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Functional properties of a novel mutant of staphylokinase with platelet-targeted fibrinolysis and antiplatelet aggregation activities

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Abstract

The present study was performed to characterize the functional properties of RGD-SAK, a novel mutant of staphylokinase (SAK). Biochemical analysis indicated that RGD-SAK maintained the similar structure and the fibrinolytic function of SAK. Measurement of platelet binding activity *in vitro* demonstrated that RGD-SAK had a much higher affinity with platelets than SAK. *In vitro* platelet-rich clot lysis assay demonstrated that the engineered mutant outperformed the non-manipulated SAK. The time required for 50% platelet-rich clot lysis was reduced significantly across different concentrations of RGD-SAK comparing with SAK. Meanwhile, RGD-SAK was found to inhibit ADP-induced platelet aggregation in a concentration-dependent manner while SAK had negligible effect on platelet aggregation. In concordance, further study in a porcine coronary balloon injury model demonstrated the efficacy of RGD-SAK for the lysis of platelet-rich coronary blood clots and for the prevention of reocclusion after thrombolysis. These results suggested that RGD-SAK may serve as a potential thrombolytic agent with platelet-targeted fibrinolysis and antiplatelet aggregation activities.

Keywords: Staphylokinase; RGD motif; Platelet-targeted fibrinolysis; Antiplatelet activity

1. Introduction

Staphylokinase (SAK), a 16.5 kDa single chain protein isolated from certain strains of *Staphylococcus aureus*, has been demonstrated as a potentially effective clot-dissolving agent for lytic therapy of myocardial infarction and peripheral thrombosis (Collen and Van de Werf, 1993; Vanderschueren et al., 1995). In a plasma milieu, SAK can form 1:1 stoichiometric complex with plasmin-(ogen), which in turn activates the complex by removing a decapetide from the N-terminus of SAK. The activated SAK-plasmin complex then forms a ternary complex with another molecule of plasminogen (plg) and converts this plasminogen into plasmin (plm) to dissolve fibrin clots. Although SAK is a fibrin-specific thrombolytic agent, it has no fibrin binding ability by itself. It binds to the clots only indirectly through the interaction with any clot-bound plasmin-(ogen) (Collen, 1998). Recently, some studies have reported that, while as a promising candidate for thrombolytic therapy, SAK was limited by its incapability of mediating early reperfusion in 38% of treated patients and by rethrombosis of the very arteries being opened in a small but significant number of patients (Collen and Van de Werf, 1993; Collen, 1998).

Nowadays, there is evidence that activated platelets play a pivotal role in arterial thrombosis and rethrombosis. Platelet-rich rather than fibrin-rich thrombosis was found to be responsible for many acute complications of angioplasty (Chen et al., 2005). Meanwhile, studies indicated that platelets aggregation is closely involved in the reformed secondary clots after thrombolytic therapy (Yasuda et al., 1990; Rebello et al., 1999). Hence, it is

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interesting to hypothesize that the clot lysis efficacy of SAK can be enhanced with direct active platelet binding ability, and at the same time the rethrombosis complication can be minimized with an antiplatelet aggregation activity.

Arg–Gly–Asp (RGD) peptide, a well-known motif contained in integrin ligands, can recognize the platelet membrane integrins-glycoprotein IIb/IIIa (GPIIb/IIIa) receptor (Ruoslahti, 1996; Cauwenberghs et al., 2006). It has been found that the binding of surface glycoprotein GPIIb/IIIa to fibrinogen mediates platelet aggregation and RGD can prevent fibrinogen binding to GPIIb/IIIa on activated platelets, thus inhibiting platelets aggregation (Yamada et al., 1996; Ruoslahti and Pierschbacher, 1987). Hence, based on these studies, we hypothesized that the acquisition of the RGD sequence in SAK may result in an acquired ability to target platelet-rich clot for thrombolysis and to prevent platelet aggregation thus reducing rethrombosis. By using site-directed mutagenesis, we have substituted K^{35} with Arg to constitute a RGD motif, resulting in a novel SAK variant, designated as RGD-SAK (Su et al., 2004), which was supposed to be recognized by the activated GPIIb/IIIa on the surface of platelet membrane. However, whether RGD-SAK can maintain the similar conformation of SAK and obtain the hypothesized platelet binding and antiplatelet aggregation activities is unclear. Therefore, in this study, the hypothesized platelet-targeted fibrinolysis and antiplatelet aggregation function of the mutant RGD-SAK was determined *in vitro* and the potential recanalization and anti-reocclusion efficacy was compared with SAK in a porcine coronary balloon injury model.

2. Materials and methods

The investigation was performed according to the European Community guidelines for animal ethical care and the Guide for care and use of laboratory animals published by the US National Institute of Health (NIH publication No 85-23, revised 1985).

