Towards Effective and Safe Thrombolysis and Thromboprophylaxis

Preclinical Testing of a Novel Antibody-Targeted Recombinant Plasminogen Activator Directed Against Activated Platelets

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Rationale: Fibrinolysis is a valuable alternative for the treatment of myocardial infarction when percutaneous coronary intervention is not available in a timely fashion. For acute ischemic stroke, fibrinolysis is the only treatment option with a very narrow therapeutic window. Clinically approved thrombolytics have significant drawbacks, including bleeding complications. Thus their use is highly restricted, leaving many patients untreated.

Objective: We developed a novel targeted fibrinolytic drug that is directed against activated platelets.

Methods and Results: We fused single-chain urokinase plasminogen activator (scuPA) to a small recombinant antibody (scFvSCE5), which targets the activated form of the platelet-integrin glycoprotein IIb/IIIa. Antibody binding and scuPA activity of this recombinant fusion protein were on par with the parent molecules. Prophylactic in vivo administration of scFvSCE5–scuPA (75 U/g body weight) prevented carotid artery occlusion after ferric chloride injury in a plasminogen-dependent process compared with saline (P<0.001), and blood flow recovery was similar to high-dose nontargeted urokinase (500 U/g body weight). Tail bleeding time was significantly prolonged with this high dose of nontargeted urokinase, but not with equally effective targeted scFvSCE5–scuPA at 75 U/g body weight. Real-time in vivo molecular ultrasound imaging demonstrates significant therapeutic reduction of thrombus size after administration of 75 U/g body weight scFvSCE5–scuPA as compared with the same dose of a mutated, nontargeting scFv–scuPA or vehicle. The ability of scFvSCE5–scuPA to lyse thrombi was lost in plasminogen-deficient mice, but could be restored by intravenous injection of plasminogen.

Conclusions: Targeting of scuPA to activated glycoprotein IIb/IIIa allows effective thrombolysis and the potential novel use as a fibrinolytic agent for thromboprophylaxis without bleeding complications. (Circ Res. 2014;114:1083-1093.)

Key Words: blood platelets ■ thrombolytic therapy ■ thrombosis ■ urokinase-type plasminogen activator

Acutelyoccurringthrombosiscausingvesselocclusionandresulting

in ischemic complications, such as myocardial infarction and stroke, is a major cause of death and disability.1 Atherosclerosis, as the main underlying disease, is a progressive inflammatory process caused by the accumulation of lipids and lipid-loaded macrophages in the artery wall along with the adherence of activated platelets and monocytes.2,3 On rupture of atherosclerotic plaques, thrombogenic material is exposed, which leads to platelet activation, aggregation, and vessel occlusion.

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Restoring and maintaining perfusion to the affected tissue is crucial, and treatment of acute thrombosis using plasminogen activators (PAs, such as streptokinase), tissue plasminogen activators (tPA), and urokinase plasminogen activators (uPAs) has been proven to be beneficial.4 However, the clinical utility of these agents has been limited by side effects such as bleeding complications through lysis of hemostatic clots and

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neurotoxicity in the case of stroke treatment with tPA.5,6 In addition, thrombolitics are rapidly neutralized, and their large size hampers clot penetration, causing a delay in restoring perfusion.2

Further research is warranted to improve the safety and efficacy of fibrinolytic therapy. Antibodies as tools to deliver thrombolytic drugs specifically to developing and existing clots are a promising alternative for locally enhanced potency and reduced side effects.

Platelets play a key role in thrombus formation1 and are, therefore, a target of interest for combination with thrombolytic therapy. Targeting the fibrinogen receptor glycoprotein (GP) IIb/IIIa on platelets has been extensively studied for the prevention of platelet aggregation and has led to the reduction of ischemic complications,8–10 especially in patients undergoing percutaneous coronary intervention. However, in large clinical trials, contradictory to its anticipated therapeutic effects, the administration of GPIIb/IIIa receptor blockers in combination with fibrinolytic agents has shown little improvement in mortality, mainly because of excess bleeding.11,12 Limiting the broader utilization of the combination of GPIIb/IIIa inhibition and fibrinolysis. This can be, in part, attributed to the fact that all currently available GPIIb/IIIa inhibitors target the receptor regardless of the activation status, thereby causing complete systemic inhibition of platelet aggregation and firm adhesion. In addition, the ligand mimetic properties of clinically used GPIIb/IIIa inhibitors also can lead to paradoxical platelet activation imitating ligand-induced outside-in signaling.13,14 We have previously addressed these issues by generating conformation-specific small recombinant single-chain antibody fragments (scFv) that only target the activated GPIIb/IIIa receptor on platelets, avoiding outside-in signaling and allowing effective platelet inhibition without prolonging bleeding time.15,16 The potential of these scFvs to be used as targeting tools has been demonstrated by their use in noninvasive diagnostic molecular imaging of thrombosis and inflammation.17–19

Here, we use the targeting potential of a specific single-chain antibody SCE5, which selectively binds activated GPIIb/IIIa, to enrich the plasminogen activator scuPA at the site of platelet activation. We describe the recombinant fusion of this scFv to scuPA and the in vitro and in vivo testing of this novel targeted drug. This therapeutic approach facilitates highly effective thrombolysis without prolonging bleeding time. In addition, the described ability to inhibit thrombus formation together with the effective targeting at low systemic concentration could potentially allow the unique application of a fibrinolytic drug in a thromboprophylactic approach.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>GP</td>
<td>glycoprotein</td>
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<tr>
<td>LIBS-MBs</td>
<td>ligand-induced binding sides (LIBS)-targeted microbubbles</td>
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<td>PRP</td>
<td>platelet-rich plasma</td>
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<td>scFv</td>
<td>single-chain antibody fragments</td>
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<td>scuPA</td>
<td>single-chain urokinase plasminogen activator (uPA)</td>
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<td>tPA</td>
<td>tissue plasminogen activator</td>
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Methods

A detailed description of methods is provided in the Online Data Supplement.

Generation, Expression, and Purification of scFVs and scuPAs

Two different scFvs (activated GPIIb/IIIa-targeted scFv_{SCE5} and nontargeted scFv_{mut}) were fused with active scuPA and cloned into the pSectag2A vector system. Briefly, both scFv–scuPA plasmid constructs were produced using the human embryonic kidney cells (H293F) suspension culture. All scFv–scuPAs contain a 6x His-tag, which was used for purification with nickel-based metal affinity chromatography (Invitrogen).

Evaluation of the scFv–scuPA Constructs

The purity of the proteins was analyzed using SDS-PAGE and Western blotting. Anti-6x His-tag antibody horseradish peroxidase was used to detect the purified scFv–scuPA constructs. The scuPA in the fusion protein was converted to the active form using plasmin to cleave the Lys-158 to Ile-159 bond.

Static Adhesion Assay

The specificity of scFv_{SCE5}, targeting activated GPIIb/IIIa was demonstrated using Chinese hamster ovary (CHO) cells that were either expressing activated GPIIb/IIIa integrin, nonactivated GPIIb/IIIa, or not expressing the GPIIb/IIIa integrin. Cells were grown to confluency in 6-well plates (BD Bioscience), incubated with purified scFv–scuPAs, followed by anti-Penta-His AlexaFluor 488–conjugated monoclonal antibody (anti-His-488; Qiagen, Germany). Cells were visualized with the IX81 Olympus microscope (Olympus, Japan) and Cell^P 1692 (ANALytics Image Processing) software.

Flow Cytometry

Platelet-rich plasma (PRP) was obtained from healthy volunteers. Diluted PRP was either not activated or activated with 20 μmol/L ADP, 5 μg/mL collagen-related peptide, or 30 μmol/L thrombin receptor-activating peptide before incubation with the purified scFv constructs, followed by anti-His-488 for detection. The activity of platelets was determined by FITC-labeled fibrinogen, PAC1-FITC, and CD62P-PE. The specificity of scFv_{SCE5} targeting activated platelets was analyzed using FITC-labeled fibrinogen and PAC1-FITC. Competitive assays were performed using abeciximab (ReoPro) and FITC-labeled fibrinogen. Samples were fixed with 1x Cellfix (BD Bioscience) and analyzed by FACs Calibur (BD Bioscience). In addition, GPIIb/IIIa-expressing CHO cells were also used for flow cytometry.

