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Sustained thromboprophylaxis mediated by an RBC-targeted pro-urokinase zymogen activated at the site of clot formation

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Plasminogen activators (PAs) are used to treat life-threatening thrombosis, but not for thromboprophylaxis because of rapid clearance, risk of bleeding, and central nervous system (CNS) toxicity. We describe a novel strategy that may help to overcome these limitations by targeting a thrombin-activated PA pro-drug to circulating red blood cells (RBCs). We fused a single chain antibody (scFv Ter-119) that binds to mouse glycophorin A (GPA) with a variant human single-chain low molecular weight urokinase construct that can be activated selectively by thrombin (scFv/uPA-T). scFv/uPA-T bound specifically to mouse RBCs without altering their biocompatibility and retained its zymogenic properties until converted by thrombin into an active 2-chain molecule. As a result, RBC-bound scFv/uPA-T caused thrombin-induced fibrinolysis. One hour and 48 hours after intravenous (IV) injection in mice, approximately 70% and approximately 35% of scFv/uPA-T was retained in the blood, respectively, and approximately 95% of the circulating scFv/uPA-T remained bound to RBCs. A single IV injection of scFv/uPA-T provided effective prophylaxis against arterial and venous thrombosis for up to 24 hours. Thus, prophylactic delivery of RBC-targeted PA pro-drugs activated selectively at the site of clot formation represents a new approach to prevent thrombosis in clinical settings where the risk of clotting is high. (*Blood*. 2010;115(25): 5241-5248)

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Introduction

Plasminogen activators (PAs; tissue-type, tPA; urokinase, uPA) can provide urgent thrombolysis within a narrow therapeutic window of time in the setting of life- or limb-threatening thrombosis.[1,2] However, their efficacy is limited by plasma inhibitors (eg, PAI-1) and inadequate delivery into impermeable occlusive clots, a situation that is exacerbated by delays in initiating treatment. Endowing tPA derivatives with higher affinity for fibrin[3,4] further impairs clot permeation,[5] while increased dosing and constitutive lytic activity enhances the risk of bleeding and central nervous system (CNS) toxicity.

Coupling tPA to carrier red blood cells (RBCs) followed by re-infusion of the RBC/tPA conjugates in animals provides protracted thromboprophylaxis in arteries and veins, including the vulnerable cerebrovascular circulation.[6-8] RBC carriage prolongs the half-life of tPAs in the circulation from minutes to many hours and prevents drug extravasation and access to hemostatic plugs, thereby reducing the risk of bleeding episodes.[7] In the prophylactic setting where tPAs lacked efficacy but caused bleeding and CNS toxicity, RBCs/tPAs mediated timely reperfusion, reducing morbidity and mortality.[8]

Translation of RBC/PA thromboprophylaxis into the clinical domain could improve management of patients known to be at high risk of thrombosis or rethrombosis, including after acute myocardial infarction (AMI), transient ischemic attack, pulmonary embolism, angioplasty, and abdominal or other surgical procedures such as knee replacements, where the efficacy of thromboprophylaxis is low and/or the risk of bleeding is high. The goal of this study was to modify an existing prototypic approach (ex vivo coupling of PAs to RBCs followed by RBC/PA re-infusion) into a more clinically applicable approach to thromboprophylaxis. To anchor the injected PAs to circulating RBCs, we used a scFv fragment of the monoclonal antibody Ter-119 that recognizes mouse glycophorin A (GPA), an abundant RBC-specific surface molecule (~ 10^6 copies/RBC,[9,10] similar to its human homologue[11]). We fused scFv Ter-119 to a recombinant low molecular weight single chain uPA lacking the kringle and growth factor-like domains (lmw scuPA). Lmw scuPA is a zymogen that is naturally activated by plasmin, but does not interact with urokinase receptors on vascular cells.[12] To design a latent pro-drug more suitable for prophylaxis, we converted the plasmin cleavage site into a thrombin cleavage site (by deleting amino acids Phe^{157} and Lys^{158}).[13] In this work, we studied the activity of the RBC-targeted fusion protein scFv/uPA-T in vitro and in vivo, providing a proof-of-principle for the utility of such chimeric biotherapeutics in thromboprophylaxis.

Methods

The following reagents were used in our study: Fibrinogen (Enzyme Research Labs), thrombin (Calbiochem), Iodogen (Pierce), the Quick-Change Site-Directed Mutagenesis kit (Stratagene), *Drosophila* S2 cells,

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pMT/Bip/V5-His-A vector and Schneiders S2 cell medium (Invitrogen), *Drosophila* serum-free medium (SFM; Lonza), the polymerase chain reaction (PCR) core kit and Rapid DNA ligation kit (Roche), endonucleases (New England Biolabs), spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw-2-chain uPA (tcuPA) standard (American Diagnostica). Other chemicals were obtained from Sigma-Aldrich.

