APTT and PT were found in FH group, but significant prolongations were discovered in

Received: March 21, 2008; **Accepted:** April 16, 2008
Supported by: National Programs for High Technology Research and Development of China (No. 2007AA02Z158).
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HV group. This revealed that the anticoagulant activity of FH was released in thrombus-targeting way and limited in the vicinity of the thrombus, and this could be extrapolated to SFH. In conclusion, the high thrombus affinity and thrombus-targeting release of anticoagulant activity of SFH assigned low bleeding risk to SFH.

Keywords: staphylokinase, hirudin, thrombus-targeting, thrombolysis, anticoagulation

Thrombolysis therapy is one of the well established methods for treating patients with thromboembolism diseases, such as acute myocardial infarction, ischemia stroke etc[1–3]. But reocculusion of the damaged vessels frequently reduces the therapeutic effects[4, 5]. To improve thrombolytic effects and decrease the reocclusion, thrombolytic and anticoagulant agents are simultaneously administrated clinically[6]. The benefits of the combination of thrombolytic and anticoagulant therapy have been proved in many clinical trials[7–9]. This concept encourages the design of multifunctional fusion proteins containing both fibrinolytic agents and anticoagulant agents[10–13]. However, the combination of fibrinolytic activity and anticoagulant activity may lead to severe bleeding risk because both of the fibrinolytic agents and anticoagulant agents have hemorrhagic side-effect. Therefore, the improvements of the thrombolytic effects of the previous fusion proteins are often accompanied with increased hemorrhagic side-effects. To solve the dilemma of thrombolytic effect and hemorrhage event, SFH, a chimeric protein composed of SAK and HV, is designed by us.

In SFH, HV is linked to the C terminus of SAK. This can retain the intact fibrinolytic activity of SAK and block the anticoagulant activity of HV. Several studies have proved that the extension of the N terminus of HV can attenuate, even eliminate its anticoagulant activity[14,15]. To release anticoagulant activity of HV in SFH, FXa recognition sequence is inserted between SAK and HV. The high activity and enrichment of FXa at thrombus site might ensure the thrombus-targeting release of anticoagulant activity. According to this design idea, SFH will improve the thrombolytic effect because of the combination of fibrinolytic activity of SAK and anticoagulant activity of HV. At the same time, FXa cleavage and the release of anticoagulant activity at the thrombus will impair the bleeding risk of SFH. The improved fibrinolytic effect and low bleeding risk of SFH have been confirmed in animals [16].

In this study, we tried to elucidate the mechanism of the improved thrombolytic effect and low bleeding of SFH in detail. According to SFH molecular design, two thrombus-targeting mechanisms may account for the improved thrombolytic effect and low bleeding risk of SFH *in vivo*[16]. Firstly, kinetic and equilibrium studies indicate that the complex of thrombin and HV possess a binding constant as low as 2×10^{-14} mol/L. The binding of the C terminus of HV to the exosite of thrombin is the initial and rate-limiting event during the complex formation[17, 18]. In SFH, the N terminus of HV is linked with SAK and the C terminus is free. This may retain its high affinity to thrombin and will improve the selectivity of SFH for thrombus. This will partly account for the improved fibrinolytic effect and low bleeding of SFH. Secondly, the release of anticoagulant activity of SFH by FXa cleavage is considered to be the main contribution to the attenuated hemorrhage risk. To illustrate the thrombus-targeting release of anticoagulant activity of SFH, FH-a derivative of HV with only FXa recognition sequence at the N terminus of HV is designed. Herein, SAK is deleted from SFH. Because SAK can break down the clots in many anticoagulant animal models, SAK must be deleted to obviate its effect on animal models when the thrombus-targeting release of anticoagulant activity of SFH is investigated.

The anticoagulant activity and bleeding risk of FH were analyzed *in vitro* and *in vivo*. To demonstrate the thrombus-targeting release of anticoagulant activity, the following three hypothesizes must be verified. (1) If the FXa recognition sequence can block the anticoagulant activity of HV, no anticoagulant activity will be detected *in vitro*. (2) If the anticoagulant activity of FH can be successfully released by FXa cleavage *in vivo*, a definite antithrombotic effect will be found in animal test. (3) If the anticoagulant activity of FH can be released and limited in the vicinity of thrombus, lower haemorrhage will be discovered in vivo. The results of FH investigation both *in vivo* and *in vitro* confirmed the above hypothesizes. These hypothesizes and results can be extrapolated to SFH. That is to say, the anticoagulant activity of SFH is thrombus-targeting released.

