

# 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

**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)**

© 2005 by The American Society of Hematology

## 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.<sup>1-3</sup> 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.<sup>4-6</sup>

Clinical settings characterized by a high propensity for thrombosis have been identified, and means to diagnose early clot formation have been developed.<sup>1,2</sup> 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,<sup>7,8</sup> are not practical when the need to enhance fibrinolysis is acute and of short duration.<sup>9</sup> 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.<sup>10</sup> This approach may have utility in settings in which RBC transfusion is part of current management. Drug targeting to suitable endothelial-cell-surface determinants<sup>11-13</sup> 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.<sup>14,15</sup> 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 thrombosis<sup>16</sup>; (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 arteries<sup>17</sup>; and (4) endothelium does not internalize anti-PECAM and anti-ICAM, allowing antithrombotic activity to be maintained on the luminal surface.<sup>14,18</sup>

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.

Submitted May 18, 2005; accepted August 16, 2005. Prepublished online as *Blood* First Edition Paper, September 6, 2005; DOI 10.1182/blood-2005-05-2002.

Supported by grants from the National Institutes of Health (NIH) (HL71175, HL071174, HL079063, DOD PR 012262) and from the University of Pennsylvania Research Foundation (V.R.M.) and by grants from NIH (HL076406, HL076206, HL60169) and from the University of Pennsylvania Research Foundation (D.B.C.).

B.-S.D. and C.G. designed and performed the experiment and wrote the paper; A.G., A.K., and K.G. designed and performed the experiment; S.M.A. and D.B.C. helped design the experiment and wrote the paper; V.R.M. designed the study, wrote the paper, and serves as corresponding author.

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.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology

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,<sup>16,19</sup> which terminates the effectiveness of delivered enzymes despite high activity and successful targeting in vivo.<sup>11,18,20</sup> 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 Glu<sup>143</sup>-Leu<sup>144</sup> peptide bond, has little constitutive protease activity but is readily cleaved by plasmin at Lys<sup>158</sup>-Ile<sup>159</sup> to yield a disulfide-linked 2-chain enzyme with equal potency to full-length 2-chain uPA.<sup>21,22</sup> 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 proliferation<sup>23</sup>), 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.<sup>24</sup> *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 (tuPA) standard were from American Diagnostica (Stamford, CT). REN cells, a human mesothelioma cell line previously isolated in our laboratories,<sup>25</sup> were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (R10 media) containing 10 000 U penicillin and 10 000 U streptomycin. REN cells transfected with full-length mouse PECAM (REN/PECAM cells) have been described previously.<sup>26</sup> The H5V mouse endothelioma cell line was kindly provided by Dr Silvia Muro (University of Pennsylvania). These cells were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine.

### Cloning of anti-PECAM scFv and anti-PECAM scFv-uPA

P-390 is a rat monoclonal antibody (mAb) to murine PECAM-1 characterized previously.<sup>27</sup> The variable regions of the antibody heavy and light chains were cloned into the plasmid pww152 essentially as described.<sup>28</sup> The variable heavy chain and light chain were assembled into a scFv fragment by overlap extension PCR and cloned into the expression plasmid pswc4.<sup>29</sup>

The 390 scFv was amplified for cloning in the expression plasmid pMT/Bip/V5 using the upstream primer sen390 (5'-GGACTAGTCAGGT-TACTCTGAAAGCGTCTGGCCC-3'), which introduces a restriction site for *SpeI* at the 5' end, and the downstream primer rev390 (5'-ATA-AGAATGCGGCCGCGCCGGAAGAGCTACTACCCGATGAGGAAG-AACGCAATTCACCTTGG-3'), which appends the sequence of a short peptide linker (Ser<sub>4</sub>Gly)<sub>2</sub> and a *NotI* restriction site.

