A prourokinase-RGDS chimera *

——Construction, expression and characterization

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Abstract A tetrapeptide, RGDS, was inserted into proUK kringle domain G118-L119 by the construction of a mutant proUK-RGDS gene. The gene was expressed in the baculovirus expression system. Immunoaffinity chromatography was used to purify the chimera and protein with purity over 90% was achieved. The chimera was tested for its platelet membrane binding function and showed a calcium-dependent platelet binding activity. Amidolytic activity of the chimera was tested. The result indicated that specific amidolytic activity of plasmin activated chimera was 62 000 IU/mg, comparable to the previously reported 65 355 IU/mg of plasmin activated natural proUK[1]. Activation of plasminogen by the chimera after plasmin treatment followed Michieal-Menten kinetics, and the Km was 0.97 μmol/L, which was also comparable to 1.64 μmol/L of native urokinase. The chimera also showed intensive ability to inhibit platelet aggregation *in vitro*. These results indicate that this chimera might be useful as a bifunctional thrombolytic agent.

Keywords: RGDS, ProUK, bifunctional protein, thrombolysis, anti-aggregation.

One of the most advanced methods to treat myocardial infarction is thrombolytic therapy[1]. Despite their widespread use, all currently available thrombolytic agents have significant limitations, such as limited efficiency, thrombus reform and intracerebral bleeding[2]. Researches are being explored to produce safe and efficient thrombolytic agents[2]. Many efforts are concentrated on producing recombinant thrombus-specific thrombolytic agents, which contain anti-thrombus antibody fragments[3] or other thrombus binding molecules[4].

The RGD (Arg-Gly-Asp) sequence is a common characteristic of most cell adhesive proteins[5] for binding to their receptors, the integrins. Recently, many efforts were made as introducing RGD to thrombolytic agents under certain conformational restraint to target them to platelet-rich thrombi and inhibit platelet aggregation, at the same time remaining their thrombolytic activity[6]. The results suggest that the insertion of functional peptide using "loop grafting" method[7] is practical. ProUK is the proenzyme form of urokinase with the characteristics of fibrin-specification and immune to inhibition by PAI-1, and it might be an inviting medicine for treatment of myocardial infarction.

In this study, we inserted RGDS, the critical sequence of one of fibrinogen's platelet membrane glycoprotein GPIIb/IIIa binding sites[8], into proUK to get a bifunctional molecule with platelet-binding and thrombolytic activity. In order to keep the proper conformation of RGD, we chose proUK kringle domain G118-L119 (in PDB 1 ku, Kringle domain G67-L68) as the insertion site, which lies in a protein surface β-turn structure between two β-sheets, a scaffold for functional RGD conformation. We show here the construction of the RGDS containing proUK chimera and the preliminary study of its function.

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1 Materials and methods

1.1 Materials

1.1.1 Enzymes and chemicals. Chromogenic substrates Chromozym^R U and Chromozym^R PL were from Boehringer Mannheim Co. (Germany). Lipofectin was from Gibco/BRL Co. (NY, USA). All other chemicals were of analytical grade or better. Plasminogen, urokinase standard and thrombin were from the National Institute of Biological Medicine Examination (Beijing, China). Plasmin was from Sigma Co. (USA). All routine restriction enzymes, ligase, Klenow fragment were from Promega Co. (WI, USA). T7 DNA sequence kit was from Pharmacia (Sweden). Grace's powder, fetal bovine serum were from Gibco/BRL Co. (NY, USA). TC yeastolate, TC lactalbume hydrolysate were from Difco Co. (USA).

1.1.2 Cells, plasmids and gene. *E. coli* HB101, DH5α, JM101, GT1, RZ1032 were from our laboratory. Plasmids M13mp18, M13mp19, pBluescript SK+/-, pUC19 were from our laboratory. BacPAK6 virus was from Clonetech Co. (USA). Sf9 cell line and vector pBacPAK9 were gifts from Prof. Chen (Peking University, Beijing, China). Human pro-UK gene was a gift from Prof. Hu (Peking University, Beijing, China).

1.1.3 Instruments. SDS-PAGE system was from BIO-RAD Pacific (Beijing, China). Chromatography system was from Pharmacia (Sweden). DAM bi-channel platelet aggregation monitor was from Danyang Electric Research Institute (Jiangsu Province, China). Immunoaffinity column UK-McAb-Sepharose 4B was a gift from Prof. Hu (Peking University, Beijing, China).