2.1. Structural analysis of RGD-SAK

RGD-SAK with a purity >98% was expressed and purified in our lab (Su et al., 2004). SAK standard (100,000 IU/mg) was purchased from SFDA. The structural analysis of the mutant was performed using N-terminal sequencing and circular dichroism spectroscopy (CD spectra). Briefly, the purified recombinant protein were transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting for N-terminal sequencing by automated Edman degradation procedure as described previously (Matsudaira, 1987). The obtained sequence was compared with the cDNA-deduced polypeptide sequence. The secondary structure of the protein was estimated from spectral simulations based on reference CD spectra (Yang and Teng, 1998).

2.2. Fibrinolytic activity assay on fibrin plate

Fibrinolytic activity of SAK and RGD-SAK was measured on fibrin plate (Bi et al., 1998). Petri dishes containing 0.5% agar, 0.055 IU/ml thrombin, 5.5 μg/ml plasminogen (Sigma, USA) and 0.52 μg/ml fibrinogen (Sigma, USA) were prepared. On this solidified fibrin plate, wells of equal diameter were punched and 10 μl/well of diluted appropriate concentration of SAK or RGD-SAK was added and kept at 37 °C for 16 h. The diameter of the halo around the well was measured to calculate the functional activity of SAK or RGD-SAK.

2.3. Activation of human plasminogen

The kinetics of plaminogen activation was determined as described previously (Schlott et al., 1997). Plasminogen was incubated with equimolar SAK or RGD-SAK at 37 °C for 1 h to generate the SAK-plasmin or RGD-SAK-plasmin bimolecular complex. The performed activator complex (final concentration 10 nM) was then mixed with different concentration of plasminogen (final concentration 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM) and generation of plasmin was continuously monitored at 405 nm with S-2251 (3 mM, American Diagnostica, American). The kinetics constants were determined from Lineweaver-Burk plots.

2.4. Measurement of platelet binding activity

An ELISA method was used to assess the platelet binding activity of SAK and RGD-SAK (Zhang et al., 2004). The activated human platelets induced by thrombin solution (0.2 IU/ml) were transferred to microtiter plate. SAK or RGD-SAK in the buffer A (50 mM Tris–HCl, 100 mM NaCl, 2 mM $CaCl_2$, pH 7.4) was added to the wells at a final concentration of 25 nM, 50 nM, 100 nM, or 200 nM. After incubated in 37 °C for 3 h, the plate was washed with PBST (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2, 0.1% Tween 20) for three times to remove the unbound materials. SAK or RGD-SAK retained on the well was probed with polyclonal antibodies against SAK followed by horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies. The amount of HRP retained was assessed by using TMB as HRP substrate. Color development was determined at 450 nm using a microplate reader (Perkin Elmer 1420, VICTOR 3). As a control, some wells were layered only with buffer A but treated otherwise the same. The experiment was repeated three times.

2.5. Platelet-rich clot lysis assay

Platelet-rich clot was formed using the modified procedure (Zhang et al., 2004). Clotting was initiated by mixing human thrombin (to 0.6 NIH unit/ml), human fibrinogen (1 mg/ml) and $CaCl_2$ (to 20 mM) in human platelet. Each clot in the microtiter plate was layered with 100 μl aliquot containing freshly mixed human plasminogen (1.5 μM) and varied concentration of SAK or RGD-SAK (50, 100, 200 nM). After 30 min the surface of each clot was washed three times with HEPES-buffered saline (HBS; 0.01 M HEPES, 0.13 M NaCl, pH 7.4) to remove unbound SAK or RGD-SAK and 100 μl human plasminogen (1.5 μM) were layered on each clot. The clot lysis process was monitored by measuring the turbidity using a microtiter plate reader. Duplicate wells were taken in each one of the three

repeated experiments. As a control, some clots were layered only with HBS but treated otherwise the same.

2.6. Inhibition of platelet aggregation

Platelet aggregation assay was performed with a modified method as described previously (Seymour et al., 1990). Isolated human platelet-rich plasma for *in vitro* platelet aggregation assay was incubated for 15 min at 37 °C with either SAK (diluted by saline, 2.5, 5, 10, 20, 40 μM) or equimolar concentrations of RGD-SAK or RGD (Sigma, USA) prior to the platelet aggregation stimulation with ADP (10 μM). The aggregation response was recorded using a dual-channel aggregometer (APACT Labor, Germany) and aggregation results were expressed as percent inhibition of platelet aggregation.

