

Construction, Expression, and Characterization of a Recombinant Annexin B1-Low Molecular Weight Urokinase Chimera in *Escherichia coli*

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Abstract To produce a thrombi-targeting plasminogen activator, low molecular weight single-chain urokinase gene (*scuPA32k*) was spliced with the full-length cDNA of annexin B1 gene (*anxB1*) by overlap extension method. The fused gene *anxB1scuPA* was ligated into pET28a vector, transformed into *E. coli* BL21-RIL, and then induced to express under the control of T7 promoter. The AnxB1ScuPA protein expressed amounted to 22% of the total bacterial proteins. The product was refolded, and then purified by using DEAE Sepharose fast flow ion-exchange column and Superdex S-200 gel-filtration column. HPLC analysis revealed that the final purity is about 95%. The specific activity of AnxB1ScuPA, measured as amidolytic activity, reached 100,000 IU/mg. It had a similar S2444 catalytic efficiency (k_{cat}/K_m) to ScuPA32k, and also showed high activated-platelet membrane-binding activity and anticoagulant activity, indicating that the chimera fully retained the components of enzymatic and membrane-binding activities of the parent molecules. *In vivo* test revealed that, the dogs administered with AnxB1ScuPA had less reperfusion time, higher reperfusion ratio, and less bleeding effects than those with urokinase. These findings indicated that AnxB1ScuPA might have advantages over current available thrombolytic agents.

Key words annexin B1; thrombolytic agent; low molecular weight single-chain urokinase; chimera

Thrombolytic therapy has been a major advance in the treatment of myocardial infarction over the last two decades. Urokinase (UK), streptokinase (SK), and tissue plasminogen activator (t-PA) are the common available thrombolytic agents. These agents, however, have certain limitations due to the insufficient binding affinity for the fibrin clot and/or short half-lives in circulation. The thrombus may be resistant to lysis in some patients or reform after initial lysis; and systemic hemorrhage is a serious problem, especially when large doses are administered. To solve these problems, improved fibrinolytic agents that have a higher binding affinity to thrombi are needed. One effective approach is to prepare chimeric proteins composed of plasminogen activator and thrombus-specific

antibody. Chemical chimeras, which have prourokinase attached to antibodies against fibrin or platelet membrane proteins, have a higher affinity for thrombi and showed an enhanced fibrinolytic activity [1].

Annexins are a large family of calcium-dependent phospholipid-binding proteins widely distributed in eukaryotes. In the presence of Ca^{2+} , proteins of this family can bind to negatively charged phospholipids like phosphatidylserine (PS) with high affinity. PS is the major component of the platelet membrane and plays a key role in the process of coagulation cascade. Under viable and unperturbed conditions, PS localizes predominantly in the membrane leaflets facing the cytosol. When platelets are activated by agonists like thrombin and collagen, PS becomes surface-exposed and thereby provides a catalytic surface for the procoagulant reactions. AnxB1, a member of a novel annexin family isolated from *Cysticercus cellulosae*, has a high Ca^{2+} -dependent PS binding activity [2]. Therefore, it can inhibit the activation of coagulant factor Xa by shielding PS on the surface of the activated platelets and subse-

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quently inhibit the thrombin generation [3]. Based on these findings, we proposed that a chimera composed of AnxB1 and a thrombolytic agent might both have the Ca^{2+} -dependent PS binding property and the thrombolytic activity, which might have certain advantages when compared with current thrombolytic reagents.

ScuPA32k, a 32 kD polypeptide composed of the 144–411 amino acids of prourokinase, retains the proenzyme character of full-length prourokinase [4]. Due to its small size, it might have certain advantages in production or clinical use. Therefore, we choose it as the thrombolytic moiety to design the chimera. Our goal in this study was to design and express an AnxB1ScuPA32k chimera, and to test its activities *in vivo* and *in vitro*. We show that this chimera can be expressed and purified in active form; they fully retain the thrombolytic activity and platelet-membrane binding activities of the parent molecules.