Lmw-scuPA (Leu144-Leu411) was amplified with the primers senUK (5'-ATAAGAATGCGGCCGCATTAATAATTTTCAGTGTGGCC-3'), which introduces a *NotI* restriction site at the 5' end, and downstream revUK (5'-CCGCTCGAGTCAGAGGGCCAGGCCATTC-3') to introduce an *XhoI* 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 *SpeI*, *NotI*, and *NotI*, *XhoI*, respectively. Second, the 2 digested fragments were ligated and cloned into *SpeI* 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 19:1, and stable transfectants were established by adding hygromycin (300 µg/mL). Anti-PECAM scFv-uPA, wild-type scuPA, and active site mutant scuPA-Ser<sup>356</sup>Ala were expressed using the *Drosophila* Expression System (Invitrogen) and purified from cell media, as described.<sup>24</sup>

### 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.<sup>14</sup> Proteins were radiolabeled with <sup>125</sup>I-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 × 10<sup>5</sup>/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  $\mu\text{g}/\text{mL}$  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  $\mu\text{g}/\text{mL}$  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  $\mu\text{M}$  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. Fibrinolysis using fibrin-coated plates was performed as previously described.<sup>20</sup> Briefly, 5 mg/mL human fibrinogen in PBS was mixed with thrombin (final concentration 1  $\mu\text{g}/\text{mL}$ ) 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  $\mu\text{g}/\text{mL}$  catalytically inactive scuPA-Ser<sup>356</sup>Ala 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  $\mu\text{g}/\text{mL}$  fusion protein.

### Kinetics of cell-bound scFv-uPA and its activity

H5V and REN/PECAM cells were seeded in a 48-well plate at a density of  $2 \times 10^5$  cells/mL. scFv-uPA (25  $\mu\text{g}/\text{mL}$ ) 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-uPA ELISA and the Spectrozyme substrate as described under "Urokinase activity."

### Biodistribution of fusion protein and lmw-scuPA in vivo

Male and female C57BL/6 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<sup>30</sup> was kindly provided by Dr Joseph Madri (Yale University). These mice were backcrossed for more than 10 generations onto the C57BL/6 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  $\mu\text{g}$ ) 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 <sup>125</sup>I 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.<sup>31</sup>

### 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.<sup>10,32</sup> One hour later, mice were killed and residual isotope was measured in the lungs. As in previous studies,<sup>10,32</sup> 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 390<sup>14,27</sup> and a (Gly<sub>4</sub>Ser)<sub>3</sub> linker sequence (Figure 1B).

DNA encoding scFv was fused with the DNA encoding lmw-scuPA using a (Ser<sub>2</sub>Gly)<sub>2</sub>Ala<sub>3</sub> linker, yielding the plasmid pMT-BD1 encoding for the fusion protein scFv-uPA (Figure 1C). scFv-uPA expression was induced in S2 *Drosophila* cells as described,<sup>24</sup> 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 identically sized fragments, the N-terminal portion of fusion protein comprising scFv linked to amino acids Leu<sup>144</sup>-Lys<sup>158</sup> of uPA (30 kDa) and the B-chain of uPA (amino acids Ile<sup>159</sup>-Leu<sup>411</sup>; 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 line<sup>33</sup> 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).