2.7. Pharmacodynamic research of RGD-SAK

Chinese micro-pigs (20–25 kg) were sourced from the Xi Shan Animal Breeding House (Suzhou, China). The porcine coronary balloon injury model was a modification of one as described before (Rosenthal et al., 2001). After overnight fasting, micro-pigs were anesthetized with phenobarbital (40 mg/kg), the femoral artery was isolated and cannulated to establish an extracorporeal circuit. Invasive arterial pressure measurement, oxygen saturation and ECG were monitored and a calibrated flow probe was placed on the coronary artery to monitor blood flow. Balloon injury was performed in the coronary artery. To induce coronary artery thrombosis expeditious, the model made use of cutting balloon (Cordis, USA) to denude the endothelial surface of the vessel that invariably resulted in the formation of an intravascular thrombus. Animals included in the final protocol satisfied the following preestablished criteria: 1) A circulating platelet count of no less than 100,000/ml 2) The existence of occlusive thrombus (except control group) confirmed by coronary angiograph at the site of injury. Thirty-six micro-pigs were randomized among six different groups as follows: group 1 ($n=6$) received sham operation as control; group 2 ($n=6$) received saline 1 ml/kg; group 3 ($n=6$) received SAK 50,000 IU/kg (Lijnen et al., 1991); group 4–6 ($n=6$, each group) received RGD-SAK 10,000 IU/kg, 30,000 IU/kg, 50,000 IU/kg. All the drugs were administered in an intravenous bolus dose in 1 h after the confirmed thrombus formation in injured site. The coronary angiograph was detected by digital substrate angiography (GE, USA) to observe the vessel patency before thrombosis (baseline), thrombus formation (designated as before administration), 90 min, 24 h and 30 days after thrombolytic therapy. Blood samples were collected for analysis of D-dimer, activated partial thromboplastin time, prothrombin time, thrombin time, fibrinogen and platelet aggregation as described elsewhere (Schlott et al., 1997).

2.8. Statistics

Data are expressed as means ± S.E.M.. One-way analysis of variance (ANOVA) or Student's *t* test was used where applicable. The differences with *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Structural analysis of purified RGD-SAK

To explore the structure of RGD-SAK, the purified sample was subjected to both N-terminal sequencing by the Edman degradation procedure and far-ultraviolet CD spectrum analysis. Sequencing of the first fifteen amino acid residues from the RGD-SAK (SSSFDKGKYKKGDDA) matched exactly with the mature SAK sequence. Analysis of the far-ultraviolet CD spectra showed that SAK comprised 18.2% α-Helix, 36.7% β-Sheet, 45.0% turns and random coils while RGD-SAK comprised 19.3% α-Helix, 36.9% β-Sheet, 43.9% turns and random coils. These results revealed that the secondary structure of the two proteins were very similar and suggested that the substitution of K^{35} with Arg did not change the secondary structure of SAK significantly.

3.2. Fibrinolytic properties of RGD-SAK

The produced RGD-SAK was evaluated for fibrinolytic potencies determined on the fibrin plate (Fig. 1). Compared with the standard SAK, the fibrinolytic activity of RGD-SAK was 120,000 IU/mg, which was at least equal to or slightly higher than SAK. The result revealed that the introduction of RGD sequence into SAK had not altered its fibrinolytic activity. The plasminogen activation rate (determined by k_{cat}/K_m, K_m represents the Michaelis constant, k_{cat} represents the catalytic rate constant) was also examined. Using the formed SAK-plasmin complex, the kinetic parameters for the activation of plasminogen were

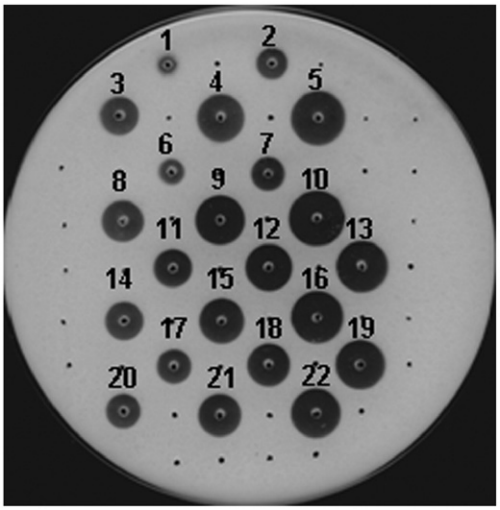

Fig. 1. RGD-SAK fibrinolysis activity assayed on fibrin plate. The numbers above the wells represent various concentrations of samples. 1–5 represent various concentrations of standard SAK (6.5 IU, 12.5 IU, 25 IU, 50 IU, 100 IU/well, respectively). Duplicate wells (6–10) corresponding to 1–5 were included. 11–13 represent various diluted RGD-SAK determined by comparing with the standard SAK. Three duplicated sets (14–16, 17–19, 20–22) of RGD-SAK corresponding to 11–13 were also shown.