Proteins were labeled with Na[^{125}I] (Perkin-Elmer) using the standard Iodogen method. The free iodine was removed using a Bio-Spin 6 column (Bio-Rad Laboratory). RBCs were obtained from fresh anticoagulated mouse blood and labeled with [^{51}Cr]Cl_2 (Perkin-Elmer), as described.[6]

Construction and expression of anti-GPA scFv/uPA-T

We followed the template developed by us for fusing plasminogen activators with scFv's using a serine-rich linker peptide.[14,15] Ter-119 is a rat monoclonal antibody (mAb) to mouse GPA that has been characterized previously.[9] The pNscTDdSeY plasmid served as the source of the scFv Ter-119 cDNA sequence and has been described earlier.[10] Briefly, the variable heavy and light chain regions of Ter-119 were joined by PCR with a $(GGGGS)_3$ linker to assemble the scFv Ter-119. cDNA encoding anti-GPA scFv was amplified for cloning in the expression vector pMT/Bip/V5-HisA using the upstream primer 5′-cgt acg act agt cag gtg aag ctg cag gag tca gga gga ggc-3′, which introduces a restriction site for *SpeI* at the 5′ end, and the downstream primer 5′-ata aga atg cgg ccg cgc cgg aag agc tac tac ccg atg agg aag aag ccc gtt tca gtt cca gct tgg tcc c-3′, which appends the sequence of a short peptide linker $(SSSSG)_2$ and a *Not I* restriction site at 3′ end. cDNA encoding the C-terminal fragment of human single-chain urokinase plasminogen activator (scuPA) lacking the kringle and EGF-domains (ie, low molecular weight scuPA, lmw-scuPA), Leu^{144}-Leu^{411} was further modified by deleting Phe^{157} and Lys^{158} that converts lmw-scuPA from plasmin-activated into thrombin-activated zymogen indicated thereafter as uPA-T.[13,15] Briefly, cDNA encoding lmw-scuPA was amplified with the primers sen-uPA (5′-ata aga atg cgg ccg cat taa aat ttc agt gtg gcc-3′), which introduces a *Not*I restriction site at the 5′ end, and downstream rev-uPA (5′-ccg ctc gag tca gag ggc cag gcc att c-3′) to introduce an *Xho*I restriction site at the 3′ end.

Anti-GPA scFv/uPA-T (hereafter designated scFv/uPA-T unless specified otherwise) was assembled as follows: First, the 2 PCR products were purified and digested with *SpeI*, *NotI*, and *Xho*I, respectively. Second, the 2 digested fragments were ligated and cloned into the SpeI and *Xho*I sites of the vector pMT/Bip/V5-HisA. Successful cloning was confirmed by restriction analysis of plasmid and by automated sequencing.

We then established a stable drosophila cell line expressing scFv/uPA-T and nontargeted lmw-scuPA (indicated hereafter as uPA). The proteins were purified from cell media by affinity chromatography using an M2 (anti-flag) affinity column (Sigma-Aldrich) followed gel filtration chromatography on Sephacryl SHR100 (Amersham) to greater than 95% purity confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; data not shown), with a yield approximately 3 to 5 mg/L medium as described previously.[15] Proteins were concentrated in phosphate buffer to a level not to exceed 2 mg/mL, separated into aliquots and stored at -80°C until use.

Biochemical characterization of anti-GPA scFv/uPA-T

The size and homogeneity of the fusion protein preparations were analyzed using a 4% to 12% gradient SDS-PAGE and Western blotting (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). For Western blot analysis, the separated proteins were transferred to a nitrocellulose membrane (NitroBind; Osmonics) blocked with tris(hydroxymethyl)aminomethane (Tris)-buffered saline containing 5% nonfat milk powder and 0.1% Tween–20. A horseradish peroxidase (HRP) conjugated monoclonal antibody against flag tag (Sigma-Aldrich) served as the primary antibody and the antigen-antibody complex was detected with ECL Plus (Amersham Biosciences).

The intrinsic and thrombin-induced plasminogen activator activities of scFv/uPA-T were measured using casein zymography. Aliquots of serum free media (SFM) from scFv/uPA-T–expressing cells, intact or pre-incubated with 20nM thrombin, were mixed with nonreducing Tris-glycine SDS sample buffer before zymography. The samples were resolved on a 7.5% gel cast with 1% nonfat dry milk and 20 μg/mL plasminogen to detect PA activity.[16] Thereafter, the gels were renatured in Novex Zymogram Renaturating buffer (Invitrogen) and developed in Novex Zymogram Developing buffer (Invitrogen) per the manufacturer. EDTA (5mM) was used to block potential metalloproteinase activity. Gels were stained with GelCode Blue stain (Pierce). Gels run in parallel in the absence of added plasminogen served as a control for PA activity (not shown). Recombinant human tPA (Alteplase; Genentec) served as a positive control. This assay confirmed the induction of plasminogen conversion activity of scFv/uPA-T by thrombin (supplemental Figure 1B).

The specificity of scFv/uPA-T binding to RBC was tested by immunocapture and Western blotting (WB). Mouse and rat RBC ghosts prepared as described[17] were incubated for 1 hour with SFM from the induced S2 cells expressing scFv/uPA-T, washed 3 times with PBS, and lysed in a sample buffer (Invitrogen). The lysates (normalized for total protein) were separated on 4% to 12% SDS-PAGE under nonreducing conditions. WB analysis to detect captured scFv/uPA-T was performed as described for supplemental Figure 1A. An aliquot of the SFM from the induced S2 cells expressing scFv/uPA-T served as a positive control to detect the fusion protein. Results of this analysis showed that scFv/uPA-T bound to mouse, but not to rat, RBC (supplemental Figure 1C) or to human RBC (data not shown), which served thereafter as the negative control.

Binding of anti-GPA scFv/uPA-T to the RBC membrane

We measured the binding of ^{125}I- scFv/uPA-T to mouse (target cells) versus human (negative control) RBCs as previously described.[18] Briefly, RBCs were washed by centrifugation (1200*g*) with PBS/3% BSA, resuspended in the same buffer to a 1% or 10% hematocrit and incubated with several concentrations of ^{125}I-scFv/uPA-T for 1 hour at 37°C (loading) with gentle rotation. Unbound reagent was eliminated by washing the RBC 4 times with a 20-fold volume of PBS-BSA. The residual radioactivity in the RBC pellets was measured in a γ-counter (Perkin Elmer).