The results in this research revealed that SFH displayed two thrombus-targeting characteristics: SAK was targeted to thrombus because of the high affinity of free C terminus of HV toward thrombin; and the anticoagulant activity of SFH was thrombus-targeting released because of FXa cleavage at the site of thrombus.

1 Materials and methods

1.1 Host strain and plasmids

Plasmid pBV-*SFH* (our lab) was used as the

template for PCR to amplify gene *FH* (with *Xho* I and *Eco*R I restriction site). Plasmid pPIC9 was used as FH expression vector. *Picha pastoris* GS115 was used as host strain for FH expression.

1.2 Enzymes and reagents

Human α-thrombin was purchased from Sigma (St, Louis, Missouri, USA). FXa was from Roch (Basel, Switzerland). Restriction enzymes and ligase, as well as *Taq* DNA polymerase were from TaKaRa Biotechnology Company (Dalian, China). Human fibrinogen was obtained from China Academy of Medical Sciences (Beijing, China). All other reagents used were of analytical grade.

1.3 Construction of expression plasmid pPIC9-*FH*

The following two primers were used for *FH* gene cloning, Primer 1: upstream primer of *FH* with *Xho* I restriction site (underlined) and FXa recognition peptide IEGR (double underlined)

5′-CCG CTC GAG AAA AGA TTG GGT CCA AGA ATT ACT TAC-3′;

Primer 2: downstream primer of *FH* with *EcoR* I restriction site (underlined)

5′-CCG GAA TTC TTA TTG TAA ATA TTC TTC TGG-3′

The amplification conditions for *FH* were as follows: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s with primers 1 and 2, totally for 30 cycles, followed by extension for 7 min at 72°C. The amplified *FH* was digested with *Xho* I and *Eco*R I and inserted into pPIC9 to construct plasmid pPIC9-*FH*. All recombinants were verified by DNA sequencing.

1.4 Expression and purification of FH

For expression of FH, pPIC9-*FH* was digested and linearized with *Sal* I and then electroporated into the competent cells of *P. pastoris* GS115. A single clone was picked out from MD plate for fed batch cultivation in which methanol was used to induce the expression of FH. The culture supernatant was collected for further ultrafiltration and concentration. SP Sepharose Fast Flow chromatography was involved in the first step and followed by Source 15Q chromatography. The targeted fraction was examined by SDS-PAGE, and the anticoagulant activity was analyzed after FXa cleavage in purification. The detailed procedure of expression and purification were described by Shi BX *et al*[19].

1.5 Cleavage of FH by FXa and anticoagulant activity assay *in vitro*

To release free HV from FH with FXa cleavage, 1u FXa was added to 100 μmol/L FH in 50 μL TA buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 1 mmol/L $CaCl_2$, pH 8.0). The cleavage reaction was performed at 37°C for 30 min. The anticoagulant activities of FH before and after cleavage by FXa *in vitro* were monitored with the fibrin-clot method. Briefly, 10 μL of serially diluted samples were added to 20 μL thrombin solution (8 NIHU/mL) followed by addition of 50 μL fibrinogen (5 mg/mL) and clot formation was monitored. The anticoagulant activity was expressed as antithrombin units (ATU).

1.6 High affinity of SFH for thrombin

ELISA and immunohistochemistry assay were used to detect the affinity of SFH for thrombin or thrombin-rich thrombus.

ELISA was performed as routine method. Briefly, the microtiter plates were coated with thrombin (2 NIHU/mL) at 4°C overnight. After washed and blocked with blocking buffer (2% BSA), SFH, SAK, the mixture of SAK and HV, and HV at equal molar concentration (10 nmol/L) were applied to the plates and incubated at room temperature for 1 hour. After washing, the first antibody against SAK was added and incubated at 4°C overnight. After washing, the second antibody tagged with horseradish peroxidase (HRP) was applied and incubated at room temperature for 1 hour. Then after washing, TMB was added to the plates and the coloration was monitored at 405 nm.

