Endothelial targeting of a recombinant construct fusing a PECAM-1 single-chain variable antibody fragment (scFv) with prourokinase facilitates prophylactic thrombolysis in the pulmonary vasculature

Bi-Sen Ding, Claudia Gottstein, Andrea Grunow, Alice Kuo, Kumkum Ganguly, Steven M. Albeida, Douglas B. Cines, and Vladimir R. Muzykantov

Means to prevent thrombus extension and local recurrence remain suboptimal, in part because of the limited effectiveness of existing thrombolytics. In theory, plasminogen activators could be used for this purpose if they could be anchored to the vascular lumen by targeting stably expressed, noninternalized determinants such as platelet-endothelial-cell adhesion molecule 1 (PECAM-1). We designed a recombinant molecule fusing low-molecular-weight single-chain prourokinase plasminogen activator (lmw-scuPA) with a single-chain variable fragment (scFv) of a PECAM-1 antibody to generate the prodrug scFv/lmw-scuPA. Cleavage by plasmin generated fibrinolytically active 2-chain lmw-uPA. This fusion protein (1) bound specifically to PECAM-1-expressing cells; (2) was rapidly cleared from blood after intravenous injection; (3) accumulated in the lungs of wild-type C57BL6/J, but not PECAM-1 null mice; and (4) lysed pulmonary emboli formed subsequently more effectively than lmw-scuPA, thereby providing support for the concept of thromboprophylaxis using recombinant scFv-fibrinolytic fusion proteins that target endothelium. (Blood. 2005;106:4191-4198)

Introduction

Plasminogen activators (PAs; eg, uPA, urokinase plasminogen activator) help to restore perfusion after thrombotic vascular occlusion, the leading cause of human morbidity and mortality.1-3 However, the clinical utility of PAs is limited by (1) inadequate delivery because of rapid elimination and inactivation en route and ineffective penetration into formed clots; (2) side effects, including extravasation leading to collateral damage in the central nervous system and other tissues; (3) lysis of “physiologic” (hemostatic) clots leading to hemorrhage; and, (4) reperfusion injury following a delay in restoring perfusion, where morbidity correlates with the duration of ischemia.4,6

Clinical settings characterized by a high propensity for thrombosis have been identified, and means to diagnose early clot formation have been developed.1,2 Although the indications for prophylaxis are known, PAs are not used prophylactically because of their unfavorable pharmacokinetics and side effects. Gene therapy approaches, effective in cell-culture and animal experiments,7,8 are not practical when the need to enhance fibrinolysis is acute and of short duration.9 Conceivably, prophylactic delivery of a PA derivative that rapidly restricts and sustains its activity in the vascular lumen can help to lyse nascent clots expeditiously, inhibit propagation of mural thrombi, and reduce the duration of ischemia.

For example, PAs can be used for thromboprophylaxis by coupling to carrier red blood cells (RBCs), prolonging circulation and limiting extravasation.10 This approach may have utility in settings in which RBC transfusion is part of current management. Drug targeting to suitable endothelial-cell–surface determinants11-13 may provide an alternative approach and, in theory, localize PA activity in the affected intravascular compartment.

For example, drugs coupled with antibodies to platelet-endothelial cell adhesion molecule 1 (PECAM-1) and intercellular adhesion molecule 1 (ICAM-1) bind to the endothelium and exert therapeutic effects in vivo.14,15 These molecules are especially attractive targets for delivering antithrombotic agents, because (1) they are expressed by endothelial cells and are actively involved in sites of inflammation and thrombosis16; (2) after intravenous injection, anti-PECAM and anti-ICAM conjugates accumulate in the pulmonary vasculature, a common site of thromboembolism; (3) these conjugates can also deliver drugs to endothelium in other organs, such as, in cardiac or cerebral vasculature, through infusion via their respective afferent arteries17; and (4) endothelium does not internalize anti-PECAM and anti-ICAM, allowing antithrombotic activity to be maintained on the luminal surface.14,18

From the Department of Pharmacology, University of Pennsylvania, Philadelphia, PA; the Department of Internal Medicine I, Experimental Oncology and Vascular Biology, University of Cologne, Germany; the Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA; the Department of Pulmonary, Allergy, and Critical Care Medicine, University of Pennsylvania, Philadelphia, PA; and the Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, PA.