We tested whether anti-GPA scFv/uPA-T caused RBC aggregation in V-shaped plates.[19] Aliquots of mouse RBC suspension were transferred to a 96 well V-shaped plate (Corning) wells (1% hematocrit, 100 μL per well), incubated for 1 hour with increasing concentrations of scFv/uPA-T or parental scFv antibody Ter119 (eBioscience), and the wells were inspected for aggregation.

Thrombin activation of scFv/uPA-T and in vitro fibrinolysis

To measure activation of the fusion protein by thrombin, a 150nM solution of scFv/uPA-T was incubated with 25nM thrombin ranging from 5 minutes to 2 hours, the thrombin was quenched with hirudin (33 U/mL), and uPA activity was measured using Spectrozyme UK as a substrate at a wavelength of λ405 nm, as described.[15] A standard curve was generated with varying concentrations of lmw-tcuPA (American Diagnostica).

The fibrinolytic activity of RBC-bound scFv/uPA-T was measured using ^{125}I-labeled fibrin clots, as described.[6] RBCs were incubated with SFM from the induced S2 cells expressing scFv/uPA-T in the absence of or after adding 20nM thrombin for 1 hour, washed 3 times with PBS and added to a solution of bovine fibrinogen (3 mg/mL in PBS) containing trace labeled ^{125}I-fibrinogen. Clotting was induced by addition of $CaCl_2$ and thrombin (20mM and 0.2 units/mL final concentrations, respectively). The clots were then overlaid with 200 μL of PBS, incubated at 37°C, and the radioactivity in the supernatants was measured.

To compare the fibrinolytic activity of free versus RBC-bound scFv/uPA-T, mouse RBCs were sensitized with approximately 4×10^4 molecules of purified unlabeled scFv/uPA-T/RBCs as determined in a parallel experiments using ^{125}I-scFv/uPA-T. Equimolar amounts of free versus RBC-bound scFv/uPA-T were added to citrated human platelet pure plasma (PPP) containing ^{125}I-labeled fibrinogen and clots were formed by addition of calcium and thrombin as described in the previous paragraph. To help maintain the initial clot structure, the same number of human RBCs was added to the PPP containing free scFv/uPA-T. The clots were then overlaid with 200 μL of PBS, incubated at 37°C, and the radioactivity in the supernatants was measured.

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In vivo tracing of RBCs and RBC-anchored anti-GPA scFv/uPA-T

Adult C57BL/6 mice (The Jackson Laboratory) were studied using protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Washed RBCs obtained from fresh anticoagulated mouse blood were labeled with ^{51}Cr, as described previously.[18] ^{51}Cr-RBCs were injected into anesthetized mice via the jugular vein. Animals were then immediately injected with scFv/uPA-T (4 mg/kg, the dose that lead to binding of $\sim 4 \times 10^4$ scFv/uPA-T copies per RBC) via the jugular vein. At predesignated times, aliquots of blood were withdrawn in heparin, the animals were killed, and the radioactivity in the blood and organs was measured. In other experiments, ^{125}I-labeled scFv/uPA-T or ^{125}I-uPA (3-5 μg) was injected into anesthetized mice via the jugular vein. At the same predesignated times, 100 to 200 μL of blood was withdrawn in heparin, centrifuged at 1200*g*, and the radioactivity in the plasma and RBC pellets was measured. The animals were killed and the radioactivity in the organs was measured.

Fibrinolytic activity of anti-GPA scFv/uPA-T in vivo

We followed the protocol described previously for a prototype RBC/tPA conjugate.[7] Briefly, equimolar amounts of scFv/uPA-T or uPA that gave an initial blood concentration of approximately 0.95μM (2 mg/kg uPA or 4 mg/kg scFv/uPA-T) were injected in 200 μL of saline vehicle into anesthetized mice via the jugular vein (drug-free saline was injected as a placebo control). At predesignated times, 100-μL aliquots of blood were withdrawn in the absence of anticoagulant, mixed rapidly with tracer ^{125}I-fibrinogen and allowed to clot in borosilicate tubes at 20°C. After 20 minutes of maturation ex vivo, clots were overlaid with saline and incubated at 37°C for 24 hours and the release of ^{125}I was measured.

Effect of anti-GPA scFv/uPA-T in a mouse model of vascular thrombosis

To test thromboprophylaxis, we used a mouse model of acute thrombotic occlusion in response to vascular injury described previously.[6] Equimolar amounts of scFv/uPA-T, uPA, or saline were injected into anesthetized mice via the jugular vein. At a prespecified time, thrombosis was induced in the exposed contralateral jugular vein or carotid artery by applying a 1 × 2-mm piece of filter paper (Whatman No. 1) saturated with 15% $FeCl_3$ to the adventitia for 2 minutes. Time to vascular occlusion and total blood flow over the ensuing 30 minutes was measured by Doppler ultrasound using a 0.5VB flow probe connected to a recording system (Transonic Systems). In these studies, 0.95μM scFv/uPA-T or uPA was injected 1 hour before inducing carotid artery thrombosis, whereas 0.35μM and 0.95μM of these agents were injected 1 and 24 hours before jugular vein injury.

Data analysis

All data are presented as the mean plus or minus SEM of at least 3 separate experiments. Differences between groups were tested for statistical significance using Student *t* test or analysis of variance (ANOVA). Statistical significance was set at *P* less than .05.