1.2 Methods

1.2.1 Construction and verification of expression vector. Standard molecular biological methods were followed[9]. First we utilized Kunkel's method to construct an StuI site in proUK gene for convenient insertion of the RGDS fragment. Plasmid pBluescript-SK/proUK, which encodes full-length proUK, was digested by PstI and EcoRI. The PstI-EcoRI fragment (184 bp) was retrieved and ligated with PstI-EcoRI digested M13mp19 larger fragment. After introducing an StuI site by oligonucleotide primer 5'-cttt<u>aggcct</u>acctgcatag-3' at site 426—431 of pro-UK gene, the mutant fragment was digested with BalI-PstI, and the BalI-PstI fragment was cloned back into pBluescript-SK/proUK. DNA sequencing was performed to verify the StuI site. Synthesized DNA fragment (5'-ccgcggcgacag-3', 3'-ggcgccgctgtc-5') was phosphorylated at 5' end, and ligated with StuI digested pBluescript-SK/proUK (StuI). This plasmid, named pBluescript-SK/proUK-RGDS, was then digested with XbaI, followed by Klenow fragment extension and KpnI digestion. Retrieve the 1.4 kb fragment. pBacPAK9 was digested with BamHI, followed by Klenow fragment extension and KpnI digestion, and then ligated with the 1.4 kb fragment to obtain pBacPAK9/proUK-RGDS. pBacPAK9/proUK-RGDS was cotransfected into Sf9 cells along with Bsu36I digested BacPAK6 virus. The culture supernatant that contained virus particles was identified and purified using limiting dilution and phage plaque method to achieve pure mutant viruses.

1.2.2 Expression and purification of chimeric protein. Sf9 cells were transfected by mutant viruses with MOI = 5. The cells were grown at 27℃ in TFN-FH media containing 10% fetal bovine serum

for 96 h. Western blotting was used to check the expression product. Then the culture media were collected, centrifuged 100 000 *g* for 1 h to eliminate cell debris and virus particles. Supernatant was applied to UK-McAb-Sephrose 4B immunoaffinity column (1 × 10 cm). The column was equilibrated with 4 × PBS (560 mmol/L NaCl, 108 mmol/L KCl, 32 mmol/L $Na_2HPO_4 \cdot 12H_2O$, 6 mmol/L KH_2PO_4, pH 7.4), and eluted with 2 mol KSCN[10]. The eluant was assayed for urokinase activity with fibrin-agarose plate. The final yield of chimera protein was about 2.5 mg/L culture media.

1.2.3 Activating chimera by plasmin. Chimera was incubated in solution A (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Tween80, pH 8.0), with the presence of 1 μg/mL plasmin for 60 min at 37℃.

1.2.4 Measurement of amidolytic activity (chromozym^R U). Activated or un-activated chimera was added to solution A containing 0.5 mmol/L chromogenic substrate chromozym^R U. The rate of absorbance change was measured over a 5-min period. One amidolytic activity unit was defined as the amount of protein that causes the release of 1 μmol of p-nitroaniline ($\varepsilon_{405\ nm}$ = 9 750 $M^{-1} \cdot cm^{-1}$). Control experiments showed that under these conditions the traces of plasmin present from the activation reaction had a negligible effect on the observed absorbance changes.

1.2.5 Plasminogen activation assay. Chimera was firstly activated by incubation with 1 μg/mL plasmin for 60 min at 37℃ in solution A. The activity of generated urokinase was then quantified by chromozym^R U assay as described above. 0.5 IU urokinase or activated chimera was added to plasminogen (0—1.2 μmol/L) at room temperature in the same buffer as used in amidolytic activity assay for 2 min, and then diluted 50-fold at room temperature in solution A containing 0.5 mmol/L of chromozym^R PL. The rate of absorbance change was measured over a period of 5 min. One plasmin unit was defined as the amount of protein that causes the release of 1 μmol of p-nitroniline ($\varepsilon_{405\ nm}$ = 9 750 $M^{-1}cm^{-1}$). Control experiments showed that under these conditions the traces of plasmin present from the activation reaction had a negligible effect on the observed absorbance changes. Another Control experiment was carried out in the absence of the chimera or native urokinase to subtract the plasmin activity existing in plasminogen used. The rates of plasmin generation at each concentration of plasminogen were then determined by dividing the activity generated with time consumed. Kinetic parameters were determined by double-reciprocal plotting.

1.2.6 Platelets preparation. Human platelet rich plasma (PRP), platelet poor plasma (PPP) and gel-filtered platelets were prepared as previously described[11].