Table 1
The kinetics of equimolar mixture of human plasmin with RGD-SAK

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
SAK	0.36±0.02	0.013±0.001	0.031±0.002
RGD-SAK	0.73±0.03	0.026±0.001	0.036±0.003

While both K_m (the Michaelis constant) and k_{cat} (the catalytic rate constant) of RGD-SAK were higher than SAK, but k_{cat}/K_m which represents the catalytic efficiencies between the compared proteins was comparable. Data obtained from three experiments and were reported as mean±S.E.M.

determined. Lineweaver–Burk analysis of the hydrolysis of S-2251 by plasmin-SAK or plasmin-RGD-SAK indicated that the k_{cat}/K_m values were not significantly different (0.031±0.002, 0.036±0.003 respectively Table 1), revealing that RGD-SAK has SAK-like plasminogen activation property.

3.3. Platelet binding and platelet-rich clot lysis assay

To examine the possible platelet-targeted binding ability of RGD-SAK due to the presence of RGD sequence, the *in vitro* platelet binding assay was performed. Data showed that the platelet-targeted binding of RGD-SAK was much stronger than that of SAK at various examined concentrations ($P<0.01$, Fig. 2A). ELISA for platelet binding also indicated that RGD-SAK could bind the active platelets in a dose dependent manner, while SAK almost had no platelet-binding activity. To further examine whether the enhanced active platelet binding ability by RGD-SAK would lead to a faster clot lysis, we carried out

Table 2
Platelet-rich clot lysis mediated by various concentrations of SAK and RGD-SAK

Compound	Concentration (nM)	$T_{50\%}$ (min)
SAK	50	181±7.5
	100	143±8.9
	200	118±4.3
RGD-SAK	50	103±3.9
	100	91±2.3
	200	57±6.6

$T_{50\%}$ (The time required for 50% platelet-rich clot lysis) was significantly shortened for each corresponding concentration (RGD-SAK *vs* SAK, $P<0.01$). Results shown represent means±S.E.M. from 6 clots for each concentration of SAK or RGD-SAK.

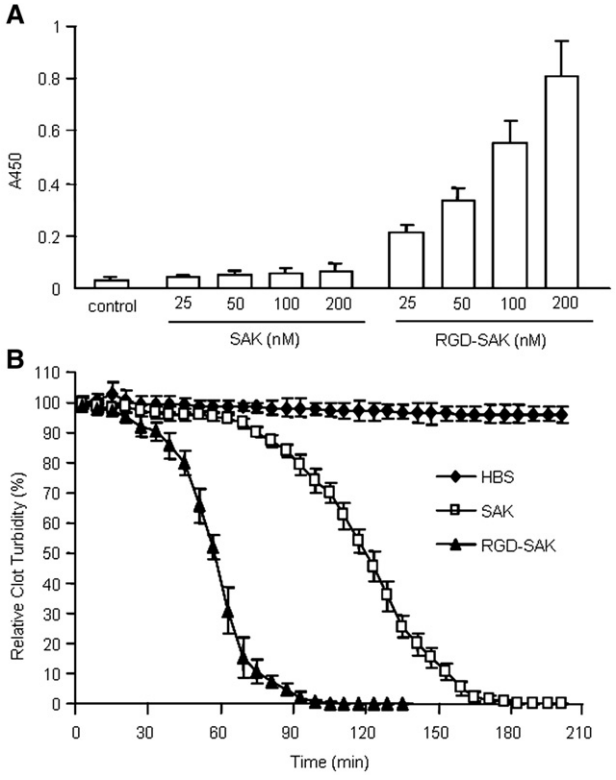

Fig. 2. Platelet binding and time course of platelet-rich clot lysis assay. (A) The means±S.E.M. came from three separate experiments. ELISA was taken to measure the OD450, which represented the quantity of SAK or RGD-SAK bound to the active platelet. The statistical differences were significant for each tested concentration (RGD-SAK *vs* SAK, $P<0.01$). (B) The relative clot turbidity was calculated by detecting the decrease of the absorbance at OD405. The clots were either acted with HBS (HEPES-buffered saline) or with SAK or RGD-SAK. The experiments were repeated for three times. $T_{50\%}$ was 118±4.3 min for SAK and 57±6.6 min for RGD-SAK at the final concentration 200 nM.