Materials and Methods

Materials

Plasmids and *E. coli* strains pUC18-*anxB1*, which contains the full cDNA sequence of *anxB1*, was previously constructed and preserved in our laboratory. The *scuPA32k* gene was artificially synthesized by Shanghai Shenergy Biocolor BioScience & Technology Co., Ltd. and inserted into pUC-118 vector to generate the plasmid pUC118-*scuPA32k*. *E. coli* strain BL21-CodonPlus™-RIL strain (BL21-RIL) and pET28a vector were kind gifts from Prof. Bing-Gen RU.

Reagents and enzymes The following reagents were obtained from commercial sources: restriction enzymes, T_4 DNA ligase were from TaKaRa (Japan); *pfu* DNA polymerase was purchased from Promega; Chelating Sepharose and SDS-PAGE standards were from Pharmacia Biotech Inc.; chromogenic substrates *L*-pyroglutamyl-Glu-Gly-Arg-*P*-nitroalide (S2444), DTT, and IPTG were from Sigma; agarose was from Gibco Co.. Urokinase standard, thrombin, human fibrinogen, and human plasminogen were from National Institute for the Control of Pharmaceutical and Biological Products (NICPBR). Other chemicals used in this study were of analytical or higher grade. Recombinant AnxB1 and ScuPA32k proteins, and polyclonal antibody against AnxB1 were prepared in our laboratory. The HRP-conjugated goat anti-rabbit IgG was from Sigma.

Instruments The chromatography systems for protein purification were from Pharmacia. ChemiImager™5500

fluorescence and chemiluminescence imaging system from Alpha Innotech., USA. RM-6300 polygraph physiology recorder system was from NIHON KOHDEN KOGYO Co.. SC-3 electrical respirator was from Shanghai Medical Instrument Factory.

Animals Healthy mongrel dogs ($n = 30$, weight range 10–14 kg) of either sex, obtained from Tianjin suburb, and fed more than a week before use.

Construction and verification of expression vector

Gel electrophoresis, restriction enzyme digestion, ligation of DNA and transformation of *E. coli* were performed according to the protocols described in [5]. The *anxB1* and *scuPA32k* genes were fused using PCR technique of splicing by overlap extension (SOEing) [6]. The strategy for constructing *anxB1scuPA* was illustrated in Fig. 1. The *anxB1* full-length cDNA was obtained by PCR amplification on the plasmid pUC18-*anxB1* with primers A (5'-GCCCATGGCCTACTGTCGCTCCC-3') and B (5'-GCCCACTGAAATTTTAATGCAGGGCCGAT-

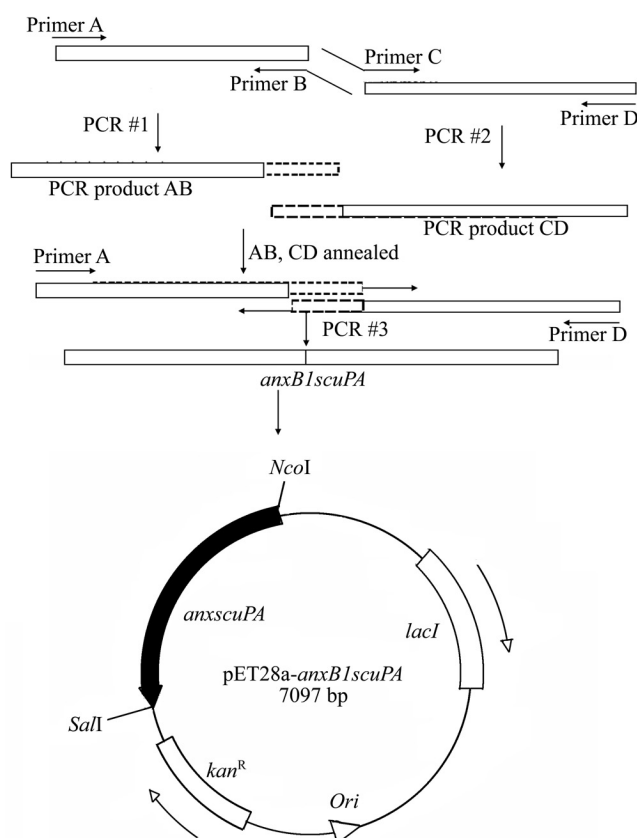


Fig. 1 Splicing of gene *anxB1* with *scuPA* by overlap extension and construction of the expression vector