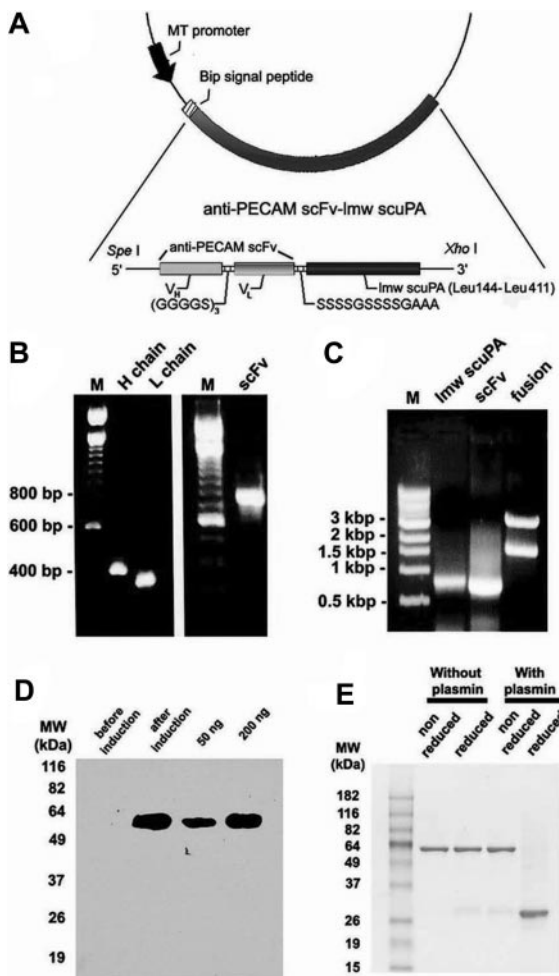
### Enzymatic and fibrinolytic activity of anti-PECAM scFv-uPA

Plasmin converted scFv-uPA fusion protein into 2-chain lmw-urokinase (Figure 1E). To verify that this produces an active urokinase from a latent prodrug, we tested its enzymatic activity using a chromogenic substrate. Indeed, plasmin induced a marked, dose-dependent increase in the amidolytic activity of scFv-uPA from a basal level of 5040 IU/mg or less to 46 590 IU/mg (Figure 3A). scFv-uPA lysed preformed fibrin clots containing plasminogen to the same extent as free lmw-scuPA (Figure 3B). Therefore,

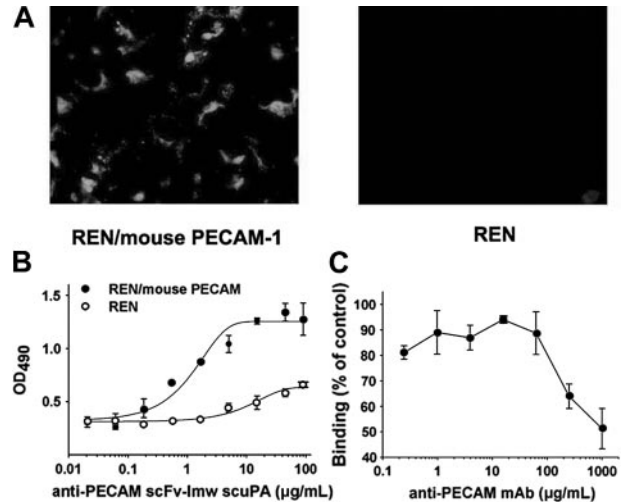
N-terminal fusion of lmw-uPA to the scFv did not compromise its folding, ability to become activated by plasmin, or ability to initiate fibrinolysis.

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



**Figure 1. Molecular design, expression, and characterization of anti-PECAM scFv-lmw scuPA.** (A) Schematic diagram describing the cloning strategy for the fusion construct pMT-BD1. Variable domains of heavy chain and light chain were linked by a (Gly<sub>4</sub>Ser)<sub>3</sub> linker and then fused to the N-terminus of lmw-scuPA by a (Ser<sub>4</sub>Gly)<sub>2</sub>Ala<sub>3</sub> linker. (B) Variable domains of heavy chain (H chain) and light chain (L chain) of P-390 were amplified and assembled into full-length scFv. M indicates DNA standards. (C) Lmw-scuPA and anti-PECAM scFv were ligated and cloned into *Spe*I and *Xho*I sites of the pMT expression vector. *Xho*I and *Spe*I digestion of the fusion construct (fusion). (D) Western blot analysis of 40  $\mu$ L culture medium alone or after induction by 0.5 mM CuSO<sub>4</sub>. Purified fusion protein (50 ng and 200 ng) was blotted to compare expression levels. (E) Four percent to 12% gradient SDS-PAGE of purified fusion protein with or without plasmin treatment under unreduced or reduced conditions.