Urokinase Activity Assay

Urokinase activity was determined with a chromogenic substrate assay. A comparison between clinically used uPA (Medac GmbH, Germany) and scFv–scuPA was made on the basis of equal urokinase activity. About 100 nmol/L of scFv–scuPA was monitored against urokinase standards (0–100 U/mL) used as positive controls. Plasmin was added to activate scuPA. S2444 (Chromogenix, Italy) was added, and samples were measured on a Victor3V Multi-label counter (PerkinElmer) at a wavelength of 405 nm.

Plasmin Activity Assay

The conversion of plasminogen to plasmin using commercial uPA or the 2 scFv–scuPA versions was determined in microtiter plates using a chromogenic substrate. Ten nmol/L of commercial uPA and scFv–scuPAs was incubated with 400 nmol/L of human glu-plasminogen (Sigma-Aldrich) and 1 mmol/L of S2251 (Chromogenix, Italy). Samples were measured using the Bio-Rad Benchmark Plus at a wavelength of 405 nm every 30 seconds over a period of 60 minutes.

Fibrin Zymography

SDS-PAGE–based fibrin zymography was performed to evaluate plasminogen-dependent fibrinolytic activity of the targeted and nontargeted scFv–scuPA. Briefly, the commercial uPA and scuPA...
were subjected to SDS-PAGE. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1.5 hours and then placed on top of a fibrin/agarose–plasminogen matrix. The washed SDS-PAGE gel was then overlaid onto the exposed agarose gel and incubated in a humidified 37°C oven until lytic zones were evident. Images were captured at various incubation times using a flatbed document scanner.

Light Transmission Aggregometry
Ninety-six–well plate light transmission aggregometry was performed using 100 μL of PRP. PRP were incubated with abecimab, scFv–scuPA, scFvSCE5 alone, or commercial uPA and then activated with 10 μmol/L ADP. Platelet-poor plasma was obtained by centrifugation of blood at 1000g for 10 minutes at room temperature. Light transmission was adjusted to 0% with PRP and 100% with platelet-poor plasma. To differentiate the effects of scFv from those of urokinase, 200 μmol/L of the urokinase blocker amiloride was added. Light transmission aggregometry was measured using the Bio-Rad Benchmark Plus at wavelength of 595 nm. Samples were measured every 30 seconds for 60 minutes.

Flow Chamber Adhesion Assay
Flow chamber in vitro adhesion assays were performed with glass capillaries or microfluidic flow channels, which were coated over-night with collagen. Whole blood was perfused through the capillaries or channels to form microthrombi. Binding of scFv–scuPAs was observed via staining with anti-His-488. Fibrin degradation was demonstrated using Oregon-Green Fibrinogen (Invitrogen). The microthrombi were visualized with the IX81 Olympus microscope and Cell^P 1692 software.

In Vivo Mouse Experiments
Male C57BL/6 mice and plasminogen-knockout mice (plg−/− mice; Jackson Laboratories) were maintained at the Alfred Medical Research and Education Precinct Animal Services and assigned randomly to the different groups. The amount of targeted and nontargeted scFv–scuPA for injection was calculated according to units per block amiloride 21 was added. Local transmission aggregometry was measured using the Bio-Rad Benchmark Plus at wavelength of 595 nm. Samples were measured every 30 seconds for 60 minutes.

Femoral Vein Catheterization and Ferric Chloride Injury Model for Doppler Flow Velocity Measurement
A catheter was placed into the femoral vein to facilitate injection. A small filter paper saturated with 10% ferric chloride was placed under the carotid artery of the animal for 3 minutes to induce an occlusive thrombus. Animals were injected with either constructs or controls 1 minute before the injury. The nano-Doppler flow-probe (0.5VB; Transonic, Japan) was placed under the carotid artery postinjury to monitor thrombotic occlusion.

Intravital Microscopy of the Mesenteric Arterioles in Mice
Intravital microscopy was performed as previously described. Briefly, the mesentery was exteriorized through a midline abdominal incision. Six percent ferric chloride was used to induce thrombus formation on mesenteric arterioles. Binding of the scFv–scuPA conjugated with Cy3 fluorescence dye (Lumiprobe) was monitored using the fluorescence channel on the Nikon A1r confocal microscope (Nikon, Japan).

Assessment of Tail Bleeding Time
An incision to reveal the left jugular vein was made to insert a catheter to facilitate injections. One minute after injecting commercial uPA, scFv–scuPAs, or vehicle, the tail was transected 5 mm from the tip and immediately submersed in saline at 37°C. The bleeding time was monitored and recorded as the time needed for the cessation of visible blood stream for 1 minute.

In Vivo Ultrasound Molecular Imaging of Carotid Artery Thrombolysis
Ultrasound of animals was performed with a Vevo770 high-resolution imaging system (VisualSonics Inc, Canada) using a 40-MHz RMV704 transducer. Animals were placed on the imaging station after 6% ferric chloride injury was performed to the left carotid artery. Videos and images were acquired before, during, and at several time points after injecting 1.5×10^6 targeted microbubbles (ligand-induced binding sides–targeted microbubbles, LIBS-MBs) specific for activated platelets (targeting the ligand-induced binding site on activated GPIIb/IIIa) in a total volume of 100 μL. We have recently established this ultrasound imaging methodology for the assessment of thrombosis and thrombolysis.19 We injected 500 U/g of uPA (Medac, Germany), 75 U/g of scFv–scuPA, or saline as vehicle control. Repetitive ultrasound imaging sequences were performed every 5 minutes for an hour after thrombolysis. Analysis was performed using a linear contrast agent imaging software (VisualSonics Inc).

Statistical Analysis
Unless otherwise specified, data are expressed as mean±SEM. Flow cytometry, flow chamber, and data for thrombolysis were analyzed with 2-way repeated-measures ANOVA using Bonferroni multiple comparison post-test. All analyses containing >2 groups were corrected by post hoc analysis, and the corrected P values are given. Statistical analyses were performed using GraphPad Prism 5.0.

Results
Cloning and Purification of scFv–scuPA Constructs
The success of DNA amplification and restriction digest of scFv–scuPA fragments was evaluated by electrophoresis (Online Figure I). Both constructs were visualized between 1.5 and 2 kbp marker after amplification with PCR and restriction digest. The pSectag2A plasmid was visualized at ±5 kbps after single-cut restriction digest. After the respective constructs were cloned into the pSectag2A plasmid, transformed, and purified, they were analyzed by gel electrophoresis. The sequences of both fusion constructs were confirmed via DNA sequencing. After production of the scFvs, SDS-PAGE and Western blot were used to prove successful purification (Online Figure IIA and IIB). Western blot was also used to demonstrate the digestion of scuPA after the addition of plasmin (Online Figure IIB).

Binding of Targeted scuPA to CHO Cells In Vitro
The specificity of scFvSCE5–scuPA was also observed under static adhesion conditions. Direct fluorescence staining using anti-His-488 demonstrated binding of scFvSCE5–scuPA to the activated GPIIb/IIIa-expressing CHO cells but not on either
Evaluation of the Functionality of scFv–scuPA by Flow Cytometry

To confirm retained binding capacity of scFv to ADP-activated platelets, the functionality of scFv–scuPA was evaluated with anti-His-488 (Figure 2). No binding was observed for control nontargeted scFv
mut to either activated or nonactivated platelets (2.69±0.18 versus 2.84±0.25 arbitrary units [AU]; mean±SD; ns). The incubation of activated platelets with scFv
mut resulted in an increase in fluorescence intensity as compared with nonactivated platelets (2.69±0.16 versus 23.88±8.22 AU; mean±SD; P<0.01). Competitive assays were performed using FITC-labeled fibrinogen to demonstrate the binding of scFv
SCE5–scuPA. After incubation with scFv
SCE5–scuPA, fibrinogen was not able to bind to activated platelets anymore. However, after incubation with scFv
mut–scuPA, fibrinogen binding was not inhibited (57.11±5.82 versus 75.54±23.2 versus 4.67±2.92 AU; P<0.001). Competitive assays using PAC1 showed similar results as with FITC-labeled fibrinogen (39.03±6.5 versus 41.74±6.45 versus 3.03±0.21 AU; P<0.01). The binding of scFv
SCE5 was also confirmed with collagen-related peptide and thrombin receptor-activating peptide–activated platelets (Online Figure IVA). Platelet activation by these platelet agonists was demonstrated using PAC1 and anti-CD62P fluorescence staining (Online Figure IVB and IVC). The specificity of scFv
SCE5–scuPA binding to activated GPIIb/IIIa on activated platelets was demonstrated via competition with abciximab (Online Figure VA). The specificity of scFv
SCE5–scuPA toward fibrinogen-binding sites on activated GPIIb/IIIa was demonstrated by decreased binding of fibrinogen on increased concentrations of scFv
SCE5–scuPA in flow cytometry (Online Figure VB).