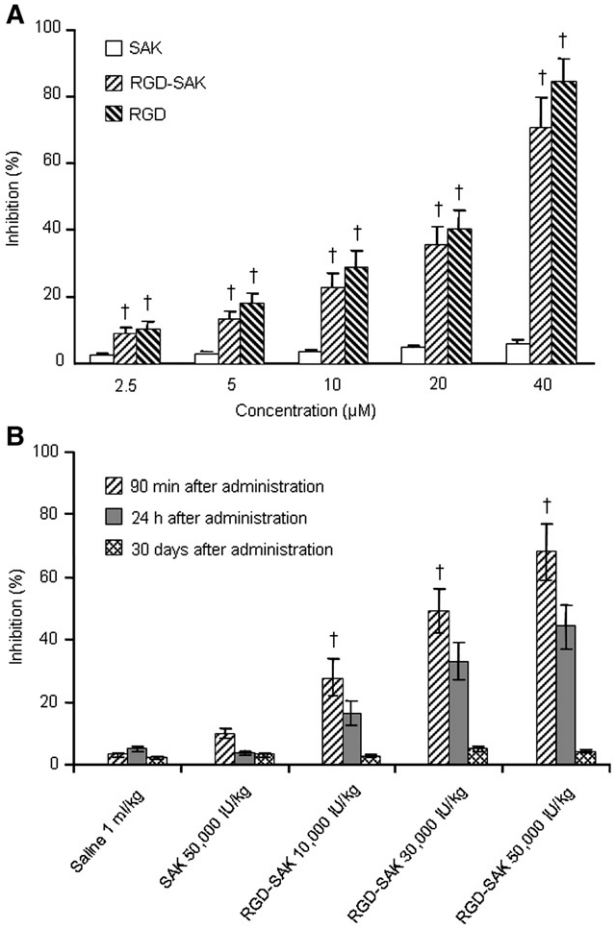

Fig. 3. Inhibition of platelet aggregation by RGD-SAK. (A) The means±S.E.M. came from six independent *in vitro* experiments. The statistical differences between the inhibitory effects of RGD-SAK and SAK for each tested concentration were significant (super †$P<0.01$). RGD in the figure represented the samples incubated with Arg–Gly–Asp (RGD) peptide prior to the platelet aggregation stimulation with ADP (10 μM). (B) In the porcine coronary balloon injury model, RGD-SAK (10,000–50,000) IU/kg inhibited platelet aggregation significantly (super †$P<0.01$ *vs* SAK 50,000 IU/kg group) in 90 min and 24 h after thrombolysis, whereas SAK 50,000 IU/kg had no effect on the platelet aggregation.