For immunohistochemistry test, thrombin-rich thrombi were produced as described [12]. Briefly, the plasma from healthy people was collected, and $CaCl_2$ and thrombin were added to 200 μL plasma to final concentration of 20 mmol/L and 0.8 NIHU/mL, respectively. After incubation for 2 h at room temperature, the thrombin-rich thrombi were washed with PBST for 3 times. 100 μL of SFH, SAK, the mixture of SAK and HV, and HV at equal molar concentration (10 nmol/L) were added and incubated with the thrombus at room temperature for 1 h. After washing with PBST for 3 times, frozen sections were made and fixed in the precooled methanol at −20°C for 30 min, and then permeabilized in 0.2% Triton X-100 for 30 min. After blocking and washing for three times, antibody against SAK was used as the first antibody and HRP-tagged IgG was used as the second antibody. After washing, DAB was added and the coloration was detected by microscopy.

1.7 Thrombus-targeting release of FH anticoagulant activity *in vivo*

To verify the thrombus-targeting release of FH anticoagulant activity *in vivo*, two features must be observed in animals: definite antithrombotic effect and lower bleeding. Herein, two models were involved in

the research: rat inferior vena cava (IVC) thrombosis model and mouse tail-transected model.

1.7.1 *Inferior vena cava (IVC) thrombosis model in rats*

Clot weights were chosen to assess the antithrombotic effect of FH in the modified IVC thrombosis model[20,21]. In brief, the rats were anaesthetized and the abdomen was opened by a midline incision. The left jugular vein was cannulated for drug administration and blood collection. At 10 min after drug (FH 0.5 mg/kg, HV 0.5 mg/kg) administration, IVC thrombosis was performed with IVC ligation below the level of the renal veins. Clots were harvested and weighed at 4 hours after drug administration.

To assess the bleeding risk of FH, blood samples were taken to prepare plasma at the following time points: prior to drug administration, 0.5 h, 1 h, 2 h and 4 h after drug administration, respectively. Activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) were monitored by coagulometer according to the guidelines of the manufacturer.

1.7.2 *Bleeding time of FH in tail-transection mouse model*

To assess the bleeding risk of FH more directly, bleeding time was measured in a tail-transection mouse model [22]. Briefly, mice were anaesthetized with 0.1 g/kg Amobarbital sodium and put on a table thermostated at 37°C to maintain the body temperature. Normal saline, HV (1.5 mg/kg), FH (1.5 mg/kg, 3.0 mg/kg and 6.0 mg/kg) were injected *via* tail vein. At 20 min after drug administration, the mouse tail was transected with a razor blade at approximately 6 mm from the tail end. The bleeding tail was immediately immersed in normal saline and the time from the beginning of bleeding to its arresting was recorded as the bleeding time.

1.8 Data analysis

Data from animals in each group were expressed as mean ± SEM. Statistical significance was determined using t-test or one-way analysis of variance (ANOVA). Significance was defined at a level of $P<0.05$ or $P<0.01$.

All animal studies were approved by Chinese Animal Research Ethics Board and all animals received care in compliance with Chinese Convention on Animal Care.

2 Results

2.1 Expression and purification of FH

The fermentation broth of FH was centrifuged and the supernatant (about 20 L) was cleared, ultrafiltered by membrane of cutoff 20 kD and 3 kD. The concentrate was applied to chromatography for separation. The final product was authenticated by mass spectrogram (MOLDI-TOF) and the molecular weight was 7363.75 (the theoretic molecular weight is 7362.04). The result of HPLC showed that the purity of the purified FH was up to 98%.

2.2 Cleavage of FH by FXa and anticoagulant activity assay *in vitro*

To monitor the release of anticoagulant activity of FH by FXa cleavage, the antithrombin activity of FH before and after cleavage was detected. No antithrombin activity of intact FH was detected, and the cleaved product showed a certain antithrombin activity. Compared with equal molar concentration of HV, about 22% antithrombotic activity was released from FH (Fig. 1).