Results

Binding of thrombin-inducible latent anti-GPA scFv/uPA-T fusion protein to target RBC

Figure 1A shows the design of the cDNA encoding a plasmin-resistant, thrombin-activated scFv/uPA-T targeted to mouse GPA. S2 cells transfected with this construct secrete a protein that migrates as a single band (MW ~ 61 kDa) on Western blotting (supplemental Figure 1A), expresses thrombin-inducible plasminogen activator activity detectable by zymography (supplemental Figure 1B), and binds to mouse, but not to rat or human, RBC membranes (supplemental Figure 1C).

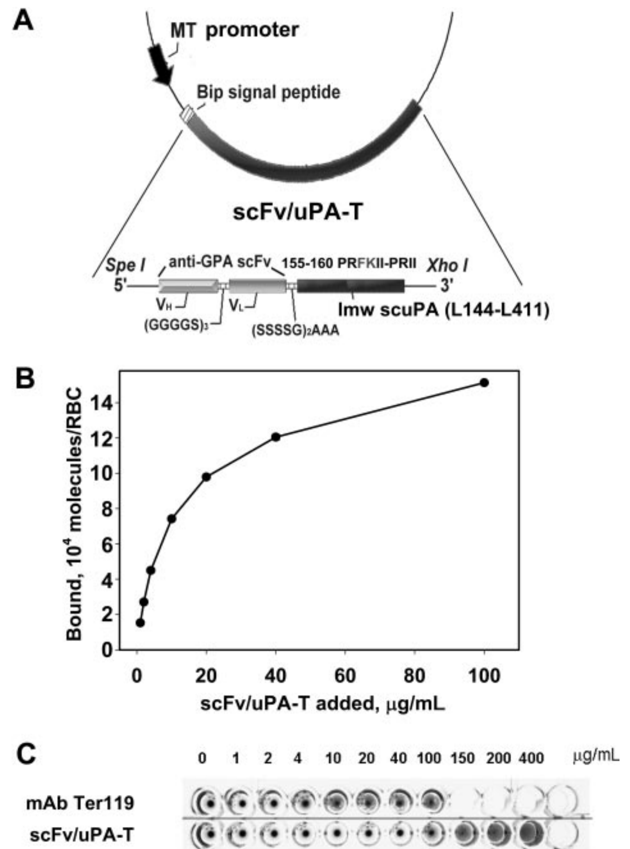

Figure 1. Molecular design and specific binding of scFv/uPA-T fusion protein to the mouse RBC. (A) Schematic diagram describing the cloning strategy for the fusion construct scFv/uPA-T. The final construct contains a triple FLAG tag at the C-terminus introduced for purification purposes (not shown). (B) Binding of ^{125}I-scFv/uPA-T fusion protein to a 1% suspension of washed mouse RBCs. Unless indicated otherwise, data in figures are shown as mean plus or minus SEM (n = 3, deviation bars are smaller than symbols). (C) Mouse RBC agglutination visualized in a V-shaped plate by incubating a 1% suspension of mRBC with indicated concentrations of either monovalent scFv/uPA-T fusion protein or bivalent anti-GPA mAb Ter119. Sharp dots at the bottom of the V-shaped well indicate absence of RBC aggregation. Horizontal lines have been inserted to indicate repositioned images.

Purified ^{125}I-labeled scFv/uPA-T bound to mouse RBCs in a dose-dependent manner, reaching a level of 1.4×10^5 molecules per RBC at the maximal concentration used (100 μg/mL; Figure 1B), whereas fewer than 100 copies bound per human RBC at this dose (data not shown). Monovalent scFv/uPA-T did not cause mouse RBCs to aggregate at the maximal concentration, whereas the bivalent parental mAb TER-119 caused RBC aggregation at concentrations as low as 4 μg/mL (Figure 1C).

Fibrinolytic activity of RBC-bound scFv/uPA-T

Next, we tested the fibrinolytic properties of RBC-bound scFv/uPA-T (Figure 2A). Mouse RBCs pre-incubated with scFv/uPA-T and thrombin lysed fibrin clots within 30 minutes. In the absence of preactivation by thrombin, mouse RBC-bound scFv/uPA-T also lysed clots, but within 60 minutes, indicating that thrombin required to initiate clotting is also capable to activate the fusion protein. Human RBCs preincubated with scFv/uPA-T caused no fibrinolysis whether or not thrombin was present, indicating that specific anchoring to RBCs is necessary (Figure 2A). The half-maximal activity of scFv/uPA-T was attained within 15 minutes of incubation with thrombin, while unactivated scFv/uPA-T conferred only background activity (Figure 2B).