In Vitro Evaluation of scFv–scuPA Activity in Platelet Aggregation

Light transmission aggregometry in a 96-well plate assay was performed to determine the ability of recombinant...
fusion proteins to inhibit platelet aggregation. High concentrations of scFvSCE5 alone (5 and 10 μg/mL) and equimolar amounts of scFvSCE5–scuPA (10 and 20 μg/mL) demonstrated a strong inhibition of ADP-induced platelet aggregation as opposed to scFvmut–scuPA, which showed no inhibitory effect (Figure 3A; n=4; P<0.001). At lower concentrations, scFvSCE5 alone (0.1 and 1 μg/mL) and equimolar amounts of scFvSCE5–scuPA (0.2 and 2 μg/mL) would not inhibit platelet aggregation. Platelet aggregation also was not inhibited with 100 or 200 U of commercial uPA (P<0.001). Similar results were obtained when 200 μmol/L of amiloride was used to block the function of scuPA (Figure 3B), demonstrating that urokinase has no effect on thrombus formation in this assay.

In Vitro Evaluation of the Urokinase Activity of scFv–scuPA

Urokinase activity was monitored by incubating scFv–scuPA with urokinase substrate S2444L (Online Figure VIA) in comparison with commercial uPA. Both scFv–scuPAs and standards using commercial uPA at different concentrations resulted in linear enzymatic activity >60 minutes.

In Vitro Evaluation for the Conversion of Plasminogen to Plasmin Using scFv–scuPA

The conversion of plasminogen to plasmin was monitored using the S2251 amidolytic assay (Online Figure VIB). Both scFv–scuPA versions and the commercial uPA at 10 nmol/L generated plasmin activity. uPA-dependent plasmin generation was blocked in the presence of 200 μmol/L of the urokinase inhibitor amiloride. SDS-PAGE fibrin zymology was also performed to demonstrate the direct digestion of fibrin (Online Figure VIC). Commercial uPA produced a lytic zone as expected at ≈55 kDa. scFvSCE5–scuPA and scFvmut–scuPA produced a lytic zone at ≈70 kDa.

Binding to Activated Platelets and Fibrin Degradation With Targeted scuPA to Microthrombi In Vitro

Targeting of scFvSCE5–scuPA was determined by binding performance in vitro in a flow chamber adhesion experiment with microthrombi. Fluorescence staining using anti-His-488 demonstrated binding of scFvSCE5–scuPA but not with scFvmut–scuPA (Figure 4A). Fibrin degradation was observed when scFvSCE5–scuPA and a high dose of commercial uPA was used, but not with scFvmut–scuPA (Figure 4B). Using 2 μg/mL of scFvSCE5–scuPA, fibrin degradation was observed specifically around platelet aggregates (Online Figure VIB).

Evaluation of scFv–scuPA Binding to Thrombi In Vivo

Binding of scFv–scuPA was determined by intravital microscopy in a ferric chloride–induced thrombosis model in the mesenteric arterioles of mice. The binding of scFvSCE5–scuPA conjugated with the fluorescent dye Cy3 to developing thrombi could be demonstrated, whereas no fluorescence was observed with scFvmut–scuPA (Figure 4C).

In Vivo Evaluation of scFv–scuPA for Prophylactic Fibrinolysis

Thrombi were induced in the carotid artery of mice using 10% ferric chloride for 3 minutes. Blood flow was measured by a nano-Doppler flow-probe and used as an indicator of occlusive thrombus (Figure 5). Saline was injected as a negative control, and 500 U/g of commercial uPA was used as a positive control. The baseline Doppler velocity was set to 100%. At 20 minutes, Doppler flow velocities obtained from mice treated with 75 U/g targeted scFvSCE5–scuPA were significantly higher than those treated with saline, the equimolar concentration of scFvSCE5 alone, 75 U/g of nontargeted scFvmut–scuPA, the combination of scFvSCE5 and 75 U/g of nontargeted scFvmut–scuPA, or 75 U/g of commercial uPA (84.0±9.4 versus 5.4±2.7 versus 23.8±11.8 versus 45.3±13.9 versus 38.5±11.8 versus 21.6±11.4 AU, respectively; mean±SEM; P<0.05; n=6). No difference was observed in groups treated with 75 U/g body weight of nontargeted scFvmut–scuPA, the equimolar concentration of scFvSCE5 alone, or the combination of both scFvSCE5 and 75 U/g body weight of nontargeted scFvmut–scuPA. Doppler flow velocities obtained from mice treated with 75 U/g targeted scFvSCE5–scuPA were similar to those treated with 500 U/g of commercial.
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DIC indicates differential interference contrast; channel. No specific fluorescence/binding was observed using activated platelets/thrombus was detected on the fluorescence of stable thrombi. Increased binding of scFvSCE5–scuPA to fluorescence dye were injected into the mice after the formation (n=3 each). scFv–scuPA fusion proteins conjugated with Cy-3 induced by ferric chloride injury in the mesenteric arteriole demonstrating the attachment of scFv SCE5–scuPA to the microthrombi. No fluorescence was detected on microthrombi with scFvmut–scuPA (n=3 each). B, scFvSCE5–scuPA caused fibrin degradation in vitro on microfluidic flow channels. Fibrin degradation was observed at platelet aggregation perfused with scFvSCE5–scuPA but not with scFvmut–scuPA. Fibrin degradation was also observed in platelet aggregates perfused with a high dose of commercial uPA (n=3 each). Image analysis was done with ImageJ applying a median filter (1.5 pixels) and a false false color look-up table. C, Intravital microscopy demonstrating the binding of scFvSCE5–scuPA conjugated with Cy-3 fluorescence dye to thrombi in vivo. Representative images showing the binding of scFv–scuPA fusion proteins to thrombi induced by ferric chloride injury in the mesenteric arteriole (n=3 each). scFv–scuPA fusion proteins conjugated with Cy-3 fluorescence dye were injected into the mice after the formation of stable thrombi. Increased binding of scFvSCE5–scuPA to activated platelets/thrombus was detected on the fluorescence channel. No specific fluorescence/binding was observed using scFvmut–scuPA. DIC indicates differential interference contrast; scFv, single-chain antibody fragments; and scuPA, single-chain urokinase plasminogen activator.

Figure 4. Binding of scFvSCE5–scuPA to microthrombi resulting in fibrin degradation in vitro and fluorescence staining of scFvSCE5–scuPA in vivo. A, Representative microscopy images of microthrombi with scFvSCE5–scuPA. Fluorescence-labeled anti–His-tag antibody demonstrates the attachment of scFvSCE5–scuPA to the microthrombi. No fluorescence was detected on microthrombi with scFvmut–scuPA (n=3 each). B, scFvSCE5–scuPA caused fibrin degradation in vitro on microfluidic flow channels. Fibrin degradation was observed at platelet aggregation perfused with scFvSCE5–scuPA but not with scFvmut–scuPA. Fibrin degradation was also observed in platelet aggregates perfused with a high dose of commercial uPA (n=3 each). Image analysis was done with ImageJ applying a median filter (1.5 pixels) and a false false color look-up table. C, Intravital microscopy demonstrating the binding of scFvSCE5–scuPA conjugated with Cy-3 fluorescence dye to thrombi in vivo. Representative images showing the binding of scFv–scuPA fusion proteins to thrombi induced by ferric chloride injury in the mesenteric arteriole (n=3 each). scFv–scuPA fusion proteins conjugated with Cy-3 fluorescence dye were injected into the mice after the formation of stable thrombi. Increased binding of scFvSCE5–scuPA to activated platelets/thrombus was detected on the fluorescence channel. No specific fluorescence/binding was observed using scFvmut–scuPA. DIC indicates differential interference contrast; scFv, single-chain antibody fragments; and scuPA, single-chain urokinase plasminogen activator.