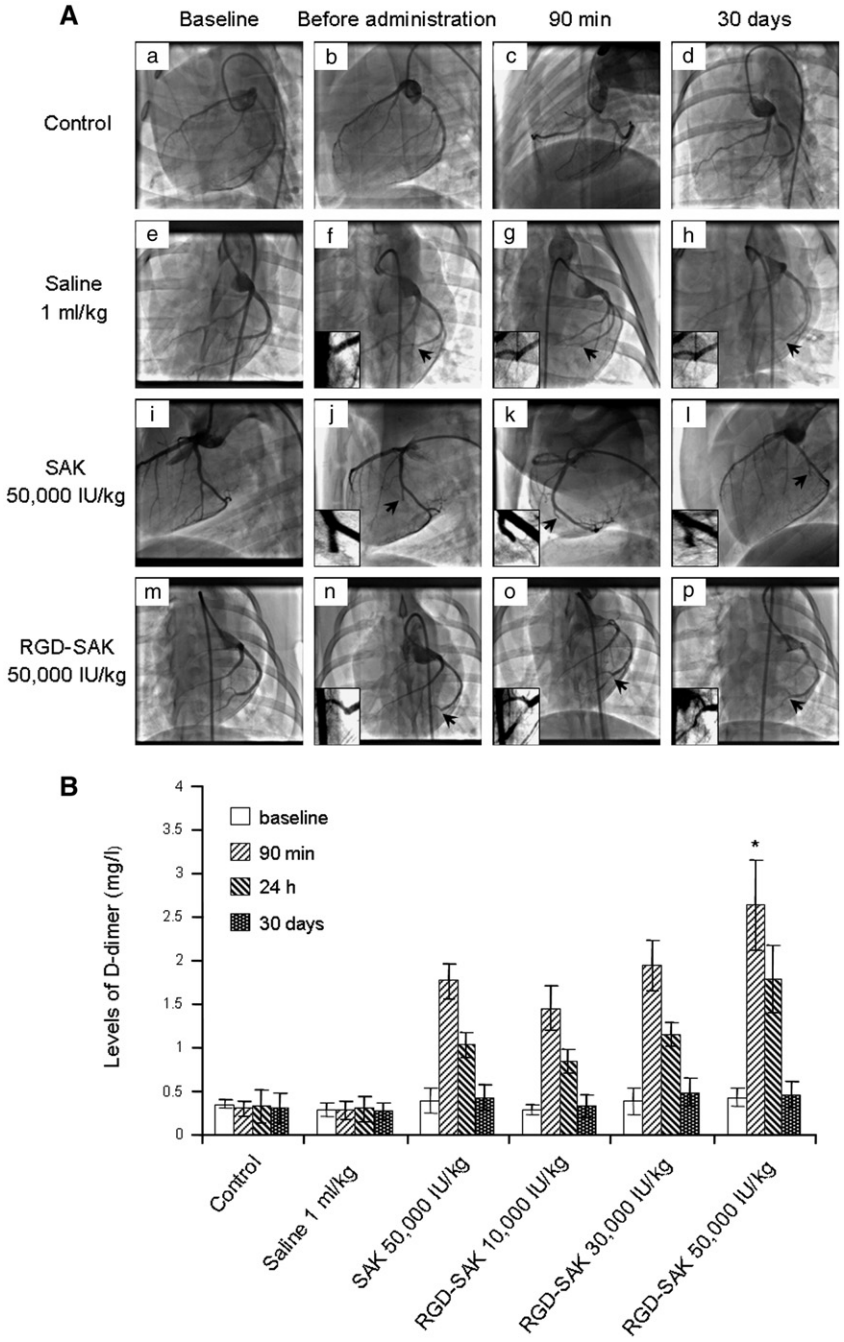

Fig. 4. Thrombolytic and anti-reocclusion efficacy of RGD-SAK observed by coronary artery angiography in the porcine coronary balloon injury model. (A) Coronary angiograms were obtained to determine patency of the injured artery at different experimental time, graded as the scale of Thrombolysis in Myocardial Infarction (TIMI) 0 to 3. A patency score of 3 was assigned to all of the vessels ($n=6$, each group) before application of the cutting balloon injury (a, e, i, m). The injured site was indicated by arrowhead (Left bottom inset represented the blowups of the injured site). The occlusion caused by thrombus was indicated (f, j, n) and there was no blood flow in the area distributed by the pre-injury coronary artery. The efficacy of reperfusion of different treatment regimen in 90 min after administration was shown (g, k, o), which indicated that the still existence of occlusion in saline (1 ml/kg) and SAK (50, 000 IU/kg) groups and disappear of occlusion in RGD-SAK (50, 000 IU/kg) group. Reocclusion in 30 days after administration was observed in saline (1 ml/kg) and SAK (50, 000 IU/kg) groups (h, l) while there remained normal blood flow in RGD-SAK (50, 000 IU/kg) group (p). (B) Levels of D-dimer as molecular markers for thrombolysis in plasma were measured. D-dimer was increased at 90 min in both SAK and RGD-SAK groups. The levels of D-dimer in RGD-SAK 50,000 IU/kg group were higher than that of SAK 50,000 IU/kg group, $^*P<0.05$.

platelet-rich clot lysis experiments which partially simulate the physiological situation. The time required for 50% platelet-rich clot lysis ($T_{50\%}$) by SAK (118±3.4 min) was obviously longer (SAK *vs* RGD-SAK, $P<0.01$) than that by RGD-SAK (57±6.6 min) at the final concentration of 200 nM (Fig. 2B). The differences in $T_{50\%}$ values between SAK and RGD-SAK were also dramatic (RGD-SAK *vs* SAK, $P<0.01$) at the lower concentrations (50, 100 nm, Table 2). The results in Table 2 indicated that in comparison with SAK, the $T_{50\%}$ of RGD-SAK was shortened by 1.75, 1.57 and 2.07 folds respectively at the corresponding concentrations (50, 100 and 200 nm). These results showed that compared with SAK, RGD-SAK could bind to the

active platelets more strongly and resolve the platelet-rich clots more efficiently, suggesting that RGD-SAK has the potential ability for platelet-targeted fibrinolysis.

3.4. Antiplatelet aggregation assay

It is predicted from the molecular design that the bifunctional RGD-SAK not only possesses the fibrinolytic activity, but may also inhibit the platelet aggregation. This was testified by the antiplatelet aggregation assay both *in vitro* and *in vivo* (Fig. 3A and B). *In vitro*, a significantly higher inhibitory effect of RGD-SAK on platelet aggregation was obtained compared with that of SAK, which had negligible effect on platelet aggregation. The dose dependent inhibition of human platelet aggregation by RGD-SAK was also indicated (Fig. 3A and B). The inhibitory effect of RGD-SAK was comparable to RGD-peptide alone. In the porcine coronary balloon injury model, RGD-SAK (10,000–50,000 IU/kg) inhibited platelet aggregation significantly in 90 min and 24 h after thrombolysis, comparatively while SAK 50,000 IU/kg did not affect platelet aggregation (Fig. 3B).