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B.-S.D. and C.G. contributed equally to the study.

Reprints: Vladimir Muzykantov, IFEM, 1 John Morgan Bldg, University of Pennsylvania, 3620 Hamilton Walk, Philadelphia, PA 19104-6068; e-mail: muzykant@mail.med.upenn.edu.

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We have also found, however, that multivalent conjugates, formed by chemical crosslinking of antibodies and drugs, cluster endothelial PECAM or ICAM, leading to endocytosis and lysosomal degradation,16,19 which terminates the effectiveness of delivered enzymes despite high activity and successful targeting in vivo.11,18,20 FC-fragment-mediated side effects (activation of leukocytes or complement) and translational hurdles also restrict the prospective utility of such conjugates.

To circumvent these problems, we developed recombinant constructs fusing PAs with monovalent single chain variable fragment, scFv. In this study we used low molecular weight single-chain prourokinase plasminogen activator (lmw-scuPA) as a prototype fibrinolytic for immunotargeting to the endothelial luminal surface. Lmw-scuPA, which is generated from native 54-kDa scuPA by hydrolysis of the Glu143-Leu144 peptide bond, has little constitutive protease activity but is readily cleaved by plasmin at Lys158-Ile159 to yield a disulfide-linked 2-chain enzyme with equal potency to full-length 2-chain uPA.21,22 Therefore, targeting lmw-scuPA offers the advantage of plasmin-mediated activation of a latent prodrug into a fully active fibrinolytic agent at sites of thrombosis. Also, lmw-scuPA lacks the domain that mediates binding to the widely expressed urokinase receptor (uPAR, CD87, capable of altering cell adhesion, migration, and proliferation23), which enhances the specificity of the anti–PECAM/lmw-scuPA targeting and effects.

We designed a fusion protein, anti–PECAM single-chain variable fragment (scFv)/lmw-scuPA (indicated hereafter for simplicity as scFv-uPA) and tested its activity in vitro and in wild-type versus PECAM knock-out (KO; null) mice. We found that scFv-uPA (1) displays constitutive antigen-binding and plasmin-inducible fibrinolytic activities; (2) accumulates in the lungs and other vascularized organs after intravenous injection in wild-type but not in PECAM knockout mice; and (3) lyses pulmonary emboli for a longer duration than nontargeted uPA.

Materials and methods
Reagents and cell lines
All chemicals were obtained from Sigma (St Louis, MO), unless otherwise specified. *Drosophila* S2 cells, pMT/Bip/V5 vector and the generation of a plasmid containing urokinase were described previously.24 *Drosophila* serum-free medium was from Invitrogen (Carlsbad, CA). Polymerase chain reaction (PCR) core kit and Rapid DNA ligation kit were purchased from Roche (Basel, Switzerland). Endonucleases were obtained from New England Biolabs (Beverly, MA). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Endonucleases were obtained from New England Biolabs (Beverly, MA). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Endonucleases were obtained from New England Biolabs (Beverly, MA). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Endonucleases were obtained from New England Biolabs (Beverly, MA). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Endonucleases were obtained from New England Biolabs (Beverly, MA). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland). Spectrozyme UK chromogenic substrate, plasmin, and plasmin-free lmw–2-chain uPA (tcuPA) standard were from Roche (Basel, Switzerland).