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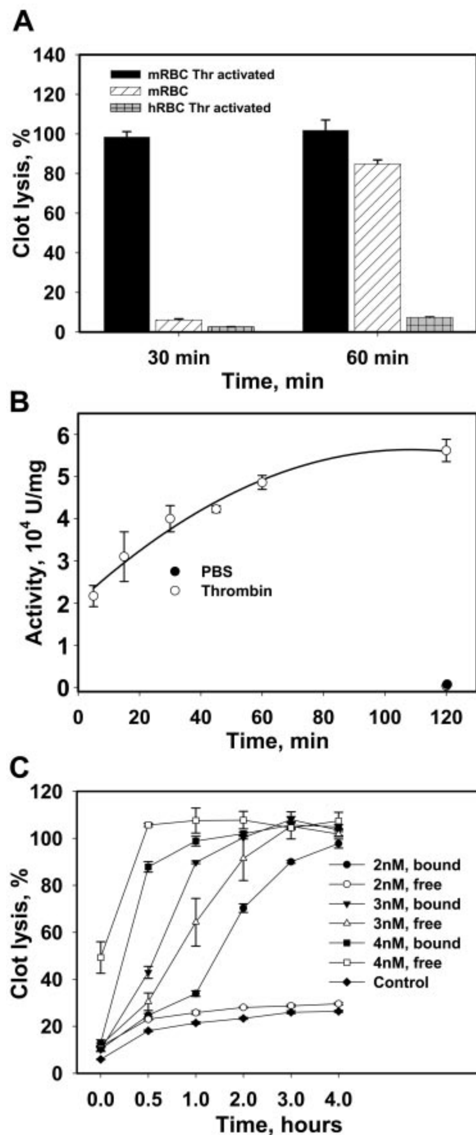

Figure 2. Thrombin activation and fibrinolytic activity of RBC-bound and soluble scFv/uPA-T in vitro. (A) Lysis of ^{125}I-labeled fibrin clots by mouse or human RBCs (mRBCs; hRBCs) preincubated for 1 hour in medium containing scFv/uPA-T, with or without activation by thrombin (n = 3). (B) Kinetics of scFv/uPA-T activation by thrombin. ● shows nonactivated scFv/uPA-T. (C) Fibrinolytic activity of RBC-bound or free scFv/uPA-T at the indicated concentrations using clots prepared from PPP. Clot lysis was monitored for 0 to 4 hours (n = 3).

To compare the fibrinolytic capacity, RBC-bound or free scFv/uPA-T that delivered 2, 3, or 4nM fusion protein were added to citrated human PPP containing ^{125}I-fibrinogen before clotting and lysis was measured as described above (Figure 2C). At the highest concentration tested (apparently exceeding the potency of plasma inhibitors), free scFv/uPA-T caused more extensive clot lysis than the RBC-bound fusion protein. However, at the lower drug dose, RBC-bound scFv/uPA-T caused greater lysis and at the lowest dose only RBC-bound scFv/uPA-T was active.

Binding of scFv/uPA-T to the RBC in vivo prolongs its circulatory half-life and confers fibrinolytic activity without damaging the target cells

Next, we tested the effect of scFv/uPA-T ($\sim 4 \times 10^4$ copies/cell) on ^{51}Cr-RBC biocompatibility. The amount of ^{51}Cr in the blood and major organs was nearly identical 1 hour (supplemental Figure 3) or 6 hours (Figure 3A) after injection of scFv/uPA-T or PBS, respectively. Only minimal amounts of ^{51}Cr were detected in plasma, while nearly 100% of the radioactivity was found in the RBC pellet, indicating that the fusion protein did not cause hemolysis (Figure 3A and supplemental Figure 3 insets). Thus, scFv/uPA-T does not damage the target cells, nor cause them to aggregate in a manner that would result in accelerated clearance by the reticuloendothelial system or retention in capillary-rich organs such as the lungs.

The next series of experiments was designed to assess the biodistribution and pharmacokinetics of scFv/uPA-T (Figure 3B). When ^{125}Iodine-labeled scFv/uPA-T was injected intravenously into mice, most of the fusion protein was detected in the blood with rather minor fractions detected in the spleen, kidneys, and lungs. A significant but minor ($< 25\%$) fraction of injected scFv/uPA-T was also found in the liver within 1 hour of injection, likely reflecting uptake of unbound drug (Figure 3B). However, scFv/uPA-T anchorage to RBCs was tight with more than 95% of the fusion protein in blood associated with the circulating RBCs throughout the study (Figure 3B inset).

As a result of binding to circulating RBCs, more than 70% of ^{125}I-labeled scFv/uPA-T was found in the blood 30 minutes after injection, after which it decreased slowly over time (Figure 4A). In fact, approximately 35% of the initially injected dose remained in the blood 2 days after injection. In contrast, only approximately 5% of nontargeted uPA was detected in blood 30 minutes after intravenous injection, in agreement with published data (Figure 4A closed symbol in bottom left corner and inset).

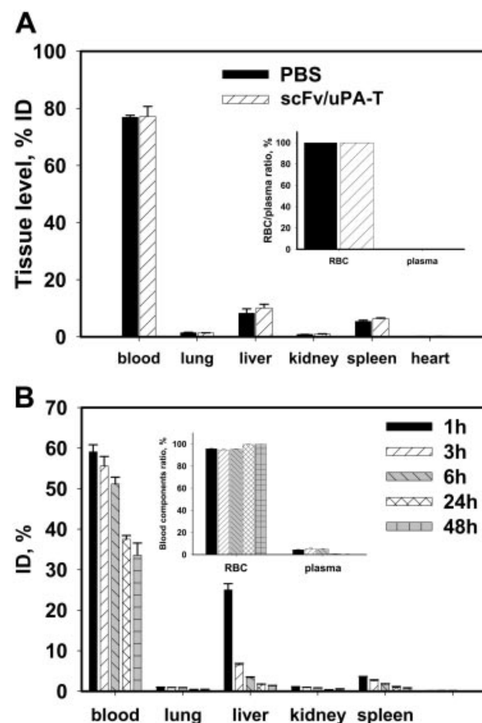

Figure 3. Effect of scFv/uPA-T on circulating RBCs and its organ distribution in vivo. (A) Distribution of radioactivity in mice after administration of ^{51}Cr-labeled mouse RBCs followed by the intravenous injection of 4mg/kg scFv/uPA-T or PBS. The animals were killed 1 hour (supplemental Figure 3) or 6 hours (panel A) later and ^{51}Cr was measured in the blood and major peripheral organs (n = 4). The inset indicates ^{51}Cr distribution between blood plasma and cells at the indicated times. (B) Distribution of ^{125}I-labeled scFv/uPA-T in organs at the indicated times after intravenous injection in mice. Inset: Distribution of ^{125}I-scFv/uPA-T between blood plasma and cells (n = 5).