GAG-3'). An *NcoI* restriction site was introduced to the 5'-end. The DNA fragment encoding *ScuPA32k* was PCR amplified on the plasmid pUC118-*scuPA32k* with the primers C (5'-*CTCATCGGCCCTGCATTTAAATTT-CAGTGTGGC*-3') and D (5'-*TTGTCGACTTAGAGG-GCCAGGCC*-3'). *SalI* restriction site was introduced to the 3'-end. A 1067 bp DNA fragment and an 824 bp DNA fragment were produced with primers A, B, and primers C, D, respectively. Primer B and C are complementary to each other: the nucleotides shown in italics are derived from the *anxB1* while the underlined nucleotides are from the *scuPA32k* sequence. Therefore, the two DNA fragments have overlapping ends and could serve as a primer for the other. Extension of the overlap by *pfu* DNA polymerase yielded a new 1849 bp PCR product, which is the chimeric gene *anxB1scuPA*.

PCR fragments were purified by agarose gel electrophoresis, digested with *NcoI/SalI*, and ligated into the *NcoI/SalI* sites of pET28a to produce plasmid pET28a-*anxB1scuPA*. The final recombinant plasmid was characterized by restriction enzyme digestion and regions around the cloning sites were resequenced to ensure that the desired constructions were prepared. GenBank™ reference sequences were AF147955 for the *anxB1* cDNA and M15476 for the prourokinase cDNA.

Expression and purification of chimeric proteins

The recombinant plasmid was transformed into *E. coli* strain BL21-RIL for large-scale expression. Cells were grown at 37 °C with shaking in 2% LB broth (pH 7.4). When the turbidity (A_{600}) reached 0.6, IPTG was added to 0.5 mM and growth was continued for 3 h. The final A_{600} was about 1.2. Cells were then harvested by centrifugation, washed once with 50 mM pH 8.0 Tris-HCl and then stored frozen at -20 °C.

The inclusion body was prepared according to [7] and renatured as follows: 5 g inclusion body was suspended in 75 ml of buffer A (8 M urea, 0.5 M NH_4Cl , 20 mM pH 8.5 Tris-HCl, 10 mM DTT) and shaken for 4 h at room temperature. After centrifugation, the supernatant was dialyzed against buffer B (8 M urea, 0.1 M NH_4Cl , 20 mM pH 8.5 Tris-HCl, 5 mM DTT) for 12 h and dropped slowly into 800 ml buffer C (1.5 mM urea, 0.4 mM *L*-Arg, 0.5 mM EDTA, 10 g/L Glycerol, 0.2 mM GSSG, 0.05 M GSH, 20 mM pH 8.5 Tris-HCl). Then the sample was dialyzed against buffer D (10 mM Gly/NaOH, pH 9.0) with slightly stirring at 4 °C for 24 h. During the dialyzation, buffer D was exchanged for about three times. Finally, insoluble materials were removed by centrifugation and the supernatant was stored for further purification.

The renaturation sample (about 900 ml) was applied to a DEAE-Sepharose fast flow column (500 ml bed volume) at a flow rate of 5 ml/min. Then the column was equilibrated with buffer D and firstly washed with buffer E (10 mM pH 8.5 Tris-HCl, 100 mM NaCl) to remove the unfolded proteins. The absorbed fractions was eluted with buffer F (10 mM pH 8.5 Tris-HCl, 300 mM NaCl) at a flow rate of 5 ml/min. The fractions (about 50 ml) were dialyzed overnight against 10 volumes of buffer G (10 mM pH 8.5 Tris-HCl) and applied to Superdex-200 filtration column (2.6 cm × 80 cm) at a flow rate of 2 ml/min. The column is equilibrated and eluted with buffer G. The fractions that contained the renatured protein was pooled and stored at -20 °C for further assay.

HPLC analysis

The purity of the final purified protein was assayed by HPLC performed on a 300 mm × 7.8 mm Protein-Pak™ 125 column (Waters Co., USA). The column was eluted with sodium phosphate (pH 7.4) for 30 min at a flow rate 1 ml/min. Detection was measured at 280 nm.

Anticoagulant activity assay

The anticoagulant activity of recombinant AnxB1ScuPA was assayed using modified kaolin partial thromboplastin time (KPTT) as described in [2]. Recombinant AnxB1 and bovine serum albumin were used as control.