Cloning of anti-PECAM scFv and anti-PECAM scFv-uPA
P-390 is a rat monoclonal antibody (mAb) to murine PECAM-1 characterized previously.27 The variable regions of the antibody heavy and light chains were cloned into the plasmid pww152 essentially as described.28 The variable heavy chain and light chain were assembled into a scFv fragment by overlap extension PCR and cloned into the expression plasmid pswc4.29 The 390 scFv was amplified in cloning the expression plasmid pMT/Bip/V5 using the upstream primer sen390 (5’-GGACTAGTCAGGT-TACTCTGAAACCTGCTGGC-3’), which introduces a restriction site for *Sspe* I at the 5’ end, and the downstream primer rev390 (5’-AAGAATCGGCGGCGATAAATTTCAGTGGGCC-3’), which introduces a NotI restriction site at the 3’ end. The 390 scFv-lmw scuPA (pMT-BD1) construct was assembled as follows: first, 2 PCR products were purified and digested with *Sspe*, NotI, and NotI, XhoI, respectively. Second, the 2 digested fragments were ligated and cloned into *Sspe* I and XhoI sites of the *Drosophila* expression vector pMT/Bip/V5. Successful cloning was confirmed by restriction analysis of plasmid and by automated sequencing.

Expression and purification of scuPA and anti-PECAM scFv-uPA
*Drosophila* S2 cells were cotransfected with the pMT-BD1 plasmid and pCoHygro (Invitrogen) at the ratio (wt/wt) of 1:1. Stable transfectants were established by adding hygromycin (300 μg/mL). Anti-PECAM scFv-uPA, wild-type scuPA, and active site mutant scuPA-Ser356Ala were expressed using the *Drosophila* Expression System (Invitrogen) and purified from cell media, as described.24

Biochemical characterization of anti-PECAM scFv-uPA
The size and homogeneity of the fusion protein was analyzed on 4% to 12% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with or without addition of plasmin. Conversion to its 2-chain derivative was determined after treatment with 50 mM dithiothreitol (DTT). For Western blot analysis, fractionated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen), and unspecific binding was blocked with tris(hydroxymethyl)aminomethane (Tris)–buffered saline containing 10% nonfat milk powder and 0.1% Tween-20. A rabbit antibody against human uPA (American Diagnostica) served as the primary antibody. The secondary antibody was conjugated with peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA), and the antigen-antibody complex was detected with ECL Plus (Amersham Biosciences, Piscataway, NJ).

Protein modifications
The purified fusion protein was biotinylated with Biotin-LC-NHS ester (Pierce, Rockford, IL), as previously described.15 Proteins were radiolabeled with 125I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce). The purified fusion protein was labeled with 125I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce). The purified fusion protein was labeled with 125I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce). The purified fusion protein was labeled with 125I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce). The purified fusion protein was labeled with 125I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce).

Immunofluorescence microscopy
Human mesothelioma REN cells transfected with cDNA encoding murine PECAM-1 (REN/PECAM) were used to study the binding of scFv-uPA. Untransfected REN cells served as the cell-type control. Cells were seeded in 8-well chamber slides at a density of 1 × 10⁵/mL. After blocking with 5% bovine serum albumin/phosphate-buffered saline (BSA/PBS) and 5 μg/mL scuPA to eliminate background signals, cells were incubated with 25 μg/mL biotinylated fusion protein at 4°C for 2 hours and then with fluorescein-conjugated streptavidin (Calbiochem, Darmstadt, Germany) after fixation with ice-cold paraformaldehyde. Staining was visualized at 40 × magnification. Fluorescence microscopy was performed with an Olympus IX-70 inverted fluorescence microscope (Chroma Technology, Brattleboro, VT). The samples were analyzed with a 40 ×/1.4 NA PlanApo objective (Nikon, Melville, NY). Images were captured with a Hamamatsu Orca-1 CCD camera and Image Pro 3.0 software (Media Cybernetics, Silver Spring, MD).
Cell-bound ELISA

Cells were seeded in 48-well plates, fixed with ice-cold methanol, and blocked with 5% BSA/PBS and 1 μg/mL scuPA. Various concentrations of biotinylated fusion proteins were added. After washing with PBS, cells were further incubated with peroxidase-conjugated streptavidin (Pierce). The colorimetric reaction was carried out with o-phenylenediamine (OPD) substrate (Sigma), and absorbance at 490 nm was measured. A competition enzyme-linked immunosorbent assay (ELISA) was used to determine the specificity of the fusion protein. Serial dilutions of P-390 monoclonal antibody were mixed with 20 μg/mL scFv-uPA and incubated with methanol-fixed cells. Signals were developed as described above.