1.2.7 Platelet adhesion assay. Platelet adhesion activity was determined using gel-filtered platelets. Chimera was added to 24-well bacteriological plates at different concentrations (0—1 μmol/L), incubated at 4℃ for 12 h, and washed 3—5 times with PBS-Tween 20 buffer (140 mmol/L NaCl, 27 mmol/L KCl, 8 mmol/L $Na_2HPO_4 \cdot 12H_2O$, 1.5 mmol/L KH_2PO_4, 0.1% Tween20, pH 7.4). Then the gel-filtered platelets in 2.5 mmol/L $CaCl_2$ solution were added to the chimera coated wells at final concentration of 2 300 000 platelets per well. Platelets in each well were activated with 5 mmol/L ADP, incubated at 37℃ for 1 h and washed with PBS-Tween20 buffer for 3—5 times. The bound platelets were counted with a microscope. Nonspecific binding was measured in the presence of 5 mmol/L EDTA.

1.2.8 Platelet aggregation test. As previously described[12], PRP was incubated with the chimera at 37℃ for 5 min, then ADP was added at final concentration of 5 mmol/L as activator. The aggregation trace was recorded for 5 min. To subtract the effect of fibrin degradation by pro-UK moiety of the chimera, we also conducted control experiment using natural UK or 0.9% NaCl solution.

2 Results

2.1 Design and construction of expression vector

We constructed a proUK-RGDS chimeric gene by inserting a DNA fragment encoding RGDS into prourokinase gene StuI site, which was introduced by Kunkel's method. The expression system that we chose was baculovirus expression vector system, allowing for high level expression in cultured log phase Sf9 cells. We chose this system based on the previous work of King et al.[13], showing that prourokinase could be produced efficiently by this method.

2.2 Expression and purification of the chimera

The result of Western blotting for 96 -h culture media using rabbit antiserum against urokinase is shown in fig.1. Lane A indicates that expression culture media displayed a single band at 50 ku, as previously reported by King et al.[13] and Xu et al.[10]. Fibrolytic activity of this culture measured with fibrin-agarose plate method was about 1 000 IU/mL. The yield of purified protein was about 2.5 mg/L. SDS-PAGE analysis of the purified chimera under reducing or nonreducing conditions was performed. Lane B in fig.2 shows non-reduced samples stained with Coomassie brilliant blue. The only significant band was at Mr. 50 ku (> %90), which corresponded to the chimera. Lane A in fig. 2 shows that the reduced sample mainly rendered three bands by Coomassie brilliant blue staining. By densitometry of the Coomassie blue stained bands, 64% of the material was accounted for by the 50 ku chimera. The 32 and 18 ku bands, which account for 15% and 11% of the reduced sample, respectively, corresponded to the predicted size of the proteolytic fragments after cleavage by either thrombin (at Arg156-Phe157) or plasmin (at Lys158-Ile159). Because the predicted products of

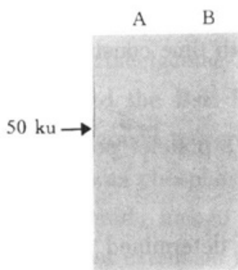

Fig. 1. Western blotting of SDS-PAGE under reducing conditions using 12% gels and staining with a polyclonal rabbit antiserum against urokinase. Lane A, 96-h expression culture media; lane B, negative control using 96-h Sf9 cell culture.

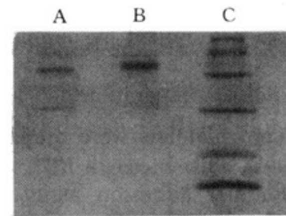

Fig. 2. SDS-PAGE of reduced or non-reduced pure chimera using 12% gel and stained with Coomassie brilliant blue. Lane A, Reduced chimera; lane B, non-reduced chimera; lane C, low molecular weight marker consisting of phosphorylase b (94 ku), bovine serum albumin (67 ku), ovalbumin (45 ku), carbonic anhydrase (30 ku), soybean trypsin inhibitor (20.1 ku), and α-lactalbumin (14.4 ku).

thrombin and plasmin cleavage would vary in molecular mass by only two amino acids and would not be distinguished by electrophoresis, we have not ascertained the relative contributions of thrombin and plasmin to proteolytic cleavage of the chimera.

2.3 Measurement of amidolytic activity

The amidolytic activity of the chimera before and after activation by plasmin was measured using substrate Chromozym^R U. Un-activated chimera had specific amidolytic activity of 20 700 IU/mg, which was about 30% of that of activated sample (62 000 IU/mg). Because prourokinase has as 1% amidolytic activity before activation by plasmin as after activation[14], this result was consistent with the amount distribution of pro-UK and UK in the purified protein as the result of SDS-PAGE indicated. At the same time, the activity value is comparable to previously reported specific activity of activated native prourokinase (65 355 IU/mg)[14].