Measurement of amidolytic activity

The amidolytic activity and the kinetic parameters of the AnxB1ScuPA/UK on the chromogenic substrate S2444 was determined as described in [8].

Platelet binding assay

Gel-filtered human platelet rich plasma (PRP) was prepared as previously described [9]. All the samples for test, AnxB1ScuPA, urokinase (negative control) and AnxB1 (positive control), were diluted with PBS to 6 different concentrations (0, 0.3, 0.6, 0.9, 1.2 and 1.5 μM). 200 μl samples were added to 24-well bacteriological plates, incubated at 4 °C for 12 h, and washed 3 times with PBS-Tween 20 buffer (140 mM NaCl, 10 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.5 mM KH_2PO_4 , 0.1% Tween 20, pH 7.4). Then the gel-filtered platelets in 2.5 mM CaCl_2 solution were added at final concentration of 3×10^5 platelets per well. Platelets in each well were activated with 5 mM ADP, incubated at 37 °C for 60 min and washed with PBS-Tween 20 buffer for 3 times. The bound platelets were counted with a microscope. Nonspecific binding was measured in the presence of 5 mM EDTA.

Thrombolytic therapy with AnxB1ScuPA for canine coronary artery thrombosis

Thirty dogs were randomly divided into five groups, six dogs per group. 1-hour bolus infusion with intravenous AnxB1ScuPA 2.25, 5.0, 10.0 $\times 10^4$ IU \cdot kg $^{-1}$, was given by microinfusion pump 30 min after coronary artery embolization. The control group was given vehicle, UK with 5 $\times 10^4$ IU \cdot kg $^{-1}$ as positive control group, the fluid velocity remained 0.5 ml/min, total accumulated volume was up to 30 ml. The surgical preparation and the detection criterion for reperfusion have been described in detail in previous publications [10], as well as the wound blood loss measures.

SDS-PAGE analysis and protein concentration measurement

10% SDS-polyacrylamide gel electrophoresis was performed according to lammeli [11]. Protein concentrations were determined by the method of Bradford [12] using bovine serum albumin as a standard.

Western blot analysis

Western blot analysis was performed according to [13].

Statistical analysis

Data were expressed as the mean \pm standard deviation ($\bar{x} \pm s$). Statistical analysis was done using Student's *t*-test. $P < 0.05$ indicated a significant difference.

Results

Expression and purification of chimeric AnxB1ScuPA

The *anxB1* and *scuPA32k* genes were spliced using the PCR technique of SOEing. The recombinant expression plasmid pET28a-*anxB1scuPA* containing the chimeric *anxB1scuPA* gene was transformed into *E. coli* strain BL21-RIL. As shown in Fig. 2, a distinguishable extra band was present with the sample of cells harboring pET28a-*anxB1scuPA* after IPTG induction. Molecular weight of this band is about 66 kD, consisting with the calculated value of AnxB1ScuPA. Western blot analysis indicated that this band could be detected by polyclonal antibody against AnxB1, which provided evidence that this band was recombinant AnxB1ScuPA. The densitometric scanning results revealed that the expression level of AnxB1ScuPA amounted to 22% of total cellular proteins. AnxB1ScuPA was expressed essentially in an insoluble denatured form,

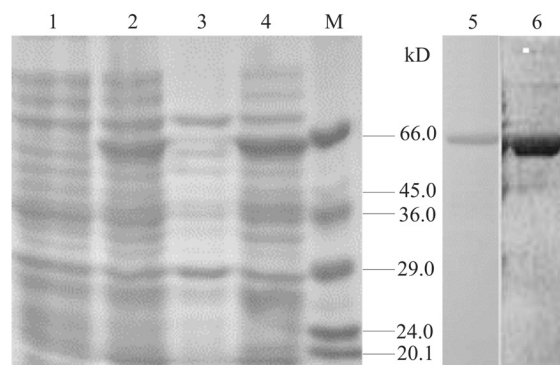


Fig. 2 Analysis of expression and purification of recombinant AnxB1ScuPA by SDS-PAGE

1, total bacterial proteins before induced; 2, total bacterial proteins after induced 3 h by 0.8 mM IPTG; 3, the supernatant of the induced cells was sonicated and centrifuged; 4, the pellet of the induced cells was sonicated and centrifuged; M, protein marker; 5, purified AnxB1ScuPA; 6, Western blot analysis result.

therefore the inclusion body fraction was isolated and subjected to a variety of denaturation and refolding procedures. We varied the denaturant (urea or guanidine), pH, salt concentration, reducing agent and protein concentration during refolding, the yield of renatured material was then determined by S2444 assay. The final optimized refolding procedure is described in "Materials and Methods". According to this procedure, the yield of active protein is about 100 mg/g inclusion body and of about 75% purity at this stage.