Urokinase activity

Plasmin was added to a solution containing 0.2 μM purified fusion protein at different molar ratios (1%, 2.5%, 5%, 7.5%). At various times thereafter, a chromogenic assay was performed by adding Spectrozyme UK (American Diagnostica) in assay buffer (50 mM Tris-HCl, 0.01% Tween 80, and 10 KIU [kilo international units]/mL aprotinin, pH 8.5). The same range of concentrations of plasmin was incubated with substrate as a control. The amidolytic activity was determined by comparing the absorbance at 405 nm with that obtained with lmw-tcuPA standards. Fibrolysis using fibrin-coated plates was performed as previously described. Briefly, 5 mg/mL human fibrinogen in PBS was mixed with thrombin (final concentration 1 μg/mL) and plasminogen (final concentration 250 nM). The mixture was poured onto the cover lid of a 24-well plate to form a fibrin gel 5 mm in thickness. The indicated amounts of lmw-tcuPA, lmw-scuPA, or the fusion protein were applied onto the surface of the fibrin clots and incubated at 37°C for 5 hours.

In a separate series of experiments, scFv-uPA was incubated with REN/PECAM or control REN cells for 2 hours in the presence of 1 μg/mL catalytically inactive scuPA-Ser106Ala to block unspecific binding via the lmw-scuPA portion of the molecule. After washing with PBS and incubation with 20 nM plasmin, the cell-associated amidolytic activity was assayed by adding Spectrozyme UK substrate as described above. The specificity of the binding was demonstrated by a competition assay in which various amounts of anti-PECAM immunoglobulin G (IgG) were added to each sample containing 20 μg/mL fusion protein.

Kinetics of cell-bound scFv-uPA and its activity

HSV and REN/PECAM cells were seeded in a 48-well plate at a density of 2 × 10⁶ cells/mL. scFv-uPA (25 μg/mL) was incubated with PBS-washed cells for 2 hours at room temperature. After unbound scFv-uPA was removed by washing in PBS, cells were further incubated for various times at 37°C, and cell-bound fusion protein and urokinase amidolytic activity were determined by using the anti-uhPA ELISA and the Spectrozyme substrate as described under “Urokinase activity.”

Biodistribution of fusion protein and lmw-scuPA in vivo

Male and female C57BL/B6 mice of ages 6 to 10 weeks were used throughout this study, except where noted. A breeding pair of PECAM-1 null mice originally created by Dr Tak Mak was kindly provided by Dr Joseph Madri (Yale University). These mice were backcrossed for more than 10 generations onto the C57BL/B6 background. All protocols were performed in accordance with National Institutes of Health guidelines and with the approval of the University of Pennsylvania Animal Use Committee.

Cocktails containing different amounts of unlabeled protein and a trace amount of radiolabeled protein (0.25 μg) were injected intravenously into anesthetized mice. At the indicated time points, blood was drawn and mice were killed. Organs of interests were harvested, rinsed, and weighed, and the 125I activity in tissues and blood was measured in a gamma counter. The parameters of targeting, including percentage of injected dose per gram tissue (%ID/g), organ-to-blood ratio, and the immunospecificity index (ISI) were calculated after subtracting residual radioactivity in tubes and syringes as described.

Prophylactic fibrinolysis in a model of pulmonary embolism

Ten minutes after injection of thrombolytic agents, a suspension of radiolabeled fibrin emboli was injected into anesthetized mice through the jugular vein as described. One hour later, mice were killed and residual isotope was measured in the lungs. As in previous studies, spontaneous dissolution of emboli in control mice at this time was approximately 50%.

Data analysis

All data are presented as the mean plus or minus standard error of the mean (SEM) of at least 3 separate experiments. Data were compared by analysis of variance (ANOVA). P value less than .025 was considered statistically different.

Results

Design and synthesis of anti-PECAM scFv-uPA

We designed and cloned an expression vector to generate anti-PECAM scFv as shown in Figure 1A. Anti-PECAM scFv was assembled from PCR-amplified cDNAs encoding the variable heavy and light chain regions of the rat mAb to mouse PECAM-1 using hybridoma clone mAb 39014,27 and a (Gly)3 linker sequence (Figure 1B).