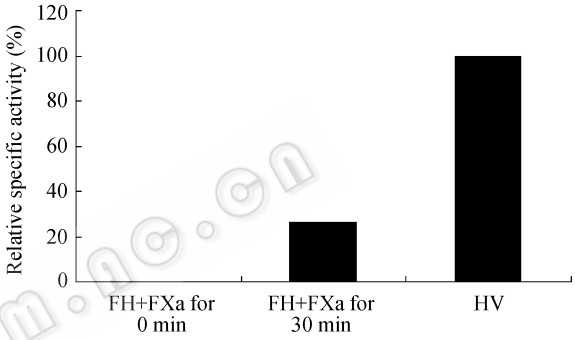

Fig. 1 Anticoagulant activity of FH before and after cleavage by FXa

Ten μL of serially diluted samples were mixed with 20 μL thrombin solution (8 NIHU/mL) followed by addition of 50 μL fibrinogen (5 mg/mL) and clot formation was monitored. Relative specific activities of cleaved products were the ratio compared on equimolar specific activity of HV

2.3 Thrombus-targeting of SFH because of the high affinity of free C terminus of HV for thrombin

To validate the thrombus-selectivity of SFH, ELISA was adopted in this research. SFH showed higher affinity toward thrombin in ELISA than that of SAK ($P<0.01$) (Fig. 2A). The affinity curve of SFH for thrombin was in the form of "S" (Fig. 2B). We presume that the efficient affinity of HV in SFH for thrombin needs a rational configuration or a rational space of SFH for thrombin combination.

To further illustrate the thrombus-targeting of SFH, thrombin-rich thrombi were formed and immunohistochemistry assay was performed with the thrombi (Fig. 3). Consistent with the results in ELISA, SFH showed positive coloration. Compared with SAK, SFH showed significantly higher affinity for the thrombin-rich thrombi.

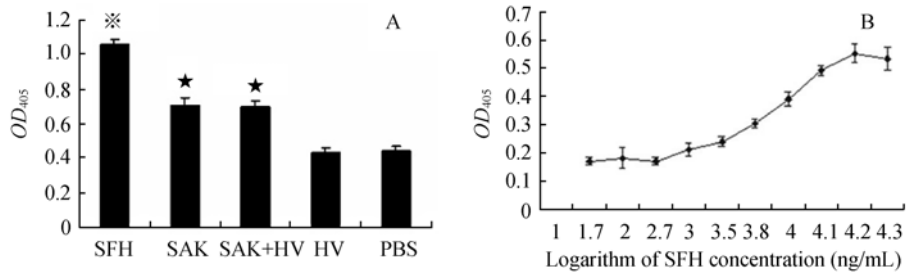

Fig. 2 High affinity of SFH for thrombin

(A) Comparison of thrombin-affinity of SFH and SAK in ELISA. plates were coated with thrombin (2NIHU/ mL) and after washing and blocking, SFH, SAK, the mixture of SAK and HV, and HV were added to the plates. After incubating at room temperature for 1 h and washing, antibody against SAK was used as the first antibody and HRP- goat anti-rabbit IgG was used as the second antibody. After washing, TMB was added to the plates and the coloration was monitored at 405 nm. (n=3, ※ P<0.01, vs all other group; ★ P<0.05, vs HV and PBS, student's t-test.) (B) The affinity curve of SFH toward thrombin. SFH were serially diluted and the range was from 50 ng/ mL to 20 000 ng/ mL. The absorbance at 405 nm was plotted against the logarithm of SFH concentration (n=4)

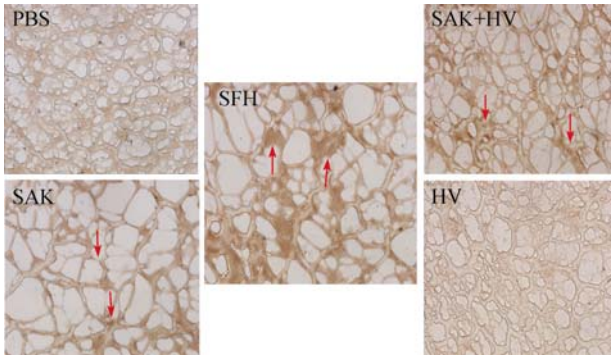

Fig. 3 A higher thrombus-selectivity of SFH than SAK toward thrombin-rich thrombus

The thrombin-rich thrombus was produced by adding $CaCl_2$ (final concentration 20 mmol/L) and thrombin (final concentration 0.8 NIHU/mL) to healthy human plasma at room temperature. After washing, SFH, SAK, the mixture of SAK and HV, HV and PBS were added to thrombus and incubated for 1 h. After washing, frozen sections of the thrombus were used for immunohistochemistry assay. Antibody against SAK was used as the first antibody and HRP- goat anti-rabbit IgG was used as the second antibody. After washing, DAB was added and the coloration was detected by microscopy