After refolding, the chimeric protein was then purified by DEAE anion-exchange column and Superdex-200

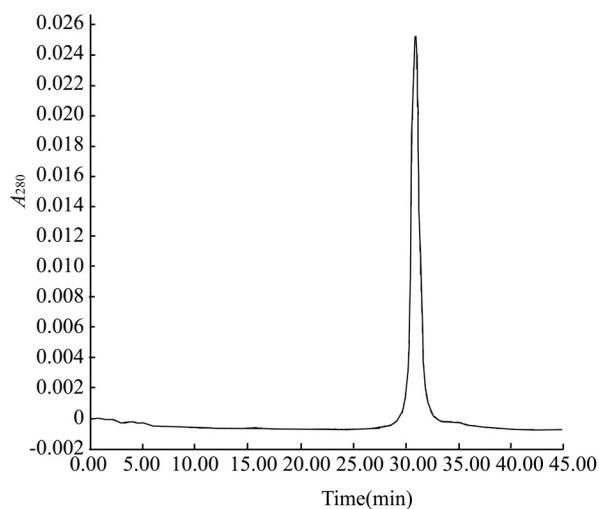


Fig. 3 HPLC analysis of purified AnxB1ScuPA

filtration column, purity of the final product is about 95% (HPLC assay, Fig. 3) and yield was about 20 mg/g inclusion body. The specific amidolytic activity of the purified protein was about 100,000 IU/mg, a little lower than that of UK (120,000 IU/mg).

Measurement of amidolytic activity

After activated by plasmin, the kinetic parameters of AnxB1ScuPA and ScuPA32k amidolytic activity were determined by S2444 incubation. As shown in Table 1, AnxB1ScuPA had no statistically significant difference in both K_m and k_{cat} values with ScuPA32k. Therefore, AnxB1ScuPA displayed a similar catalytic efficiency of plasmin activatability to ScuPA32k, indicating that AnxB1ScuPA retained the amidolytic activity of ScuPA32k moiety.

Table 1 Kinetic constant of the amidolytic activities of AnxB1ScuPA and ScuPA32k

	$K_m/\mu\text{mol}\cdot\text{L}^{-1}$	k_{cat}/s^{-1}	$k_{cat}/K_m (\mu\text{mol}^{-1}\cdot\text{s}^{-1}\cdot\text{L})$
AnxB1ScuPA	52.3 ± 3.6	5.80 ± 1.3	0.096 ± 0.008^a
ScuPA32k	55.1 ± 2.8	5.78 ± 0.9	0.105 ± 0.006

Data were expressed as $\bar{x} \pm s$ of 3 independent tests. ^a $P < 0.05$ vs. ScuPA32k.

Plate adhesion and anticoagulant activity

Binding activity of AnxB1ScuPA to platelet was determined by platelet adhesion assay described above. As shown in Fig. 4, both AnxB1ScuPA and AnxB1 displayed

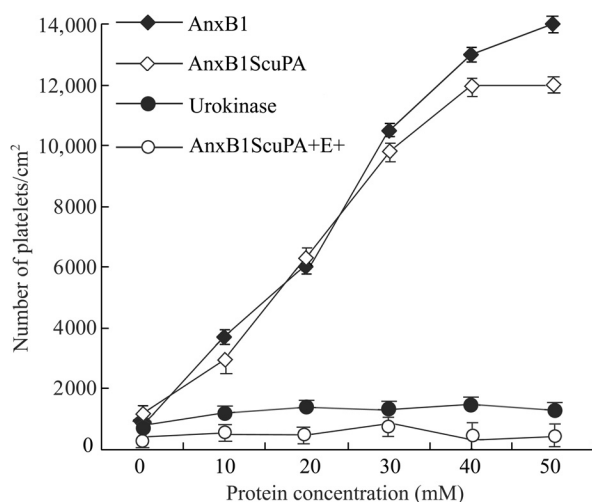


Fig. 4 Membrane-binding analysis of AnxB1ScuPA with activated-platelet

Error bars indicate the standard deviation of three separate experiments.

calcium dependent affinity for ADP activated platelets at a dose-dependent manner. However, AnxB1 was unable to bind inactivated platelets (in present 5 mM EDTA).