DNA encoding scFv was fused with the DNA encoding lmw-scuPA using a (Ser)3Ala linker, yielding the plasmid pMT-BD1 encoding for the fusion protein scFv-scuPA (Figure 1C). scFv-scuPA expression was induced in S2 Drosophila cells as described, and the fusion protein was purified from cell media with a yield of 5 mg/L (Figure 1D).

The protein migrated as a single band at the predicted size (~60 kDa) on SDS-PAGE under reducing conditions, and its identity was confirmed by Western blotting using an anti-uPA antibody (Figure 1D). The fusion protein was cleaved by plasmin into a 2-chain derivative (lmw-tcuPA) composed of 2 nearly identical sized fragments, the N-terminal portion of fusion protein comprising scFv linked to amino acids Leu144-Lys158 of uPA (30 kDa) and the B-chain of uPA (amino acids Ile359-Leu411; molecular weight [MW], 30 kDa), which comigrate and therefore appear as a single band (Figure 1E).

Binding of scFv-uPA to PECAM-expressing cells

Immunofluorescence microscopy using fluorescein isothiocyanate (FITC)–labeled streptavidin showed that biotinylated scFv-uPA bound to a human mesothelioma cell line51 transfected with cDNA encoding murine PECAM-1 (REN/PECAM) but not to control REN cells (Figure 2A). Specific binding of scFv-uPA to PECAM-expressing cells was confirmed by ELISA (Figure 2B). Addition of free anti-PECAM IgG inhibited scFv-uPA binding to REN/ PECAM cells, confirming the specificity of targeting (Figure 2C).
N-terminal fusion of lmw-uPA to the scFv did not compromise its folding, ability to become activated by plasmin, or ability to initiate fibrinolysis.

REN/PECAM, but not control REN cells, incubated with scFv-uPA developed cell-surface enzymatic activity (Figure 3C). Parental anti-PECAM inhibited the delivery of uPA activity to target cells, confirming the specificity of targeting (Figure 3D).

The binding of scFv-uPA to the surface of mouse endothelial cells was relatively stable as determined by ELISA. The half-life of bound scFv-uPA was approximately 5 hours, but the protein was not detected at 24 hours (Figure 3E). The enzymatic activity of cell-bound scFv-uPA declined by 50% within 30 minutes, but it was sustained thereafter at this level for approximately 3 hours (Figure 3E). The longevity of enzymatically active scFv-uPA anchored to the surface of human REN/PECAM cells was even more prolonged: approximately 40% of initial levels of bound scFv-uPA antigen and its activity remained on the cell surface 24 hours after binding (Figure 3F).

Vascular immunotargeting of scFv-uPA in mice

To assess blood clearance, biodistribution, and endothelial targeting, 125I-labeled scFv-uPA versus control scuPA was injected into mice. One hour after intravenous injection, 125I–lmw-scuPA was distributed similarly in organs of wild-type and PECAM KO mice (Figure 4A). Notably, the organ-to-blood ratio, a parameter reflecting targeting, did not exceed 1.0 in any organ, except for the liver, which clears plasminogen activators from the blood. Therefore, lmw-scuPA injected in circulation does not target endothelial cells (Figure 4B).

The organ distribution of 125I-labeled scFv-uPA in PECAM KO mice was nearly identical to that of nontargeted lmw-scuPA (Figure 4A). In contrast, the fusion protein accumulated preferentially in the lungs and, to a somewhat lesser extent, in other highly vascularized organs of wild-type mice expressing PECAM on the surface of endothelium (Figure 4A). The scFv-uPA immunospecificity index (ISI, ratio of tissue uptake of targeted versus nontargeted counterparts, characterizing targeting specificity) was 10.0 in the lungs and 5.0 in the heart of wild-type mice (Figure 4C). In contrast, the ISI of scFv-uPA was less than 1.0 in any organ in PECAM null mice, indicating specific binding to PECAM in endothelial targeting.