2.4 Thrombus-targeting release of the anticoagulant activity of FH *in vivo*

2.4.1 *Antithrombotic effect same as HV and lower bleeding of FH in rat IVC thrombosis model*

In this model, the antithrombotic effect of FH was evaluated by the clot size (Fig. 4A). Compared with normal saline group (7.81 mg±4.07 mg), the clot size of FH group (3.38 mg±2.77 mg, P<0.05) and HV group (2.99 mg±2.93 mg, P<0.01) was reduced markedly. FH had equal antithrombotic activity to HV (P=0.79) at the same dose (0.5 mg/kg).

TT, APTT and PT were employed to monitor the bleeding risk of FH (Figs. 4B, 4C and 4D). There were no differences in TT, APTT and PT values in FH group at different time points. However, after drug administration, significantly prolonged TT (62.34 s ± 12.13 s at 30 min, P<0.01; 51.20 s ± 9.67 s at 60 min, P<0.01, Fig. 4B), APTT (58.19 s ± 12.48 s at 30 min, P<0.01; 39.01 s ± 9.77 s at 60 min, P<0.01; 37.05 s ± 7.77 s at 120 min, P<0.05; 38.67 s ± 8.22 s at 240 min, P<0.05, Fig. 4C), and PT (20.05 s ± 1.63 s at 30 min, P<0.05, Fig. 4D) were observed in equimolar concentration of HV group.

2.4.2 *Low bleeding of FH in mouse tail transection model*

Despite TT, APTT and PT reflected the low antithrombotic activity of FH in peripheral circulation, the general bleeding risk of FH was further determined by bleeding time in tail-transected mice (Fig. 5). Compared with normal saline group (156.14 s ± 125.08 s), only high dose of FH (6 mg/kg) leads to significantly prolonged bleeding time (569.14 s ± 430.10 s, P<0.05). Moreover, bleeding time in high dose of FH (6 mg/kg) was less prolonged than that in low dose of HV (1.5 mg/kg) (1532.50 s ± 420.74 s, P<0.01).

3 Discussion

The fibrin clot-targeting behavior of HV-containing chimeric protein has been investigated by Lian Q *et al*[12]. They have investigated the inhibition of different levels of thrombin by a HV-containing chimeric protein (HE-SAKK) and the results indicate that HE-SAKK can be targeted to fibrin clots depending on the level of the clot-bound thrombin. However, they don't provide direct results to prove the targeting mechanism. In our study, ELISA and immunohistochemistry assay were performed to illustrate the thrombus- targeting of SFH. Compared with SAK, SFH displayed higher affinity for thrombin or thrombin-rich thrombus. The improved thrombus-targeting performance of SFH is partly, at least, resulted from the high affinity of the free C terminus of HV to thrombin. The increased affinity of SFH toward thrombin-rich thrombus will improve thrombolytic effect

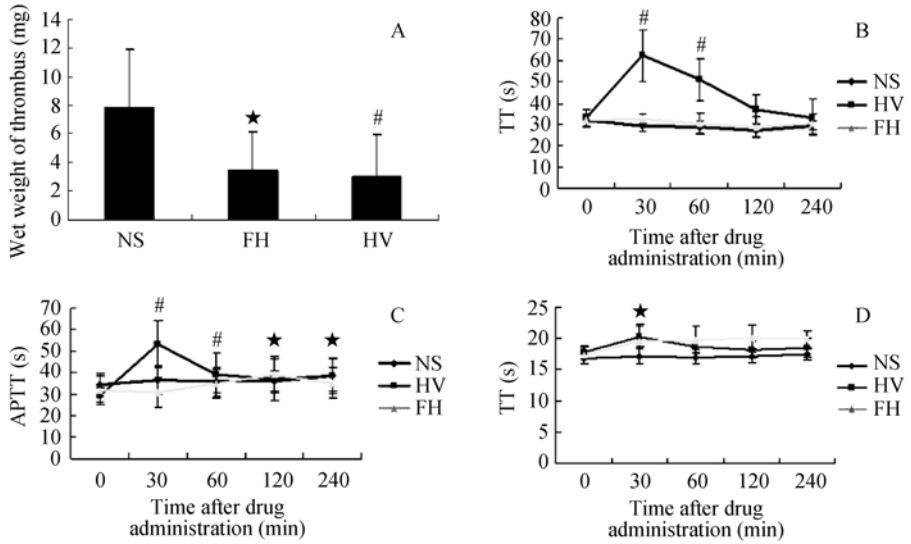

Fig. 4 Antithrombotic effect same as HV and lower bleeding of FH in rat IVC thrombosis model