Anticoagulant activity of chimeric AnxB1ScuPA was assayed using kaolin partial thromboplastin time test. As shown in Fig. 5, AnxB1ScuPA prolonged KPTT time significantly at the dose of 30 and 40 mM ($P < 0.01$ vs. BSA control), similar to that of AnxB1 at the same dose. These results indicated that AnxB1ScuPA retained the calcium-dependent binding ability to activated-platelet and the anticoagulant activity of AnxB1 moiety.

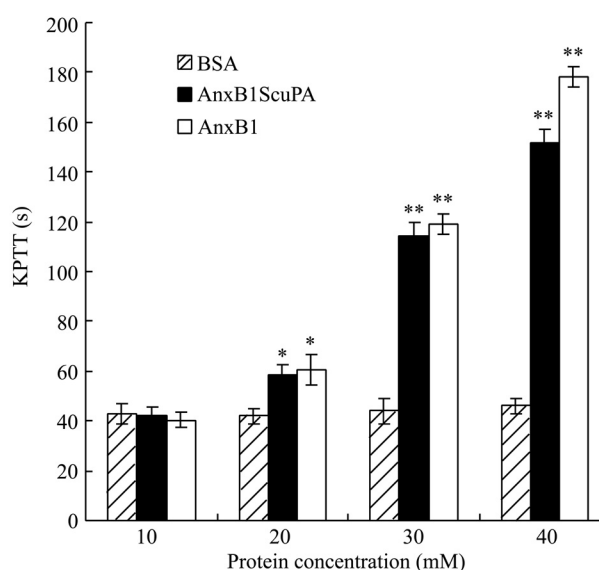


Fig. 5 Anticoagulant activity of AnxB1ScuPA

Error bars indicate the standard deviation of three separate experiments. ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. BSA.

Influence of AnxB1ScuPA on the reperfusion of coronary artery occlusion in dogs

Using coronary thrombosis of anaesthetized open chest canine model by electrical stimulation methods, the thrombolytic efficacy of intravenous AnxB1ScuPA was evaluated.

As it was shown in Table 2, the coronary blood flow in three out of six animals in UK group significantly increased, the reperfusion ratio is 50 percent and the reperfusion time is 72.7 ± 9.1 min. While in the groups of $2.5, 5.0, 10.0 \times 10^4$ IU \cdot kg⁻¹ AnxB1-ScuPA administration, the reperfusion time was $51.3 \pm 19.0, 45.0 \pm 16.8, 30.0 \pm 18.4$ min, and the reperfusion ratios were 67%, 100%, 100%, respectively. These results suggested that the reperfusion ratio and the blood flow of AnxB1ScuPA group increased significantly compared to UK group, and the average of reperfusion

Table 2 Influence of AnxB1ScuPA on the reperfusion of coronary artery occlusion in dogs

Group	Dose ($\times 10^4$ IU \cdot kg $^{-1}$)	<i>n</i>	Reperfusion ratio (%)	Reperfusion time
Control	–	6	0	>120
AnxB1ScuPA	2.25	6	67	51.3 \pm 19.0
AnxB1ScuPA	5.00	6	100	45.0 \pm 16.8*
AnxB1ScuPA	10.0	6	100	30.0 \pm 18.4
UK	5.00	6	50	72.7 \pm 9.1

Data were expressed as $\bar{x} \pm s$, * $P < 0.05$ compared with UK.

time shortened more than twenty minutes.

Influence of AnxB1ScuPA on the wound blood loss

As Table 3 showed, the blood loss of control group had no obvious change, and the blood loss increased during 30–120 min in UK group. After 2.25×10^4 IU \cdot kg $^{-1}$ AnxB1ScuPA administration, the blood loss remained the baseline, 5.0, 10.0×10^4 IU \cdot kg $^{-1}$, the blood loss also increased, mainly at 30, 60 min, but less than that of UK group.