Anti-PECAM scFv-uPA was cleared from the circulation more rapidly than nontargeted lmw-scuPA (Figures 4A, 5A), suggesting depletion of the circulating pool as a result of endothelial binding of the fusion protein. In agreement with this interpretation, the blood levels of scFv-uPA and scuPA were identical in PECAM KO mice (Figure 4A).

Pulmonary uptake of the fusion protein in the wild-type mice was maximal at the earliest inspected time point, that is, 5 minutes after injection (Figure 5B). The lung-to-blood ratio of scFv-uPA peaked at 15 to 30 minutes as a result of blood clearance and was relatively stable over the next several hours. Three hours after injection, pulmonary retention of scFv-uPA remained approximately 6-fold higher than nontargeted uPA (Figure 5C).
Endothelial targeting of anti-PECAM scFv-uPA facilitates lysis of pulmonary emboli

Given these favorable targeting and pharmacokinetic characteristics, we tested the effect of scFv-uPA delivery to endothelial PECAM in a mouse model of acute pulmonary thrombosis induced by injecting radiolabeled fibrin emboli (3-5 mm in diameter). After tail vein injection, these emboli form larger aggregates by incorporating additional fibrin and lodge in the pulmonary precapillary bed.32

To model prophylactic fibrinolysis, we injected various doses of scFv-uPA and the same amount of nontargeted lmw-scuPA prior to injecting125I-emboli and measured the residual isotope in the lungs 1 hour later. At all doses tested, the fusion protein produced significantly greater clot lysis than enzymatically identical doses of nontargeted lmw-scuPA (P < .025) (Figure 6A). This effect could not be attributed to the potential benefit of blocking of PECAM-1, because fibrinolysis by a mixture of lmw-scuPA and anti-PECAM did not exceed that produced by lmw-scuPA alone.

Discussion

Recent studies using in vivo fluorescence microscopy suggest that mural fibrin contributes to explosive formation of occlusive intravascular thrombi via complex hemodynamic-dependent pathways involving the generation of thrombin and platelet activation.34 Anchoring a fibrinolytic agent to the endothelial surface might inhibit this amplification step in the thrombosis cascade.

Figure 3. Urokinase activity of free and cell-bound anti-PECAM scFv-uPA. (A) Amidolytic activities of fusion protein generated at different molar ratios of plasmin to scFv-uPA. (B) Fibrinolytic activity using a fibrin plate. From left to right: 1:3 serial dilutions of lmw-tcuPA (50 ng), lmw-scuPA (100 ng), and scFv-lmw scuPA (200 ng) were incubated on a fibrin-coated plate at 37°C. Lytic zones were measured after staining fibrin with trypan blue. (C) Amidolytic activity associated with the cell surface of control REN (C) versus PECAM-transfected (F, REN/PECAM) REN cells was determined by conversion of chromogenic substrate after incubation with various amounts of fusion protein. Preincubation of REN/PECAM cells with parental anti-PECAM IgG, mAb 390, reduces binding of enzymatically active scFv-uPA. Kinetics of disappearance of cell-bound scFv-uPA (C) and its amidolytic activity (D) was determined by using HSV mouse endothelioma cells (E) and human REN/PECAM-1 cells (F). Basal levels of uPA antigen (C) and amidolytic activity (D) were determined by using intact cells. Amounts of fusion protein and amidolytic activity anchored to cell surface were significantly different from basal levels at 3 hours in mouse cells (P < .002) and 24 hours in human cells (P < .01). Error bars indicate SEM.

Figure 4. Biodistribution of anti-PECAM scFv-lmw scuPA and lmw-scuPA in vivo. Ten micrograms fusion protein or equal molar lmw-scuPA was mixed with 0.2 μg radiolabeled tracer protein and injected intravenously into wild-type or PECAM null mice, respectively. One hour later, tissue uptake was measured. (A) Percentage of injected dose per gram tissue (%ID/g). Note that scFv-uPA, but not scuPA, shows preferential uptake in the lungs and other vascularized organs in wild-type (WT), but not in PECAM KO mice. (B) Organ-to-blood ratio for various organs. Broken line indicates base level ratio equal to 1.0. (C) Immunospecificity index (ISI), calculated as ratio of organ-to-blood ratios of targeted and untargeted counterparts. The interrupted line shows an ISI of 1.0, reflecting equal tissue levels of targeted and untargeted counterparts. Error bars indicate SEM.
thereby inhibiting clot propagation, local recurrence, and ischemia. A strategy using prophylactic immunotargeting of plasminogen activators may, therefore, improve the clinical outcome of thrombosis.