Figure 5. Doppler flow velocity of carotid arteries of mice for monitoring of thrombolysis showing that scFvSCE5–scuPA prevents occlusion. Thrombi were induced in the carotid artery of mice using 10% ferric chloride for 3 min. The nano-Doppler flow meter was used to measure occlusion time, and the baseline Doppler velocity was set to 100%. Saline was injected as a negative control and 500 U/g body weight (BW) of clinically used commercial uPA as a positive control. The analysis of velocity 10 min postinjury showed occlusion for mice treated with saline and 75 U/g BW commercial uPA. Twenty minutes postinjury, Doppler velocity from mice treated with 75 U/g BW of targeted scFvSCE5–scuPA was significantly higher than those treated with saline, 75 U/g BW of nontargeted scFvmut–scuPA, 75 U/g BW of commercial uPA, the equimolar concentration of scFvSCE5 alone, or the combination of scFvSCE5 with 75 U/g BW of nontargeted scFv–scuPA. No difference was observed in groups treated with 75 U/g BW of nontargeted scFvSCE5 alone, or the combination of both scFvSCE5 and 75 U/g BW of nontargeted scFvmut–scuPA. Similar results were obtained for 30 min. Doppler flow velocities obtained from mice treated with 75 U/g of targeted scFvSCE5–scuPA was similar to those treated with 500 U/g BW of commercial uPA throughout the observation period. These assays were analyzed with 2-way repeated-measures ANOVA with the Bonferroni post-test. Data shown as mean±SEM; *P<0.05, **P<0.01, ***P<0.001; n=6 each. scFv indicates single-chain antibody fragments; and scuPA, single-chain urokinase plasminogen activator.

In Vivo Molecular Ultrasound Imaging of Thrombolysis

Imaging of the mouse carotid artery on ultrasound typically shows luminal blood as black or dark color, and microbubbles appear as a bright white color in the lumen. The thrombus was visualized as a white and bright signal after injection with platelet-targeted ultrasound contrast (LIBS-MB) on real-time ultrasound imaging. The baseline area before injection of uPA was set to 100%, and the area was calculated every 5 minutes for 60 minutes (Figure 7). The targeting ability of 75 U/g of scFvSCE5–scuPA is demonstrated by ultrasound imaging; as such, a reduction in thrombus size was observed after its administration. Treatment with scFvSCE5–scuPA (75 U/g) significantly reduced thrombus size after 60 minutes, whereas no significant difference was observed in the scFvmut–scuPA (75 U/g) treatment group (36.8±4.6 versus 81.1±2.6 AU; mean±SEM; P<0.001; n=3; Figure 7; Online Videos I and II). Thrombolysis was observed via ultrasound imaging using 500 U/g of commercial uPA. The ability of scFvSCE5–scuPA to

uPA throughout the observation period. Similar results were obtained at 30 minutes.

In Vivo Assessment of Bleeding Time of scFv–scuPA

Bleeding times were evaluated by surgical tail transection (Figure 6). Commercial uPA at 500 U/g considerably prolonged bleeding compared with vehicle control (saline). In contrast, a lower dose of 75 U/g of scFvSCE5–scuPA, scFvmut–scuPA, and commercial uPA minimized bleeding time. The lower dose of scFvSCE5–scuPA (75 U/g) had an antithrombolytic effect without prolonging bleeding time.
target and dissolve the thrombus was compared against mice injected with a higher dose of commercial uPA. There were no significant differences between groups of mice treated with 500 U/g commercial uPA and those treated with 75 U/g of scFvSCE5–scuPA over a period of 60 minutes (40.05±9.2 versus 36.8±4.6 AU; ns; n=3). The thrombolytic ability of scFvSCE5–scuPA was also compared with the control group where saline was administrated over a period of 60 minutes. scFvSCE5–scuPA caused a reduction in thrombus size at 60 minutes postadministration, compared with control (36.8±4.6 versus 99.2±1.3 AU; \( P < 0.001 \); n=3). This control group was also compared against the nontargeted treatment using 75 U/g of scFvmut–scuPA. Although postadministration of scFv mut–scuPA showed some reduction in the thrombus area, it was not significantly smaller than those injected with saline (81.1±2.6 versus 99.2±1.3 AU; ns; n=3).

**In Vivo Ultrasound Molecular Imaging of Carotid Artery Thrombolysis on plg−/− Mice**

plg−/− Mice were subjected to ferric chloride–induced thrombosis. Mice were then administered either scFvSCE5–scuPA (75 U/g) or scFv mut–scuPA (75 U/g). Over a 30-minute period, there was no change in thrombus size in both groups of animals (96.0±0.1 versus 98.5±0.1 AU; mean%±SEM; ns; n=3). However, when plg−/− mice were reconstituted with human plasminogen (100 μg/mL; 150 μL bolus at 30-minute time point), mice treated with scFvSCE5–scuPA developed a significant thrombus size reduction, whereas no significant difference was observed in plg−/− mice treated with scFv mut–scuPA (23.1±1.5 versus 92.1±2.2 AU; mean%±SEM; \( P < 0.001 \); n=3; Figure 8).

**Discussion**

Thrombolysis has been widely used as a therapy in acute myocardial infarction, ischemic stroke, as well as pulmonary embolism.\(^4\)\(^,\)\(^24\) However, limitations in thrombolytic efficacy, neurotoxicity of tPA,\(^25\) and bleeding complications have restricted overall use.\(^6\)\(^,\)\(^26\) The administration of anticoagulants in combination with fibrinolytic agents, in particular, has shown little improvement in mortality, safety, and efficacy.\(^27\) Therefore, research into more effective treatments is required, which could overcome the challenges associated with current fibrinolytic therapy. Targeting activated platelets with antibody-mediated delivery of thrombolytic drugs can provide an effective and safe alternative. This ideal agent would: (1) allow a low concentration in systemic circulation, thereby eliminating bleeding complications, and (2) enrich by targeting at the site of thrombus, thereby being present in localized high concentrations to lyse the clot.
or existing thrombus, thereby increasing fibrinolytic potency without increasing side effects. Through in vitro assays, we provide evidence that both scFv and scuPA retain their individual function in the fusion molecule. In vivo evaluation of these targeted fibrinolytics as both a prophylactic and a therapeutic agent showed plasminogen-dependent inhibition of thrombus growth as well as reduction in thrombus size via molecular ultrasound imaging. A low dose of 75 U/g of platelet-targeted scuPA was sufficient for localized thrombolysis, which was not achieved using nontargeted scuPA or commercial uPA at the same dose. The same effects were only achieved using urokinase at 500 U/g. We did not observe increased bleeding at the effective dose of the novel targeted scuPA, compared with an equally effective higher dose of nontargeted scuPA, which resulted in a significantly prolonged bleeding time. This antithrombotic effect of a low dose of 75 U/g of platelet-targeted scuPA was more potent than the combination of equimolar nontargeted scuPA and the activation-specific anti-GPIIb/IIIa scFvSCE5. This indicates that the superior antithrombotic effect of the fusion protein scFvSCE5–scuPA can be attributed to the antibody targeting of scuPA to activated GPIIb/IIIa on activated platelets and the resulting local enrichment of scuPA at the thrombus. If a stronger antiplatelet effect is required, a dual effect composed of effective platelet inhibition and low-dose targeted fibrinolysis would be attractive and could be achieved with the coadministration of activation-specific anti-GPIIb/IIIa scFv alone. For a potential human therapy, the optimal dosing regimen would have to be determined in clinical trials. Notably, the combination of low-dose targeted fibrinolytic and activation-specific platelet inhibition would not be expected to have similar bleeding problems as seen in clinical trials with nontargeted fibrinolytics and non–activation-specific GPIIb/IIIa inhibitors.

Early work mainly focused on the use of full monoclonal antibodies targeting specific components of the clot, such as fibrin or platelets. Indeed, in vivo studies have shown greater antithrombotic therapy with the use of fibrin-targeting thrombolysis. Early work has also been shown that potent fibrin targeting during thrombolysis has resulted in a high plasminogen concentration on the surface of the clot.