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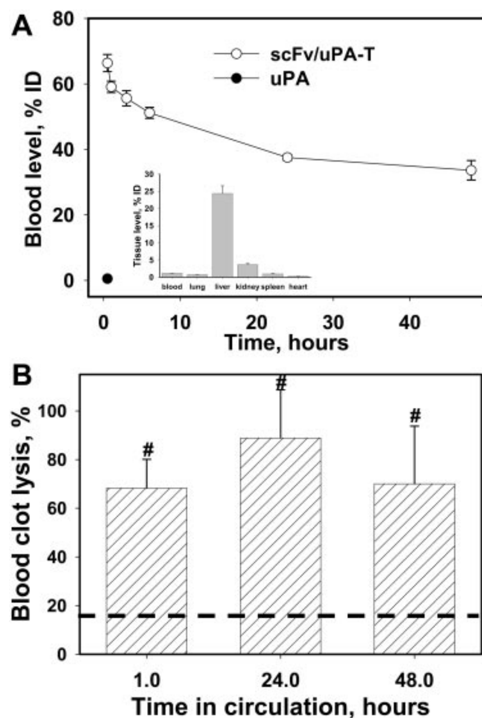

Figure 4. Pharmacokinetics and fibrinolytic activity of scFv/uPA-T fusion protein and soluble uPA in mice. (A) Blood clearance of ^{125}I-scFv/uPA-T after intravenous injection. Inset: Distribution of ^{125}I-uPA 1 hour after injection. (B) Spontaneous lysis of clots formed from blood obtained after intravenous injection of scFv/uPA-T (4 mg/kg, 0.95μM initial concentration in blood, ▨) or PBS (- - -). N = 4 per group; $P < .05$ versus saline injected control.

We then examined the duration of the fibrinolytic activity of RBC-bound scFv/uPA-T fibrinolytic activity in the blood using an ex vivo clot lysis protocol (Figure 4B). Lysis of clots formed from blood collected 1 hour after uPA injection (19% ± 1%) was indistinguishable from spontaneous lysis seen in clots formed from mice injected with PBS (dashed line in Figure 4B). In contrast, blood clots from mice injected with scFv/uPA-T lysed almost completely, even 48 hours after the initial injection (Figure 4B).

Prophylactic thrombolysis by scFv/uPA-T

We next injected equimolar doses of uPA and scFv/uPA-T into normal mice 1 hour before inducing occlusive thrombi in the carotid artery. Prophylactic injection of uPA had no effect on thrombus formation or duration of occlusion (Figure 5B) compared with the PBS-treated controls (Figure 5A). In contrast, all mice pretreated with scFv/uPA-T were protected from forming occlusive arterial clots (Figure 5C). We observed a temporary reduction of perfusion in 2 of 6 scFv/uPA-T–treated mice, but these clots lysed shortly after formation resulting in a nearly normal blood flow (eg, see Figure 5D). When the blood flow determined by Doppler was analyzed by measuring the area under the curve, animals given scFv/uPA-T retained 65% (± 9%) of normal blood flow compared with only 13% (± 2%) in the uPA-treated animals ($P < .05$; Figure 5E).

Lastly, we examined the impact of scFv/PA-T in a model of jugular venous thrombosis (Figure 6). In contrast to arterial ("white") thrombi, which are heavily populated by platelets, venous ("red") clots are predominantly composed of fibrin and RBCs. In theory, this should render them particularly amenable to thromboprophylaxis by RBC-bound drugs. The fusion protein was injected either 1 or 24 hours before causing thrombosis. In this model, scFv/uPA-T either completely prevented vascular occlusion (6 of 9 and 4 of 9 animals at 1 hour and 24 hours after treatment, respectively) or caused rapid reperfusion (3 of 9 and 5 from 9 animals at 1 hour and 24 hours after treatment, respectively), whereas uPA failed to produce reperfusion at either time point in any animal (Figure 6). Analysis of the areas under the curve of Doppler measurements of blood perfusion showed 19% (± 3%) or 29% (± 3%) of preinjury blood flow in mice injected with uPA 1 or 24 hours before injury, respectively. Therefore, nontargeted uPA provided no benefit over placebo control (21% ± 2% of initial blood flow in PBS-injected mice). In contrast, scFv/uPA-T injected 1 or 24 hours before induction of vessel injury conserved 64% (± 7%) or 63% (± 3%) of initial blood flow, respectively ($P < .05$).