In this study, we report the efficacy of a recombinant fusion protein consisting of scFv of an anti-PECAM antibody linked to lmw-scuPA. PECAM-1 is stably expressed at high density on the luminal surface of the endothelium, providing a target that can be used for thromboprophylaxis. Further, anti–PECAM-1 is not rapidly internalized (unlike selectins, vascular cell-adhesion molecule 1 [VCAM-1], angiotensin-converting enzyme [ACE], and caveolar antigens which internalize within < 15 minutes)12,13,31,35,36 which makes it a suitable carrier for drugs acting on the vascular lumen.

The data in this paper show that a single intravenous injection of anti-PECAM scFv-uPA increases uPA delivery to the pulmonary endothelium 10-fold and enhances pulmonary fibrinolysis 2- to 3-fold, thereby supporting the concept that antithrombotic agents can be delivered in effective concentrations and for a relevant duration to the endothelial surface. The proposed paradigm is modeled in Figure 6B (see the legend).

Several features in the design of this construct should be noted. First, deletion of growth factor domain of uPA from the construct restricts delivery of the proenzyme to PECAM-expressing cells and avoids the potential for unintended uPAR/CD87-mediated effects. Second, plasmin-mediated conversion of the prodrug lmw-scuPA in the fusion construct into enzymatically active lmw-tcuPA represents a prototype for a paradigm to minimize adverse effects by preventing the premature activation/inactivation of drugs (Figure 3A). Third, the recombinant fusion format offers (1) modularity of targeting and/or composition of the drug moieties; (2) feasibility of additional modifications, such as insertion of enzyme activating/release sequences37 to enhance local activation and permit the enzyme to diffuse into adjacent clots, thereby further increasing specificity and effectiveness; (3) feasibility of industrial production of homogeneous molecules; and (4) monovalent, monomeric structure of the resultant fusion protein.

Several laboratories have produced chemical and recombinant fusion conjugates of antithrombotic drugs with antibody fragments targeting components of thrombi, such as activated platelets (eg, P-selectin) and cryptic epitopes exposed on fibrin.38-45 However, delivery systems targeting determinants appearing after the thrombotic event are likely to be less suitable for prophylactic usage (in part because of their masking by thrombi) versus those targeting stably expressed endothelial determinants, such as PECAM-1, which bind to at-risk vasculature. In addition, masking the adhesion
molecules themselves by bound fusion protein may help alleviate inflammatory pathology, often intertwined with thrombosis.33

Sustained anchorage of antithrombotic drugs to the endothelial surface is a critical component in success of vascular immunotargeting for thromboprophylaxis. Monovalent scFv constructs are internalized far more slowly than polyvalent multimeric anti-PECAM conjugates, which are internalized by endothelial cells within 5 to 15 minutes.16,18 Indeed, the kinetics studies (Figures 3 and 5) showed that (1) endothelial targeting, assessed by elevated pulmonary 125I-scFv-uPA, persists for at least 3 hours after injection in mice and (2) scFv-uPA is retained for approximately 3 and approximately 24 hours on the surface of mouse and human cells, respectively. These results warrant further systematic studies of the behavior of anti-PECAM scFv-uPA and mechanisms of its disappearance from the lumen (likely because of shedding) and inactivation (likely by PA inhibitors) to improve efficacy. However, the more rapid disappearance of scFv-uPA from the surface of murine versus human cells (Figure 3E versus F) and well-known potency of murine inhibitors toward human PAs imply that data obtained in mouse models provide a minimal estimate of longevity that can be anticipated in humans.