Our study focuses on platelets, because activated platelets are an obvious target for the enrichment of thrombolysis at the clot. To explore the advantages of targeting PAs to platelets, monoclonal antibodies directed against both GPIIb/IIIa and the ligand-binding epitope of GPIIIb/IIIa have been reported. GPIIb/IIIa is the most abundant platelet receptor with 50,000 to 80,000 glycoproteins per platelet and, thus, constituting ≈2% of the amount of total platelet protein. Despite increasing the potency by fusing PAs to monoclonal antibodies or Fab fragments, chemical conjugation has several disadvantages, including the inactivation of enzymatic sites by the harsh chemical conditions for conjugating, which can reduce efficacy. In addition, the large size of the antibody fusion constructs hampered tissue penetration.

To overcome these problems, recombinant fusion technology was used to avoid chemical conjugation, thereby preserving the activity of the construct. Antibody fragments such as scFvs are preferred moieties for the construction of recombinant fusion constructs because they are small, minimally immunogenic, and the cost of production is generally low.

Antibody-targeted delivery also eliminates the dependence on the specificity of certain PAs, such as tPA, for the components of clots such as fibrin. Still the gold standard therapy for acute ischemic stroke, tPA is neurotoxic, and the use of urokinase or desmoteplase avoids these complications inherent to tPA. This has the potential to widen the currently very narrow therapeutic window for stroke thrombolysis of 3 to 4 hours. The truncated, low-molecular-weight form scuPA is the preferred entity over full-length urokinase because it provides a smaller size with improved thrombus accessibility, no immunogenicity, and similar fibrinolytic potency.

In addition to fibrin- and platelet-targeting approaches, there are several other components of the vasculature and blood that have been targeted, such as platelet endothelial cell adhesion molecule-1, glycophorin A on RBCs, and thrombomodulin, to anchor PAs to the arterial or venous lumen. Platelet endothelial cell adhesion molecule-1 fused with urokinase in a model of cerebrovascular thromboembolism mediated almost complete clot lysis without exacerbating the characteristic side effects such as intracerebral hemorrhage. Targeting RBCs was shown to have
effective prophylaxis against arterial and venous thrombosis for ≈24 hours.42

Although targeting platelet endothelial cell adhesion molecule-1 or RBCs has shown successful thrombolysis, they do not serve as exclusive targets because of ubiquitous expression. We have previously reported that scFvs against fibrin conjugated to scuPA,45 or against platelets conjugated to an anticoagulant,22 are predominantly active only at the site of the clot, allowing increased potency. In the present study, we have expanded our work to a scFv fusion molecule, which delivers scuPA activation specific to the GPIIb/IIIa platelet receptor. On activation, GPIIb/IIIa undergoes a conformation change, which allows a high-affinity state for fibrinogen binding, resulting in platelet aggregation and thrombus formation.3,15 This property is ideal for specifically targeting activated platelets. The scFv

\[ \text{scFv}_{\text{SCES}} \]

used in this work is exclusive in its specificity for the activated conformation of GPIIb/IIIa thereby, representing a unique targeting tool.15,16,23

In addition to our well-established Doppler flow measurements15,22 allowing the assessment of vessel patency, we also applied a recently developed real-time ultrasound method with scFv-targeted microbubbles used as echo enhancers.19 The small size of scFv has advantages for molecular imaging because it provides a better access to the targeted epitopes. In contrast to the anti-GPIIb/IIIa scFv used in the scuPA fusion construct, the scFv on the microbubbles binds to LIBS on GPIIb/IIIa, preserving the ligand binding function. This new molecular imaging approach allows the unique real-time and direct monitoring of the success and extent of thrombolysis, and it holds promise to substantially facilitate further research in thrombogenesis and especially thrombolysis. Furthermore, this technique is also highly promising for clinical use. It is noninvasive, inexpensive, and most importantly provides real-time analysis of vessel occlusion and reopening in an emergency setting. Direct ultrasound imaging of changes in thrombus size is a technique that could have broad clinical applications in detecting the success or failure of therapeutic interventions in thrombotic disease and, thus, provide major benefits for patients.

The potential clinical use of these targeted fusion proteins for the prevention and treatment of stroke, myocardial infarction, and venous thrombosis presents a novel strategy for highly effective thrombolysis. The expected reduction of bleeding complications could lead the way out of the current stagnation in the field of fibrinolytic therapy. Our data also have interesting implications for the potential use of a fibrinolytic drug for prophylaxis/prevention of thrombosis. Monoclonal antibodies (eg, Humira) are increasingly used as subcutaneous drugs for self-administration by patients.46 This opens the possibility that recombinant fusion proteins such as scFv

\[ \text{scFv}_{\text{SCES}} \text{–scuPA} \]

could potentially be used for thromboprophylaxis. More preclinical and clinic research is warranted to investigate the feasibility of such an application. Furthermore, the described molecular ultrasound imaging with platelet-targeted microbubbles allows precise monitoring of interventional success.

Conclusions

This study demonstrates the successful generation of a novel recombinant single-chain antibody–scuPA fusion protein, specifically targeting activated platelets. The function of both components of the fusion protein, thrombolytic activity and antibody binding, to activated GPIIb/IIIa was preserved. In vivo evaluation of this targeted fibrinolytic agent, in comparison to clinically used nontargeted fibrinolytic agents, showed that a low systemic concentration of the fusion protein allows enrichment of fibrinolytic activity at the site of the thrombus and thus the potentiation of fibrinolytic activity. Most importantly, this could be achieved without prolonging bleeding time. Therefore, this novel fibrinolytic agent promises to overcome the current limitations in thrombolytic therapy associated with the risk of bleeding complications. It has the potential to break the fatal link between increased fibrinolytic potency and bleeding complications. This targeting strategy also allows considering the application of a fibrinolytic drug for thromboprophylaxis, an approach that warrants further preclinical and potential clinical testing.

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Disclosures

A patent has been granted to protect the intellectual property of the described single-chain antibody. A patent has been filed to protect the intellectual property of the described antibody–urokinase fusion construct.

References


**What Is Known?**

- The initiating events in acute thrombotic events, such as myocardial infarction (MI) and stroke, include platelet activation, conversion of fibrinogen to fibrin, and eventual formation of an obstructing/occluding thrombus/clot.
- Thrombolytic drugs such as urokinase were developed to lyse clots through the conversion of the endogenous proenzyme plasminogen to the active fibrinolytic protease plasmin.
- Treatment with thrombolytic drugs, however, is often ineffective and associated with bleeding complications because of a lack of site specificity and, hence, disruption of normal hemostasis.

**What New Information Does This Article Contribute?**

- Molecular cloning techniques can be used to create a fusion construct of single-chain urokinase plasminogen activator (scuPA) and a small single-chain antibody (scFvSCE5) directed specifically against the activated glycoprotein (GP) IIb/IIIa receptor of platelets in a developing clot.
- The fusion construct (scFvSCE5–scuPA) allows for a small administered dose of urokinase to concentrate at the site of the developing clot, leading to a high local concentration with low systemic concentrations.
- Systemic administration of this targeted scuPA to mice subjected to arterial thrombosis leads to effective thrombolysis without negative effects on hemostasis.

**Novelty and Significance**

Thrombolytic drugs are highly effective when used for the treatment of MI and ischemic stroke. Bleeding complications due to interference with normal hemostasis, however, restrict application to a relatively small group of patients. Strategies to target the thrombolytic activity specifically to the site of thrombosis are attractive because they promise effective thrombolysis without undesired bleeding complications. We genetically fused scFvSCE5 with scuPA to form scFvSCE5–scuPA. The unique affinity of scFvSCE5 for the activated conformation of the GPIIb/IIIa receptor and thus for activated platelets means that once injected systemically, scFvSCE5–scuPA enriches in the region of a developing thrombus and exerts potent thrombolytic activity because of its high local concentration, while avoiding bleeding complications, due to its low systemic concentration. In mice with induced arterial thrombosis, a 6-fold lower dose of targeted scuPA had equivalent efficacy to clinically used uPA, but without associated bleeding time prolongation. This demonstration of targeted thrombolysis using an activation-specific platelet antibody holds promise for safer and more effective thrombolysis in patients with MI or stroke, including those that have been excluded so far based on bleeding concerns. Furthermore, the improved safety profile allows the consideration of fibrinolytics being given as thromboprophylactic drugs.
Towards Effective and Safe Thrombolysis and Thromboprophylaxis: Preclinical Testing of a Novel Antibody-Targeted Recombinant Plasminogen Activator Directed Against Activated Platelets