Discussion

This study shows the feasibility of producing thrombolytic agents in which AnxB1 provides the thrombus-targeting component. To construct a chimera protein, the most important thing is how to retain the biological activity of the parent molecules. We have previously predicted the three-dimension structure of AnxB1 by homology modeling [14]. The structure model shows that C-terminus of AnxB1 is flexible and has little influence on its calcium-dependent phospholipids binding sites. Therefore, if AnxB1 was attached to N-terminus of ScuPA32k, theoretically, the two major functional units of the chimera are still able to act independently. Our results indicated that the presence of the AnxB1 moiety does not prevent the amidolytic activity of the ScuPA32k

moiety. Likewise, the attachment of the large ScuPA32k moiety at the C-terminus of AnxB1 does not alter its affinity for activated platelet membranes. This is consistent with the expectations used to design the chimera.

Expression of human prourokinase in *E. coli* is very low, because there are several rare codons such as arginine (AGA and AGG), isoleucine (AUA), leucine (CUA), and proline (CCC) in its coding sequence. The typical solutions for enhancing human prourokinase expression are time-consuming and tedious: synonymous replacement of rare codons with more frequently used codons or moving the gene of interest into a eukaryotic expression system. In present study, we transformed the recombinant plasmid to a new bacteria *E. coli* strain BL21-RIL. BL21-RIL cells carry extra copies of the *argU*, *ileY*, and *leuW* tRNA genes. The tRNAs encoded by these genes recognize the AGA/AGG, AUA, and CUA codons, respectively. Therefore, the expression level of AnxB1ScuPA was enhanced to 22% of total cell proteins.

Thrombi-targeting fibrinolytic agents can be grouped in two categories: those with affinity for activated platelets, and those with affinity for fibrin component of the thrombus. Agents of the first group could be suitable to resolve arterial thrombi, in which aggregated platelets are the major component [15]. A chemical conjugate of urokinase with human annexin A5, which targets activated platelets, showed enhanced fibrinolytic activity [1]. In the present study, the fact that the chimeric protein

Table 3 Influence of AnxB1ScuPA on the wound blood loss in dogs

Group	Dose ($\times 10^4$ IU \cdot kg $^{-1}$)	Before drug administration	After drug administration (min)		
			30	60	120
Control	–	52.3 \pm 16.8	53.2 \pm 18.3	43.7 \pm 15.6	53.2 \pm 17.7
AnxB1ScuPA	2.25	47.5 \pm 8.8	41.8 \pm 14.4	53.8 \pm 17.7	48.0 \pm 16.0
AnxB1ScuPA	5.00	45.7 \pm 11.5	77.2 \pm 17.1 ^{###**}	106.2 \pm 27.2 ^{#*}	66.3 \pm 29.7
AnxB1ScuPA	10.00	55.3 \pm 11.9	116.0 \pm 29.5 ^{##}	122.3 \pm 28.5 ^{##}	93.8 \pm 29.3 [#]
UK	5.00	48.3 \pm 17.3	154.8 \pm 35.1 ^{###}	156.5 \pm 33.7 ^{###}	98.7 \pm 25.7 [#]

The data were expressed as $\bar{x} \pm s$, the unit of blood loss is mg; ^{*} $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs. control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. UK group.

AnxB1ScuPA has a similar amidolytic activity to ScuPA32k *in vitro* might raise doubts about the practical value of such protein for potential clinical use. However, the *in vivo* test is encouraging: the reperfusion ratio and the blood flow of AnxB1ScuPA group increased significantly compared to UK group, and the average of reperfusion time shortened more than twenty minutes. More importantly, the blood loss is much less than the UK control. Reocclusion is another side effect of current thrombolytic agents, KPTT test indicated AnxB1ScuPA could inhibit the activation of coagulant factor Xa by shielding PS on the surface of the activated platelets and subsequently inhibit the thrombin generation. Therefore, AnxB1ScuPA might have the potential of anticoagulant activity *in vivo* and reducing the incidences of reocclusion. Further *in vitro* and *in vivo* studies are needed to evaluate the fibrin specificity, thrombolytic efficacy and system half-life of AnxB1ScuPA.

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