(A) Same antithrombotic effect as HV at equal dose. Compared with normal saline group, the clot size of FH group and HV group was reduced markedly. FH had equal antithrombotic activity to HV (P=0.79) at the same dose (0.5 mg/kg). (★ P<0.05, *vs* normal saline group; # P<0.01, *vs* normal saline group, student's t-test). (B~D) FH had no effects on plasma TT, APTT and PT. There were no differences in TT, APTT and PT values at different time points in FH group. However, compared with the values before drug administration, TT at 30 min and 60 min after drug administration, APTT at all time points after drug administration, PT at 30 min after drug administration were significantly prolonged in equimolar concentration of HV group. (In HV group, ★P<0.05, vs the value before drug administration; # P<0.01, vs the value before drug administration, ANOVA)

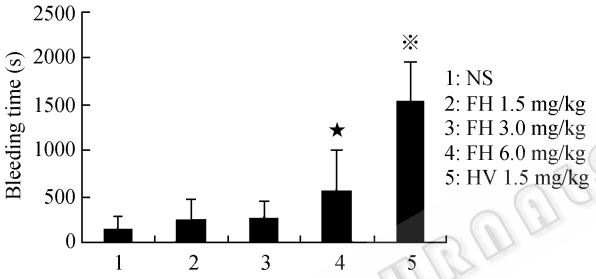

Fig. 5 Bleeding time of FH in mouse tail-transection model

Compared with normal saline group, only high dose of FH (6 mg/kg) led to significantly prolonged bleeding time. Moreover, bleeding time in high dose of FH group was less prolonged than that in HV 1.5 mg/kg group (★P<0.05, vs normal saline group; ※P<0.01, vs all other group, student's t-test)

and reduce bleeding risk of SFH *in vivo*.

Another important feature of SFH is the thrombus-targeting release of anticoagulant activity of HV. Because SAK in SFH can break down the clots in IVC thrombosis model and affect the evaluation of the anticoagulant activity release of HV in SFH, the molecular SAK in SFH was deleted, and FH-a derivative of HV only with the FXa recognition peptide at N terminus of HV was designed. To evaluate the thrombus-targeting release of HV anticoagulant activity *in vivo*, FH was employed in animal tests. Equal antithrombotic effect was observed at equal molar of FH and HV in IVC model, which indicated that FH could be successfully cleaved by FXa and release its anticoagulant activity *in vivo*. On the basis of equal antithrombotic effect in IVC model, the values of APTT, TT and PT of FH at different time points were not altered when compared with those of pre-operation and normal saline group, but the values in HV group were significantly increased at 30 min and 60 min after drug administration. The no prolongation of APTT, TT and PT, three parameters reflecting the anticoagulant activity of HV in peripheral circulation, indicated that FH did not show antithrombin activity at thrombus-free site in peripheral blood.

In the mouse tail-transection model, the bleeding time of FH at low and middle dose (1.5 mg/kg and 3 mg/kg) were not significantly prolonged. Moreover, bleeding time in high dose of FH (6 mg/kg) was less prolonged than that in low dose of HV (1.5 mg/kg). This indicated that FH was cleaved by FXa, liberating and limiting its anticoagulant activity at the site of thrombus.

As the intact FH has no anticoagulant activity in vitro, and a definite antithrombotic effect and no or less prolongation of APTT, TT, PT and bleeding time are found *in vivo*, we conclude that the anticoagulant activity of HV in FH is thrombus-targeting released and limited in the vicinity of the thrombus, and this can be extrapolated to SFH. This might be the most important reason for the low bleeding risk of SFH.

In summary, SFH displays two thrombus-targeting characteristics: high thrombus affinity and thrombus-targeting release of anticoagulant activity. These might

account for the improved thrombolytic effects and the low bleeding risk of SFH *in vivo*.

The authors thank Professors Bingxing Shi, Zhongxiong Tang, Zhonghua Jiang for their fruitful advices and discussions, Jiaxi Wang for emending the manuscript.

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