Xiaowei Wang, Jathushan Palasubramaniam, Yannik Gkanatsas, Jan David Hohmann, Erik Westein, Ruchi Kanojia, Karen Alt, Dexing Huang, Fu Jia, Ingo Ahrens, Robert L. Medcalf, Karlheinz Peter and Christoph E. Hagemeyer

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SUPPLEMENTAL MATERIAL

Supplemental Methods

**Generation, expression and purification of single-chain antibodies and single-chain urokinase plasminogen activator**

The generation of two different scFvs (activated GPIIb/IIIa-targeted scFv<sub>SCES</sub> and non-targeted scFv<sub>mut</sub>), both fused with active scuPA, were cloned into the pSectag2A vector system. Briefly, for both scFv-scuPAs, polymerase chain reaction (PCR) was performed with a sense primer that anneals at the beginning of the scFv sequence and an antisense primer that anneals directly to the 6x His-tag region at the end of the scFv. The sense strand includes the *AscI* restriction site and the antisense strand includes the *XhoI* restriction site. The scFv<sub>SCES</sub>-scuPA was generated with the following primers: sense strand: 5'- ATC TTA GGC GCG CCA TGG CGG AGG TGC AGC TGG T -3', antisense strand: 5'- GCC CGT CTC GAG TAC CGG TAC GCG TAG AAT CGA GAC C -3'. The scFv<sub>mut</sub>-scuPA was generated with the following primers: sense strand: 5'- ATC TTA GGC GCG CCA TGG CGG AAG TGC AGC TGG TG -3', antisense strand: 5'- GCC CGT CTC GAG TAC CGG TAC GCG TAG AAT CGA GAC C -3'. After amplification by PCR, the constructs were digested with the restriction enzymes *Ascl* and *XhoI* (both NEB, USA), and cloned into pAC6 vector (Avidity, USA). Electrophoresis on a 0.8% agarose gel with SYBR<sup>®</sup> Safe DNA gel stain (Invitrogen, USA) was utilized to analyze DNA amplified by PCR and restriction digests. Ligation of the plasmids was performed with T4 ligase (NEB, USA) at 16°C overnight. The resulting plasmid constructs were then transformed into BL21 Star *E.coli* cells (Invitrogen, USA).

**Expression in mammalian cells and purification of scFv-scuPA fusion constructs**

Production of mammalian cells was performed using the human embryonic kidney cells (H293F) suspension culture transfection with polyethylenimine (Polyscience Inc., Germany). This system is used for the production of proteins from pSectag vectors. DNA plasmid for transfection was diluted to a ratio of 1:4 with polyethylenimine (PEI). 24 hours prior to transfection, H293F cells were diluted with Freestyle 293 expression medium (Invitrogen, USA) to a concentration of 1 x 10<sup>6</sup> cells/ml. The cell density was approximately 2 x 10<sup>6</sup> cells/ml at time of transfection and the viability was greater than 95%. A ratio of 9:1 was used for the amount of Freestyle 293 expression medium to the PBS mixture of DNA and
PEI. Appropriate amount of cell culture medium was transferred into a shaker flask and placed in a CO₂ incubator at 37°C, shaking at 110 rpm. 1µg/ml of DNA plasmid was added to pre-warmed (37°C) PBS and vortexed gently. PEI was added at a concentration of 3µg/ml, and vortexed three times for three seconds. The mixture was incubated for 15 min at room temperature (RT). The cell culture medium was removed from the incubator. The DNA-PEI mixture was added to the medium while swirling gently. Glucose was added to a final concentration of 6g/L. The flask was returned to the incubation and cultured at 37°C, with 5% CO₂, shaking at 110 – 140rpm. The culture was supplemented with 5g/L Lupin and 0.2 mM butyric acid after one day. At day 3, 5 and 7 after transfection, the culture was supplemented with 2mmol/L glutamine. At day 5, the culture was supplemented with 5g/L Lupin. The glucose level was maintained at a final concentration of 5 – 6g/L. The cells were harvested when viability was 40 – 50%. The cells were centrifuged at 3000xg for 15 min at 4°C and supernatant was collection for protein purification. All purified single-chain antibodies carry a 6x His-tag at the C-terminal end of their amino acid sequence for purification by IMAC and for FACS analysis. Proteins were purified with a nickel-based metal affinity chromatography column, Ni-NTA column (Invitrogen, USA), according to the manufacturer’s instruction manual. Fractions of 1ml were collected and dialyzed against PBS.

**Evaluation of the scFv-scuPA fusion proteins**

Purity of the proteins was analyzed using SDS-PAGE. 30µl of each purified protein and 6µl of 5X reducing SDS loading buffer were added to 1.5ml tubes and denatured at 96°C for 5 min. The samples were run on SDS-PAGE gel in SDS running buffer at 30mA for 2 hours. The gel was then stained with Coomassie Brilliant Blue for 1 hour and subsequently destained for at least 12 hours with Coomassie destaining solution. The gel was visualized and analyzed using a BioRad Gel-Doc system with Quantity One software.

After SDS-gel electrophoresis and Western blotting, the membrane was blocked with 1% BSA and hybridized with a specific horseradish peroxidase (HRP). Anti-6x His-tag® antibody HRP was used to detect the fusion proteins. Secondary hybridization was performed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific Inc, USA), an enhanced chemiluminescent (ECL) substrate for the HRP enzyme. Plasmin was added to digest the scuPA to the two chain urokinase by cleaving the Lys-158 and Ile-159 bond.
Chinese Hamster Ovary (CHO) cells culture

The specificity of the scFv_{SCE5} targeting activated GPIIb/IIIa was demonstrated using Chinese Hamster Ovary (CHO) cells that were either expressing activated GPIIb/IIIa integrin, non-activated GPIIb/IIIa integrin or not expressing the integrin. Cells were grown to 90% confluency before washing with PBS, and addition of 0.05% Tryspin (Invitrogen, USA) for 5 min. These cells were used in flow cytometry to show the specificity of the scFv-scuPA. 1 x 10⁶ CHO cells per 50µl were used for incubated with scFv-scuPA and stained with anti-Penta-His AlexaFluor 488-conjugated monoclonal antibody. In addition, PAC1-FITC was used to show expression of activated GPIIb/IIIa receptors on the respective CHO cells. A gate was set around the cells and 10,000 cells were analyzed. The scFv binding was expressed as mean fluorescence intensity.

Static adhesion assay

Static adhesion assay were performed using CHO cells that were either expressing activated GPIIb/IIIa integrin, non-activated GPIIb/IIIa integrin or not expressing the integrin. The CHO cells (1 x 10⁶/well) were grown to confluency in 6-well plates (BD Bioscience, USA). The wells were washed twice with PBS and incubated with 10µg/ml of purified scFv for 10 min at 37°C. The wells were washed twice with PBS, followed by anti-Penta-His AlexaFluor 488-conjugated monoclonal antibody for 15 min. The wells were then washed three times with PBS and visualized with the IX81 Olympus microscope (Olympus, Tokyo, Japan) and Cell^P 1692 (ANALysis Image Processing) software, using DIC and fluorescence imaging with 20x objective.

Flow cytometry

Blood was collected from healthy volunteers who had taken no medication for at least 10 days. In an attempt to minimize platelet activation during blood collection, blood was obtained by venipuncture from an antecubital vein through a 21 gauge needle with no tourniquet. The first 2ml of blood were discarded. The collected blood was anticoagulated with 10% citric acid. Platelet rich plasma (PRP) was obtained by centrifugation at 180xg for 10 min at room temperature. The PRP was collected and diluted 1:20 with PBS containing 100mg/L calcium chloride and 100mg/L magnesium chloride. Diluted PRP was either not activated or activated with 20µM ADP, 5µg/ml collagen-related peptide (CRP) or 30µM thrombin receptor-activating peptide (TRAP) for 5 min. Activity of platelets was determined by FITC-labelled fibrinogen, PAC1-FITC and anti-CD62P-PE. As described in details below,
the platelets were analyzed by flow cytometry using either a single fluorochrome (single staining) or two fluorochromes (dual staining). For single staining, incubation with 10μg/ml of purified scFv for 10 min at 37°C was followed by 1μl of anti-Penta-His AlexaFluor 488-conjugated monoclonal antibody for 15 min. Samples were fixed with 1x Cellfix (BD Bioscience, USA) and analysed by FACS Calibur (BD Bioscience, USA). In addition, the specificity of scFv_{SCE} targeting activated platelets was analyzed in a competitive flow cytometry assay, using FITC-labelled fibrinogen and PAC1-FITC. Both FITC-labelled fibrinogen and PAC1-FITC bind to the activated GPIIb/IIIa receptors on activated platelets. For dual staining, incubation with both 10μg/ml of purified scFv and CD62P-PE for 10 min at 37°C was followed by 1μl of anti-Penta-His AlexaFluor 488-conjugated monoclonal antibody for 15 min. Competitive assays were also performed using 10μg/ml of abciximab (ReoPro®). Further dose dependent competitive assays were performed using the scFv_{SCE} and FITC-labelled fibrinogen. Samples were fixed with 1x Cellfix and analysed by FACS Calibur.