Discussion

Short-term prophylactic thrombolysis might improve the management of patients at high risk of thrombosis if it were safe and effective, for example, in patients with unstable angina and non-ST segment elevation acute myocardial infarction (NSTEMI), which account for 1 million hospitalizations annually in the US alone, with a mortality rate and incidence of recurrent AMI estimated at 8% to 16% within the first month. The pathogenesis of NSTEMI is thought to involve repetitive cycles of rethrombosis, involving incomplete clot lysis and redevelopment of occlusive coronary thrombi within a time interval ranging from few hours to few days in some patients. Percutaneous coronary intervention and stenting combined with antiplatelet therapy and heparin have improved

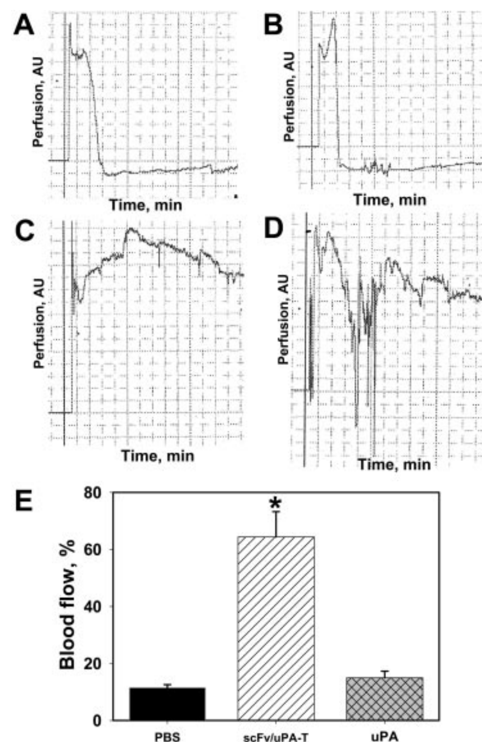

Figure 5. Prophylactic thrombolysis by scFv/uPA-T in arterial thrombosis. Panels A (saline), B (uPA), C, and D (scFv/uPA-T) show typical tracings of blood flow in the $FeCl_3$ model of carotid artery injury. Each square on the time axis equals 200 seconds. Panel E shows analysis of the data collected from each group presented as percent of the pre-injury level of blood flow in the carotid artery during the first 30 minutes after induction of injury in mice treated with uPA or scFv/uPA-T 1 hour before injury. N = 6 per group, $P < .05$.

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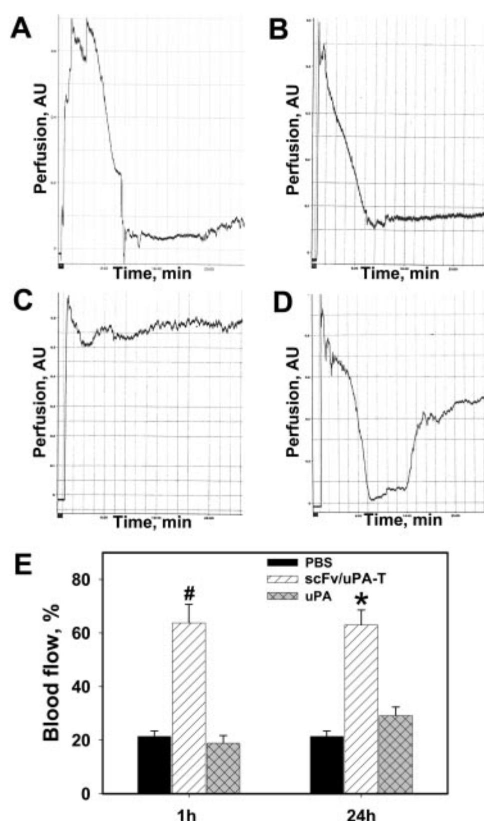

Figure 6. Prophylactic thrombolysis by scFv/uPA-T in venous thrombosis. Saline (A), untargeted uPA (B), or an equimolar dose of scFv/uPA-T (C-D) was injected intravenously in the jugular vein 1 or 24 hours before injury to the contralateral jugular vein by application of $FeCl_3$. Explanations are as in Figure 5. Panel E shows analysis of the data collected from each group presented as percent of pre-injury level of the blood flow in the jugular vein during the first 30 minutes after induction of injury in mice pretreated with uPA or scFv/uPA-T 1 or 24 hours before the injury. In panel E, n = 9 per group, $P < .05$.

survival marginally in selected subpopulations, but these approaches still do not provide protection to a substantial number of patients.[20]

Existing fibrinolytics are unsuitable for prophylactic use (half-life in blood < 20 minutes) and may cause intracranial bleeding, bleeding at sites of surgery and collateral damage in the CNS. However, coupling to RBC converts fibrinolytics into safe thromboprophylactic agents: RBC carriage restricts access of tPA to the CNS and into hemostatic clots,[8,18] while prolonged circulation allows RBC-bound tPA to enter and rapidly dissolve newly formed, potentially occlusive clots from within.[6] Infusion of RBC-bound tPA in mice, rats and pigs has been shown to prevent thrombotic occlusion in the peripheral and cerebral vasculature, without the hemorrhagic and CNS toxicities seen with soluble nontargeted tPA.[6-8,21-23]

Two major obstacles must be overcome to translate this new approach into preclinical and clinical development. First, for use as thromboprophylaxis, the respective agent should enjoy a prolonged circulation as a latent pro-drug and then be activated only when and where thrombi begin to form. Our choice of uPA in lieu of tPA as a fibrinolytic in this study was based in part on the fact that both single-chain and 2-chain tPA are constitutively active[24,25] and therefore are never entirely "latent," whereas single-chain uPA remains a zymogen until it is converted into a 2-chain molecule, in which newly formed N-terminal Ile^{159} is followed by exposure of the catalytic Ser^{356}-Asp^{255}-His^{204} triad.[26-30] Second, loading of fibrinolytics onto circulating RBCs attainable by simple injection of a uniform biotherapeutic is needed to circumvent the need for ex vivo loading and reinfusion of isolated RBC preparations. In the current study, we describe a novel path to attain both of these goals by fusing a mutant form of pro-urokinase that is activated by thrombin with a scFv that targets this zymogen to RBC. This targeting concept has been studied previously in the context of inhibiting unwanted complement activation in vitro[31] and in vivo.[10,32]