We used pulmonary microembolism as a model system to analyze fibrinolysis in vivo. However, prophylactic fibrinolysis by PECAM-targeted PA may help prevent extension/recurrence of pulmonary thromboembolism12,46 and attenuate acute lung graft rejection because of thrombotic occlusion of the pulmonary vasculature.17 Because of the pan-endothelial nature of PECAM expression and the opportunity to enrich local delivery using catheters,17 a wider utility to protect other vascular beds against imminent thrombosis can be envisioned. The cardiac accumulation of anti-PECAM scFv-uPA noted in this work (Figure 4A) is of interest in this context. Thus, these data help to support the general concept that intermittent injection of scFv/PA fusion constructs targeted to PECAM may provide local thromboprophylaxis in patients with acute or subacute risk of developing new or recurrent thrombi and preventing clot extension in clinical settings in which the risk is most intense over several hours to days (eg, transient ischemic attack, stroke in evolution, unstable angina, and acute chest syndrome in sickle cell disease, among others). Nascent iatrogenic thrombi formed in immobilized patients and after surgical interventions and transplantation (whereby drugs can be injected prophylactically into the donor graft directly, thus avoiding the systemic circulation of the recipient) also represent potential targets. Endothelial-cell adhesion molecules represent attractive, but not exclusive, targets for antithrombotic therapy. The search for differentially expressed endothelial-cell antigens has led to the identification of “vascular addresses.”12,46 Antibody-directed targeting of drugs to specific vascular areas has been reported.19,20 In theory, organ- or vessel type-specific “ZIP codes” for targeting, combined with local infusion into conduit vessels, will further enhance the specificity of drug delivery and effects and could open the possibility for local prophylaxis, such as for containment of deep vein thrombosis of the lower extremities.

In summary, we have constructed a recombinant fusion protein targeting a fibrinolytic prodrug to a luminal endothelial-cell antigen. This fusion protein specifically targets endothelial cells in vitro and in vivo and provides antigen-specific enhancement of fibrinolytic activity in a mouse model of pulmonary thrombosis, providing evidence that vascular immunotargeting can be used for prophylactic and, perhaps, therapeutic fibrinolysis.

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We thank Ms T. Krasik for invaluable help in animal experiments (both from the University of Pennsylvania) for advice in experimental procedures.

References

2. Marler JR, Goldstein LB. Stroke; tPA and the new thrombolysis for patients with acute ischemic attack, stroke in evolution, unstable angina, and acute chest syndrome in sickle cell disease, among others). Nascent iatrogenic thrombi formed in immobilized patients and after surgical interventions and transplantation (whereby drugs can be injected prophylactically into the donor graft directly, thus avoiding the systemic circulation of the recipient) also represent potential targets. Endothelial-cell adhesion molecules represent attractive, but not exclusive, targets for antithrombotic therapy. The search for differentially expressed endothelial-cell antigens has led to the identification of “vascular addresses.”12,46 Antibody-directed targeting of drugs to specific vascular areas has been reported.19,20 In theory, organ- or vessel type-specific “ZIP codes” for targeting, combined with local infusion into conduit vessels, will further enhance the specificity of drug delivery and effects and could open the possibility for local prophylaxis, such as for containment of deep vein thrombosis of the lower extremities.

In summary, we have constructed a recombinant fusion protein targeting a fibrinolytic prodrug to a luminal endothelial-cell antigen. This fusion protein specifically targets endothelial cells in vitro and in vivo and provides antigen-specific enhancement of fibrinolytic activity in a mouse model of pulmonary thrombosis, providing evidence that vascular immunotargeting can be used for prophylactic and, perhaps, therapeutic fibrinolysis.

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Endothelial targeting of a recombinant construct fusing a PECAM-1 single-chain variable antibody fragment (scFv) with prourokinase facilitates prophylactic thrombolysis in the pulmonary vasculature

Bi-Sen Ding, Claudia Gottstein, Andrea Grunow, Alice Kuo, Kumkum Ganguly, Steven M. Albelda, Douglas B. Cines and Vladimir R. Muzykantov