Samples were analyzed using a Becton Dickinson FACS Calibur flow cytometer. The platelets were distinguished using the forward and sideward light scatter profile. A gate was set around the platelets and 10,000 cells were analyzed. For single staining, the AlexaFluor 488 fluorescence is used to quantitate the amount of platelet-bound scFv. The scFv binding was expressed as mean fluorescence intensity. For dual staining, the AlexaFluor 488 fluorescence and CD62P-PE fluorescence is used to quantitate the amount of activated platelet-bound scFv.

**Urokinase activity assay**

Urokinase activity was determined in microtiter plates with a chromogenic substrate assay. Comparison between clinically used uPA (Medac GmbH, Hamburg, Germany) and scFv-scuPA was made on the basis of equal urokinase activity. A volume of 50μl scFv-scuPA, each with a final concentration of 100nmol/L, was monitored against 50μl urokinase standards (0–100U/mL) used as positive control. Single-chain urokinase was converted to the active form using 0.1U/L plasmin in assay buffer (38mmol/L of NaCl, 5mmol/L of Tris–HCl, 0.1% bovine serum albumin, pH 8.8). After incubation for 2 hours at room temperature under shaking, 0.5mmol/L of S2444 (Chromogenix, Milano, Italy) in 125μl assay buffer was added. Samples were measured on a Victor3V Multi-label counter (PerkinElmer, Massachusetts, USA) at wavelength 405nm every 5 min over a period of 60 min.
**Fibrin zymography**

SDS-PAGE-based fibrin zymography\(^1\) was performed to evaluate plasminogen dependent fibrinolytic activity of the targeted and non-targeted scFv-scuPA. Briefly, the commercial uPA and scuPA were subjected to SDS-PAGE using 10% polyacrylamide gels (see figure legends for concentrations). After electrophoresis, gels were washed in 2.5% Triton X-100 for 1.5 hour, then placed on top of a fibrin/agarose: plasminogen matrix. The matrix was prepared by first preparing a 10 ml solution of 2% low melting agarose in water (kept at 45°C). 15ml of plasminogen depleted human fibrinogen (1% in imidazole buffer) was then prepared that also contained 24µl 25% TX-100 and 25µL human glu-plasminogen (1mg/ml). Thrombin (30µl of 100U/µl) was added to the fibrinogen solution to promote polymerization, then mixed and finally added to the agarose solution before being poured into gasket sealed glass plates (15 x 16cm) separated with 1.5mm spacers. The fibrinogen was allowed to polymerize at room temperature for ~2h. The glass plates were laid flat and the top plate removed. The washed SDS-PAGE gel was then overlaid onto the exposed agarose gel and incubated in a humidified 37°C oven until lytic zones were evident (usually overnight). Images were captured at various incubation times using a flatbed document scanner.

**Flow chamber adhesion assay**

Flow chamber *in vitro* adhesion assays were performed with glass capillaries or microfluidic flow channels, which were coated with 100μg/ml of collagen. After coating with collagen overnight, a syringe pump (PhD 2000, Harvard Apparatus, USA) was used in the aspiration mode to withdraw the whole blood through the capillaries or flow channels to form microthrombi. To observe the binding of scFv-scuPA to the platelet-rich microthrombi in the capillaries, the capillaries were perfused with PBS containing 100mg/L calcium chloride and 100mg/L magnesium chloride. After washing, the syringe pump was used to withdraw the scFv-scuPA through the flow chamber at defined shear rates. The capillaries were then stained with anti-Penta-His AlexaFluor 488-conjugated monoclonal antibody. The microthrombi in the capillaries were visualized with the IX81 Olympus microscope and Cell^P 1692 software, using brightfield and fluorescence imaging with 20x objective.

Fibrin degradation was monitored using microfluidic flow channels (300µm x 54µm x 22µm width x height x length). Whole blood, anticoagulated with 3.2% w/v citrate that was supplemented with 60μg/ml Oregon Green Fibrinogen (Invitrogen, USA), was perfused at 1000s\(^{-1}\) for 6 min to generate microthrombi. On-chip microfluidic mixing was used to
reconstitute the citrate anticoagulated whole blood with 12mmol/L CaCl₂, 6mmol/L MgCl₂, 600µg/ml thromboplastin (Thromborel S; Siemens healthcare, Germany) resulting in 2mmol/L free calcium in the whole blood. Perfusion with reconstituted blood continued for 5 min at 200s⁻¹. Upon appearance of fibrin formation, 2µg/ml scFv-scuPA was added. The fibrin degradation were visualized with the IX81 Olympus microscope and Cell^P 1692 software, using DIC and fluorescence imaging with 60x objective. Image analysis was done with ImageJ applying a median filter (1.5pixel) and a “fire” false color LUT (Look up table). False color LUT is achieved according to the fluorescence intensity of the image.

In vivo mouse experiments
Male C57BL/6 mice and plasminogen deficient (plg⁻/⁻) mice were assigned randomly to different groups. For thrombolysis, animals were injected with either commercial uPA, targeted scuPA (scFVSCES-scuPA), non-targeted scuPA (scFVmut-scuPA) or saline as vehicle control after ferric chloride induced injury to the carotid artery. Animals for molecular ultrasound imaging were injected with targeted microbubbles directed against activated-platelets prior to thrombolysis treatment. The amount of commercial uPA or scFv-scuPA for injection was calculated according to units per gram (U/g) body weight of the animals. All experiments involving animals were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee.

The animals were anaesthetized with ketamine (50mg/Kg, Parnell Laboratories, NSW, Australia) and xylazine (10mg/kg, Troy Laboratories, NSW, Australia). Fur was removed by shaving cream (Dove, Australia). Mice were placed on a 37°C heater mat to prevent hypothermia.

Femoral vein catheterization
An incision was made on the left limb of the animal to expose the femoral vein. A catheter was placed into the femoral vein to facilitate injection.

Ferric chloride injury model for Doppler flow velocity measurement of the carotid artery
Male C57BL/6 mice, 6 to 7 weeks of age (20 to 25 grams), were used for this model. An incision was made on the neck and the left common carotid artery was dissected from circumferential connective tissues. A small (1x2mm) filter paper saturated with 10% ferric
chloride was placed under the carotid artery of the animal for 3 min, to induce injury, which results in the formation of an occlusive thrombus. Treatment using commercial uPA, targeted scuPA (scFv_{SCE5-scuPA}), non-targeted scuPA (scFv_{mut-scuPA}), an equimolar concentration of scFv_{SCE5} alone, a combination of both scFv_{SCE5} and scFv_{mut-scuPA}, or saline as vehicle control was injected, via the femoral vein catheter, 1 min before the injury. A nano Doppler flow probe (0.5VB, Transonic, Japan) was placed under the carotid artery post-injury and the carotid blood flow was measure by a flow meter (T106, Transonic, Japan) to determine thrombotic occlusion. Measurements of the Doppler velocities were monitored for 30 min.

**Intravital microscopy of the mesenteric arteries in mice**

Male C57BL/6 mice, 4 weeks of age (16 to 20 grams), were used for this model. Intravital microscopy was performed as previously described. Briefly, the mesentery was exteriorized through a midline abdominal incision. Mesenteric arterioles (60–160 mm) were injured via microinjection of 6% FeCl₃ into the tissue adjacent to the arteriole of interest with a microinjector needle (tip diameter 4–6 mm) which results in thrombi. ScFv-scuPA conjugated with Cy-3 NHS fluorescence dye (Lumiprobe, Florida, USA) were injected into the animal after the formation of thrombi. The accumulation of fluorescence labelled scFv-scuPA was observed using the fluorescence channel on the Nikon A1r Plus confocal microscope with 40x objective.