3.5. Thrombolytic and anti-reocclusion efficacy of RGD-SAK in the porcine coronary balloon injury model

In our present study, digital substrate angiography of coronary artery was used to evaluate the coronary patency which was quantified using a scale of 0 to 3 described by the Thrombolysis in Myocardial Infarction (TIMI) study group. The criteria of successful thrombolysis and secondary reocclusion are defined as TIMI = 3 and TIMI ≤ 1 respectively (TIMI Study Group, 1985). Before the induction of cutting balloon injury, coronary artery patency in all the groups was similar (TIMI = 3, Fig. 4A a, e, i, m). Cutting balloon injury produced thrombotic occlusion of the injured coronary artery (TIMI = 0, Fig. 4A f, j, n). Administration of SAK 50,000 IU/kg resulted in a coronary patency score ≤2 (Fig. 4k) after 90 min of infusion and reocclusion was observed after 30 days following reperfusion (Fig. 4A l). In contrast, the swine, which received RGD-SAK 50,000 IU/kg, achieved a coronary artery patency score of 3 after 90 min of infusion (Fig. 4A o) and the patency score of 3 was maintained after 30 days following recanalization (Fig. 4A p). In concordance, as a fibrinolytic marker of thrombus in plasma, the D-dimer was increased at 90 min in both SAK and RGD-SAK groups, however the levels in RGD-SAK 50,000 IU/kg were higher than SAK 50,000 IU/kg ($P<0.05$, Fig. 4B). The incidence of thrombolysis and reocclusion for each of the six groups is summarized in Table 3 which indicated that RGD-SAK 50,000 IU/kg had more thrombolytic efficacy than that of SAK 50,000 IU/kg ($P<0.05$) and RGD-SAK 30,000 IU/kg had at least the same thrombolytic efficacy with that of SAK. Meanwhile none of the angiographic vessels reoccluded in the intermediate and high-dose groups of RGD-SAK, while SAK 50,000 IU/kg could not prevent reocclusion effectively ($P<0.05$). During the study, all micro-pigs tolerated the experiment well and no bleeding was observed. Coagulation and systemic fibrinolysis profiles including thromboplastin time, prothrombin time, thrombin time and fibrinogen were determined in all the animals and no significant differences of these parameters were observed before and after administration (Supplementary data), which indicated that neither SAK nor RGD-SAK in our study influenced the blood coagulation function.

Table 3
Incidence of porcine coronary artery thrombolysis and reocclusion

Group	Coronary angiography			
	Thrombosis	Incidence of thrombolysis after 90 min		Incidence of reocclusion (TIMI ≤ 1) after 30 days
		n		
	n	TIMI = 2	TIMI = 3	*n*
Control ($n=6$)	0	/	/	/
NS (1 ml/kg, $n=6$)	6	0/6	0/6	/
SAK (50,000 IU/kg, $n=6$)	6	5/6	1/6	4/6
RGD-SAK (10,000 IU/kg, $n=6$)	6	2/6	1/6	2/6
RGD-SAK (30,000 IU/kg, $n=6$)	6	3/6	3/6	0/6 [a]
RGD-SAK (50,000 IU/kg, $n=6$)	6	1/6	5/6 [a]	0/6 [a]

Coronary angiography was used to evaluate the incidence of coronary thrombolysis and reocclusion after receiving different regimens. The coronary patency was quantified using a scale of 0 to 3 described by the Thrombolysis in Myocardial Infarction (TIMI) study group. Compared with SAK, high-dose RGD-SAK enhanced the successful recanalization (TIMI = 3) rate significantly after 90 min intravenous infusion and intermediate or high-dose RGD-SAK prevented the reocclusion significantly after 30 days following thrombolysis.
[a] $P<0.05$ *vs* SAK 50,000 IU/kg group.

4. Discussion

Although staphylokinase (SAK) is among the most promising blood dissolving agents, it is far from ideal. As three-dimensional structures of SAK and its ternary complex with microplasmin have been resolved (Parry et al., 1998), various attempts to manipulate and optimize the structure and function of SAK have been reported (Chen et al., 2005; Collen, 1998; Yasuda et al., 1990). In some research, the Arg–Gly–Asp (RGD) peptide was constructed in the SAK due to its affinity with GPIIb/IIIa on the membrane of activated platelets. However, some of these studies did not observe the anticipated function, likely attributed to the positioning of RGD, which had been blocked by unfavorable spatial structure or been cleaved off during the plasminogen activation process (Wu et al., 2003; Van Zyl et al., 2000). Further research (Su et al., 2004; Yang and Teng, 1998) found that loop 1 in SAK would be a rational introducing site for RGD due to the fact that loop 1 is exposed on the solvent accessible surface of the SAK molecule and is far from the active cleft in the SAK ternary complex (Parry et al., 1998), hence the mutation in this site may have little effect on the intrinsic activity of the SAK complex. On the basis of these studies, we have substituted K^{35} with Arg in loop 1 to constitute a RGD motif, generating a novel SAK variant, designated as RGD-SAK (Su et al., 2004). To verify the potential advantages