2.4 Activation of plasminogen by the chimera

The chimera was then tested on its intended physiological substrate, plasminogen (fig. 3). Activation of plasminogen followed Michaelis-Menten kinetics, and the chimera maintained the ability to activate plasminogen comparable to natural urokinase (table 1). This result indicated that the insertion of RGDS into kringle domain of pro-UK did not affect the activation function of the prourokinase moiety to its natural substrate plasminogen.

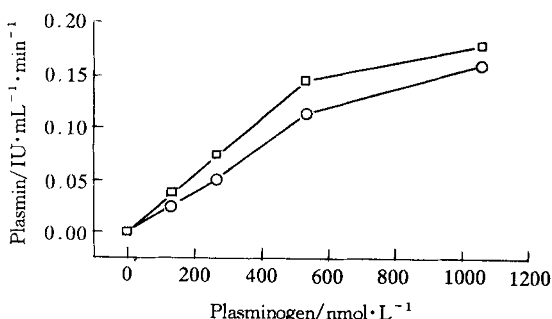

Fig. 3. Activation of plasminogen by plasmin activated chimera (□) or natural urokinase (○). Chimera was firstly activated by incubation with 1 μg/mL plasmin for 60 min at 37℃ in 50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 0.1% Tween-80. 0.5 IU urokinase or activated chimera was added to plasminogen (0—1.2 μmol/L) at room temperature in the same buffer as used in amidolytic activity assay for 2 min, and then diluted 50-fold at room temperature in the same buffer containing 0.5 mmol/L of chromozym^{R}PL. The rate of absorbance change was measured over a period of 5 min. One plasmin unit was defined as the amount of protein that causes the release of 1 μmol of p-nitroniline ($\varepsilon_{40\ nm}$ = 9750 $M^{-1}\cdot cm^{-1}$). The rates of plasmin generation at each concentration of plasminogen were then determined by dividing the activity generated with time consumed. Generated plasmin activity is plotted versus concentration of plasminogen.

2.5 Platelet adhesion assay

Binding activity of the chimera to platelet was determined by platelet adhesion assay described above (fig.4). The chimera displayed a calcium dependent affinity for ADP activated platelets. We have known that the binding of fibrinogen to activated platelet membrane glycoprotein GpIIb/IIIa relies on the presence of calcium, so the membrane binding activity of fibrinogen remained in the chimera through the inserted RGDS sequence.

2.6 Platelet aggregation assay

Platelet aggregation in the presence of the chimera or natural UK or 0.9% NaCl was tested (fig. 5). The chimera displayed a far more powerful anti-aggregation activity than natural urokinse (table 2).

Table 1 The kinetic parameters of plasmin-activated chimera and natural urokinase for activating plasminogen (determined by double-reciprocal plotting)

	$Km/\mu mol \cdot L^{-1}$	$V_{max}/IU \cdot mL^{-1} \cdot min^{-1}$
Plasmin-activated chimera	0.97	0.33
Natural urokinase	1.64	0.45

Table 2 The parameters of anti-platelet aggregation activity with 2 μmol/L chimera or natural urokinase (data were derived from two independent tests)

	Top aggregation rate	5 min anti-aggregation rate
Chimera	10.3%	89.7%
Natural urokinase	92.3%	7.7%

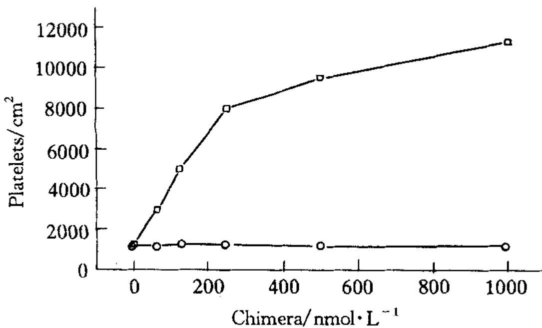

Fig. 4. Platelet binding activity of the chimera with (○) or without (□) the existence of 5 mmol/L EDTA. The density of platelets bound is plotted versus chimera concentration.

3 Discussion

Clinical experience in the treatment of myocardial infarction has revealed that the presently available thromboytic agents are efficient in terms of reducing of mortality, although significant shortcomings have been observed in terms of the occurrence of bleeding complications and platelet-mediated reocclusion. Activation of circulating platelet is directly linked to the vessel reocclusion following successful thrombolysis. The common final pathway of platelet activation involves conformational change of platelet membrane glycoprotein GpIIb/IIIa and the binding of fibrinogen to these receptors on adjacent platelets. So many antagonists to GpIIb/IIIa have been developed, and most of them contain functional RGD sequence.