**In vivo ultrasound molecular imaging of carotid artery thrombolysis**

Male C57BL/6 mice, 6 to 7 weeks of age (20 to 25 grams), were used for this model. Ultrasound of animals was performed with a Vevo770 high-resolution imaging system (VisualSonics Inc. Canada) using a 40 MHz RMV704 transducer. Animals were placed on the VisualSonics imaging station after 3 min, ferric chloride (6%) injury was performed to the left carotid artery. Videos and images were acquired before, during and at several time points after injecting 1.5x10⁷ targeted microbubbles (LIBS-MBs). LIBS-MBs bound specifically to the ligand induced binding site (LIBS) expressed on activated GPIIb/IIIa upon platelet activation. We have recently demonstrated that these LIBS-MBs specifically bind to activated platelets in vitro in flow chamber adhesion assays, and allow non-invasive, real-time molecular imaging of thrombosis via ultrasound. We have also established the ability to monitor for the success or failure of pharmacological thrombolysis in vivo. The targeted LIBS-MBs were injected, via the femoral vein catheter, in a total volume of 100μl. After
diagnostic imaging, we injected 500U/g BW of commercial urokinase plasminogen activator (uPA) (Medac, Germany), 75U/g BW of scFv-scuPA or saline as vehicle control. Repetitive ultrasound imaging sequences were performed every 5 min for an hour after thrombolysis. The acquired videos and images were then analyzed using a linear contrast agent imaging software (VisualSonics Inc.)

**In vivo ultrasound molecular imaging of carotid artery thrombolysis on plasminogen deficient animals**

Plasminogen deficient mice were placed on the VisualSonics imaging station after 6% ferric chloride injury was performed to the left carotid artery. Videos and images were acquired before, during and at several time points after injecting $1.5 \times 10^7$ targeted microbubbles (LIBS-MBs). Thereafter, we injected 75U/g of scFv$_{SCE5}$-scuPA or scFv$_{mut}$-scuPA. Repetitive ultrasound imaging sequences were performed every 5 min for 30 min. A bolus of 100µg/ml human glu-plasminogen (150µl) was injected at the 30 min time-point and repetitive imaging sequences continued for another 30 min. The acquired videos and images were then analyzed using a linear contrast agent imaging software.
Supplemental Figure I: Vector-map and generation of scFv-scuPA constructs. A. Gene-map of scFv-scuPA constructs in pSectag2A vector for mammalian expression. The restriction enzymes used to insert the constructs are *AscI* and *Xhol*. B. Electrophoresis with 0.8% agarose gel. pSectag plasmid (5076 bps) after double digest, and scFv<sub>SCES</sub> (1782 bps) and scFv<sub>mut</sub> (1719 bps) after polymerase chain reaction amplification.
Supplemental Figures II

Supplemental Figure II: SDS-PAGE and Western blot analysis. A. 12% SDS-PAGE of scFv-scuPA. A 61kD band is observed for both scFv-scuPA constructs. B. Western blot analysis of scFv-scuPA before and after plasmin digestion using a horseradish peroxidase-coupled anti-6X His-tag antibody.
Supplemental Figure III: Flow cytometry showing the specificity of \(\text{scFv}_{\text{SCE5}}\)-scuPA on CHO cells expressing activated GPIIb/IIIa receptors. Incubation of \(\text{scFv}_{\text{SCE5}}\)-scuPA with activated GPIIb/IIIa receptors expressing CHO cells resulted in an increase in fluorescence intensity as compared to both either non-expressing or non-activated GPIIb/IIIa expressing CHO cells. PAC1 staining showed an increase in fluorescence intensity for the activated GPIIb/IIIa receptors expressing CHO cells as compared to both either non-expressing or non-activated GPIIb/IIIa expressing CHO cells. There was only background fluorescence intensity for \(\text{scFv}_{\text{mut}}\)-scuPA in all three cells types. Bar graphs depict the median fluorescence intensity values of 3 independent experiments (mean±SD; *\(P<0.05, **P<0.01, ***P<0.001\)). These assays were analyzed with a 2-way repeated measures ANOVA with the Bonferroni post test.
Supplemental Figures IV

Supplemental Figure IV: Flow cytometry assay evaluating the binding properties of scFv-scuPA to platelets activated by various platelet agonists. **A.** Binding of scFv-scuPA was shown with an anti–Penta-His AlexaFluor 488–conjugated monoclonal antibody. Bar graphs depict the median fluorescence intensity values of 3 independent experiments (mean±SD; *P<0.05, **P<0.01, ***P<0.001). Representative histograms are shown underneath the bar graphs. Activated platelets were incubated with 20µM of ADP, 5µg/ml CRP or 20µM TRAP. **B.** Representative histograms showing PAC1 binding to activated platelets. PAC1-FITC binds to activated GPIIb/IIIa on activated platelets, as assessed in PRP that was incubated with three different platelet agonists. **C.** Representative histograms showing P-selectin expression on activated platelets. Anti-CD62P-PE binds to activated platelets, as assessed in PRP that was incubated with three different platelet agonists. Data were analyzed with a 2-way repeated measures ANOVA with the Bonferroni post test.
Supplemental Figure V: Flow cytometry assay demonstrating the specificity of scFvSCE5-scuPA for activated GPIIb/IIIa. A. Binding of scFvSCE5-scuPA was inhibited after addition of the clinically used GPIIb/IIIa blocker abciximab (ReoPro®). Binding of scFv-scuPA was shown with an anti–Penta-His AlexaFluor 488–conjugated monoclonal antibody. Platelets were activated with 20µM of ADP. B. A competition assay with fibrinogen demonstrates that scFvSCE5-scuPA binds to the ligand binding pocket of GPIIb/IIIa. Different concentrations of scFvSCE5-scuPA were incubated with PRP before the addition of Fibrinogen-FITC, showing that increased concentrations of scFvSCE5-scuPA resulted in decreased binding of fibrinogen. Bar graphs depict the median fluorescence intensity values of 3 independent experiments (mean±SD; *P<0.05, **P<0.01, ***P<0.001). Data were analyzed with a 2-way repeated measures ANOVA with the Bonferroni post test.
Supplemental Figure VI: Activity assay of scFvSCE5-scuPA and scFvmut-scuPA on 96-well plates and SDS-PAGE based fibrin zymogram A. Urokinase activity assay using urokinase substrate S2444L. Increase of absorption at 405 nm was measured over a 1 hour period. Both scFv-scuPAs and standards using commercial urokinase at different concentrations resulted in linear enzymatic activity. B. The conversion of plasminogen to plasmin was monitored with the S2251 amidolytic assay. Both scFv-scuPA and commercial uPA at 10nmol/L showed generation of plasmin. An increase in absorbance can be blocked by the incubation with 200µM of the urokinase inhibitor amiloride. C. SDS-PAGE based fibrin zymogram demonstrating direct digestion of fibrin. Commercial uPA produced a lytic zone close to the expected 55kD. Both scFvSCE5-scuPA and scFvmut-scuPA produced lytic zones at around 72kD.
Supplemental Figure VII: Fibrin degradation was observed specifically around platelet aggregates where scFv\textsubscript{\textsc{sce5}-scuPA} bound. Total fibrin degradation was also observed around platelet aggregates perfused with a high dose (1000U/ml) of commercial uPA. Image analysis was done with ImageJ applying a median filter (1.5pixel) and a “fire” false colour look-up table.


Video Legends

Video 1: *In vivo* real time ultrasound monitoring of successful thrombolysis in scFv\textsubscript{SCES}-scuPA treated mice. Ultrasound images were acquired every 5 min for a time period of 1 hour. The initial thrombus is successfully thrombolysed with scFv\textsubscript{SCES}-scuPA over time.

Video 2: *In vivo* real time ultrasound monitoring of thrombus size showing no change in thrombus size in scFv\textsubscript{mut}-scuPA treated mice. Ultrasound images were acquired every 5 min for a time period of 1 hour. Thrombus size of the control group remains constant throughout the 60 min ultrasound monitoring.