of this novel mutant, in the present study, the structure and biological activities of RGD-SAK were compared with that of SAK *in vitro* and *in vivo*. The uniform N terminal sequence and similar CD spectrum suggested that these two molecules had similar structure. The similar catalytic efficiencies determined by k_{cat}/K_m suggested that the RGD-SAK-plasmin bimolecular complex was able to interact optimally with the substrate plasminogen and the specific plasminogen activating potential of the mutant remained almost intact. These results indicated that the acquisition of RGD in SAK would not disrupt the structure and the fibrinolytic function of SAK. In addition, what's more important is that as an improved thrombolytic agent, RGD-SAK possesses bifunction of both platelet-targeted fibrinolytic and antiplatelet aggregation activities due to the existence of the RGD domain.

The platelet binding experiment indicated that RGD-SAK could bind the activated platelet in a dose dependent manner and with a higher affinity than SAK, which had weak ability to bind the platelet. In the platelet-rich clot lysis assay, the $T_{50\%}$ values for RGD-SAK were significantly shortened, which revealed a significant increase in platelet-rich clot lysis rate by RGD-SAK compared with SAK. Based on these *in vitro* results, we speculated that RGD-SAK may be more active toward platelet-rich arterial blood clots than SAK due to its higher affinity to the activated platelets, and thus enriching the thrombolytic activity. The porcine coronary thrombus model was used to test this hypothesis and the results revealed that the high-dose RGD-SAK resulted in a greater angiographic patency rate than that of SAK in 90 min after administraion, indicating that RGD-SAK 50,000 IU/kg had more thrombolytic efficacy than that of SAK 50,000 IU/kg. Meanwhile, the angiographically documented coronary artery recanalization rate also indicated that the intermediate-dose RGD-SAK had at least equivalent efficacy compared to that of SAK. Nowadays, it has been known that deep vessel wall injury leads to the formation of a platelet rich thrombus in coronary (Rosenthal et al., 2001). By targeting the activated platelets in thrombus, RGD-SAK may facilitate the clot dissolving process due to the increased local concentration. In addition, it would be interesting to consider that the platelets themselves can suppress clot lysis by mechanical cross-linking, promotion of clot stability as well as release of fibrinolytic inhibitors (Konstantinides et al., 2001; Lang et al., 1994) and some recent *in vivo* studies (Collet et al., 2001; Huang et al., 2001) revealed that fibrinolysis can be accelerated by conjunctive use of anti-GP IIb/IIIa antibody. Hence, these findings implied that RGD-SAK may accelerate lysis of platelet-rich clots *via* diverse mechanisms *in vivo*. Finally, our pharmacokinetics research in rat has found that the half-life of RGD-SAK was prolonged by 7-fold (data not shown) in comparison with SAK, which may result in prolongated duration of action and allow for the use of lower doses of the agent.

Another potential advantage of RGD-SAK is antiplatelet aggregation. It is well known that aggregation of activated platelets binding to fibrinogen *via* the GPIIb/IIIa receptor plays a pivotal role in the formation of arterial rethrombosis after thrombolysis and the RGD peptide is a component of ligands competitively recognizing the activated platelet GPIIb/IIIa receptor (Yamada et al., 1996; Ruoslahti and Pierschbacher, 1987). Therefore, the protein containing RGD has the potential ability to inhibit platelet aggregation and thus to be used in the prevention of arterial rethrombosis. In the present study, RGD-SAK had obvious inhibitory effects on ADP-induced platelet aggregation in a dose dependent manner both *in vitro* and *in vivo*, while the SAK had almost no effect on platelet aggregation. Then, the rethrombosis rate after 30 days following thrombolysis in different groups was compared in the porcine thrombus model by coronary angiography. Results indicated four of six swine had rethrombosis in SAK 50,000 IU/kg group while there was no rethrombosis both in intermediate and high-dose RGD-SAK group, which revealed that RGD-SAK had excellent efficacy in preventing arterial rethrombosis after thrombolysis. These results have further shown that addition of the RGD sequence to SAK resulted in acquisition of the ability to prevent platelet aggregation which may partially explain why RGD-SAK showed efficacy in reducing the incidence of rethrombosis after restoration of arterial blood flow in swine. Noticeably, the relatively long pharmacological half-life of RGD-SAK may also contribute to the efficacy of antirethrombosis after recanalization due to its potential long effective antiplatelet aggregation efficacy.

In conclusion, the present study revealed that RGD-SAK possessed the bifunction to target platelet-rich clots and to block platelets aggregation, and thus was a more potent clot-dissolving agent for thrombolytic therapy and prevention of rethrombosis in comparison with SAK in the porcine coronary thrombus model. It is worthy of future more detailed clinical trails to determine the efficacy and safety of this novel mutant of SAK.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2007.03.010.

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