We constructed a mutant prourokinase gene which contains a piece of DNA sequence encoding RGDS tetrapeptide as the function group of antagonist for activated platelets. After being expressed in the baculovirus expression system, the chimera was purified using immunoaffinity column. This chimera maintained full amidolytic activity and plasminogen activation property of its prourokinase moiety with the gain of activated platelet membrane binding activity.

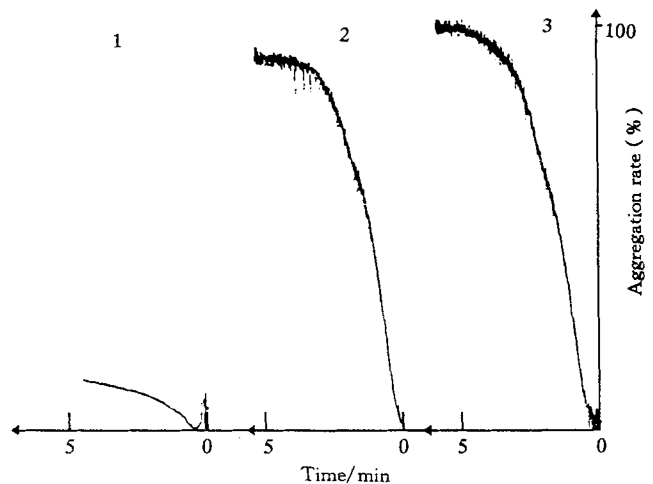

Fig. 5. Anti-platelet aggregation by the chimera or natural urokinase. Platelet aggregation was monitored with a bi-channel platelet aggregation monitor in the presence of 2 μmol/L chimera (1) or 2 μmol/L natural UK (2) or 0.9% NaCl solution (3). PRP was incubated with samples at 37℃ for 5 min, and then ADP was added in final concentration of 5 mmol/L as the activator. The aggregation trace was recorded for 5 min.

Prourokinase Kringle domain G118-L119 was chosen as the insertion site. As our results indicated, RGDS inserted here gives the chimera platelet binding activity, and affects neither the amidolytic activity nor the property of activation by plasmin of the prourokinase moiety. This confirmed our predictions that are

bases for designing the chimera. First, RGDS is the crucial sequence of fibrinogen for its binding to platelet. Second, kringle domain G118-L119 lies in a β-turn on the surface of prourokinase molecule between two β-sheets. This structure can give RGDS the proper conformational restriction (fig. 6). The computer modeling results also support our prediction (data not shown). Third, no obvious function of prourokinase kringle domain was observed, so we expected that insertion RGDS in this domain would not disrupt the normal function of prourokinase.

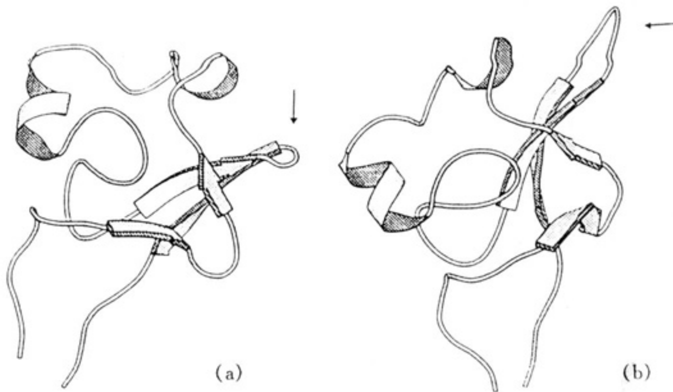

Fig. 6. Solution structure of the Kringle domain of prourokinase. (a) Wild type proUK Kringle domain structure determined by nuclear magnetic resonance; (b) RGDS-proUK Kringle domain structure determined by computer modeling.

We use natural urokinase as control in anti-aggregation test to verify that the anti-aggregation activity is not due to the fibrinolytic activity of urokinase, but to some new character of the chimera. The fact that the chimera was potent in inhibition of platelet aggregation indicates that the chimera may be valuable in the case of thrombus reformation. And although we still did not perform clot lysis assay, more specificity for platelet-rich clot of the chimera can be expected by the result of platelet membrane binding assay. *In vivo* experiment will be carried out to confirm the effectiveness of the proUK-RGDS chimera in thrombolytic therapy.

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