Single-chain uPA (scuPA) is a 54 kDa protein with negligible constitutive protease activity.[33,34] Proteolytic cleavage by metalloproteinase MMP-3 between Glu^{143} and Leu^{144} yields an approximately 30-kDa low-molecular weight zymogen (lmw-scuPA).[35] An advantage of lmw-scuPA as a prospective drug is that it lacks the growth factor and the kringle domains that binds to widely expressed CD87 (uPAR) and other cell surface receptors that may mediate some of its notable side effects.[36-38] Cleavage of scuPA or lmw-scuPA by plasmin between Lys^{158} and Ile^{159} yields disulfide-linked, active 2-chain disulfide-linked molecules (tcuPA and lmw tcuPA,[39,40] respectively) that convert plasminogen into plasmin. Yet, both scuPA and lmw-scuPA are poorly suited for thromboprophylaxis because of their short half-life, inactivation in the circulation by plasminogen activator inhibitor-1 (PAI-1)[41] and, at the site of active thrombosis, inactivation by thrombin-mediated cleavage between Arg^{156} and Phe.[157] Thus, the latter problem might be circumvented by deleting 2 critically important amino acids from scuPA (Phe^{157} and Lys^{158}), which yields uPA-T, a plasmin-resistant mutant that is instead activated by thrombin.[13] The latter mutant might be better suited for use as thromboprophylaxis as a potential candidate for targeting to carrier RBCs in clinical settings.

We fused lmw-scuPA-T with an anti-GPA scFv (Figure 1), producing a novel zymogen molecule, scFv/uPA-T that binds to mouse RBCs, thereby endowing the cells with thrombin-inducible fibrinolytic activity (Figure 2). Thrombin both initiates clotting and activates RBC-bound scFv/uPA-T, which dissolves freshly formed clots (Figure 2A). Of note, RBC-bound scFv/uPA-T causes more extensive lysis of plasma clots than free scFv/uPA-T at rate-limiting doses notwithstanding theoretical diffusional limitations imposed by RBCs binding on the interaction of scFv/uPA-T with plasminogen (Figure 2C). We attribute this result to protection against plasma inhibitors by the RBC glycocalyx, similar to our findings with tPA chemically coupled to RBCs.[22] scFv/uPA-T binds to RBCs with high affinity in vivo, does not overtly cause cell damage (Figure 3) and circulates as an activatable pro-drug for a duration that is several orders of magnitude longer than untargeted uPA (Figure 4). RBC-targeted scFv/uPA-T, but not untargeted uPA, preserved blood flow not only in a model of venous, but also arterial thrombosis, characterized by high shear stress and precipitous activation of platelets as well as of coagulation (Figures 5–6).

This work represents a preclinical proof-of-principle study including initial biochemical and pharmacokinetic characterization of a new agent compared with its currently used counterpart, uPA. Because scFv/uPA-T showed clear advantages over uPA, additional studies appear warranted to define the mechanistic basis for this outcome. Several factors may contribute to the improved performance of scFv/uPA-T including: thrombin-mediated activation of uPA-T in place of thrombin-mediated inactivation of uPA, high affinity of uPA-T for RBC prolonging its circulation, protection against plasma inhibitors by RBC glycocalyx, and a distinct mechanism of clot lysis that resists hydrodynamic forces and generates larger pores permitting

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permeation by oxygen carrying RBC.[42] Additional studies will be needed to delineate the specific contribution of altering the enzymatic activation site.

Taken together, the results of this study support the concept of generating RBC-targeted thromboprophylactic fusion proteins suitable for preclinical development and testing. Recombinant expression in diverse vectors enables large-scale, GMP-quality production of homogeneous monovalent scFv/PA fusion proteins.[1,43] Advantages of the scFv-mediated targeting approach include: (1) lack of Fc-mediated side effects; (2) absence of antigen cross-linking and RBC aggregation; (3) established techniques for humanization and reduction of immunogenicity of scFv; and (4) modular design in which the scFv domain is interchangeable and can be replaced with other specificities, allowing the synthesis of targeted variants of antithrombotic biotherapeutics tailored to specific clinical needs. For example, molecular modifications of the scFv portion can provide a series of fusion constructs with variable affinities for RBCs, thereby providing formulations with predictable circulation time and duration of thromboprophylaxis, whereas molecular remodeling of the fibrinolytic moiety of the fusion constructs would allow for additional optimization strategies and/or alterations of the activation specificities (eg, plasminogen vs other substrates). Design of a recombinant mutant pro-urokinase targeted to the RBC that lacks "nontherapeutic" domains and is activated by thrombin instead of plasmin, as described in this study, illustrates the potential versatility of this new treatment strategy to prevent pathologic clot formation.

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Authorship

Contribution: S.Z., M.P., D.B.C., and V.R.M. designed the study; D.S. and J.P.A. contributed vital new reagents; S.Z., J.-C.M., B.-S.D., S.T., M.A.K., O.A.M.-C., A.K., and V.S. performed the experiments; S.Z., D.S., J.-C.M., S.T., J.P.A., M.P., D.B.C., and V.R.M. analyzed the data; and S.Z., D.S., J.P.A., M.P., D.B.C., and V.R.M. wrote the paper.

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