**Figure 2. Specific binding of scFv-uPA fusion protein to cells expressing mouse PECAM.** (A) FITC-streptavidin staining of REN/PECAM (left) versus control REN (right) cells after incubation with biotinylated anti-PECAM scFv-scuPA (original magnification  $\times 40$ ). (B) ELISA: binding of anti-PECAM scFv-uPA to REN/PECAM (●) versus REN (○) cells. (C) ELISA: inhibition of binding of fusion protein to REN/PECAM cells by parental anti-PECAM IgG mAb 390. Error bars indicate standard error of the mean (SEM).

scFv-uPA antigen and its activity remained on the cell surface 24 hours after binding (Figure 3F).

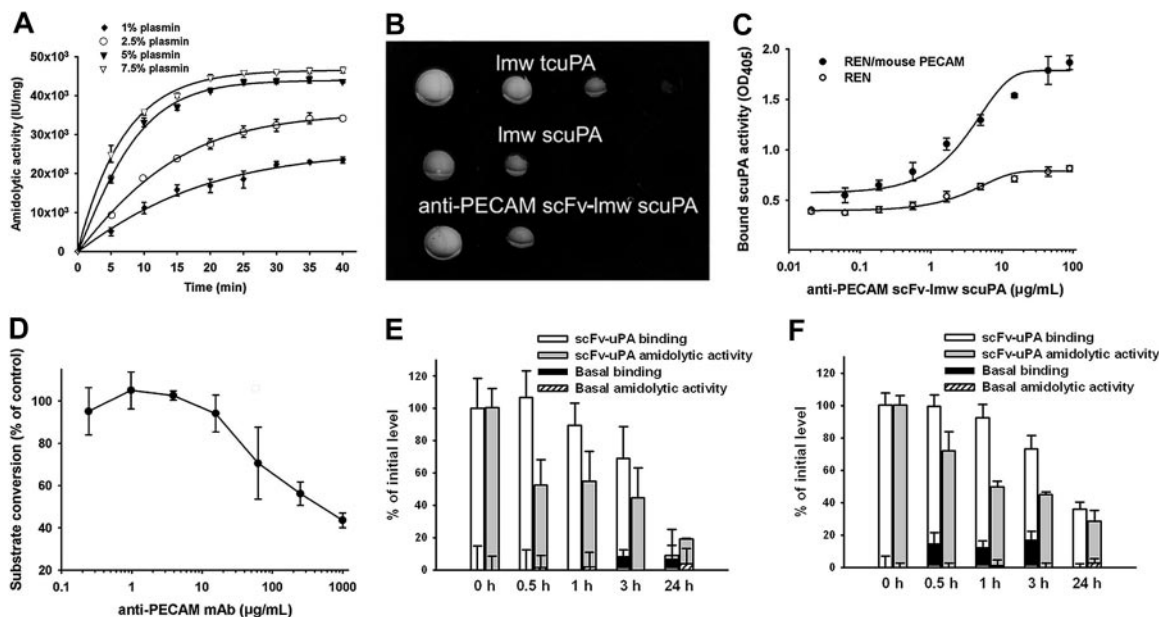
#### Vascular immunotargeting of scFv-uPA in mice

To assess blood clearance, biodistribution, and endothelial targeting, <sup>125</sup>I-labeled scFv-uPA versus control scuPA was injected into mice. One hour after intravenous injection, <sup>125</sup>I-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 <sup>125</sup>I-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).



**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 1mw-tcuPA (50 ng), 1mw-scuPA (100 ng), and scFv-1mw 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 (○) versus PECAM-transfected (●, REN/PECAM) REN cells was determined by conversion of chromogenic substrate after incubation with various amounts of fusion protein. (D) 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 (□) and its amidolytic activity (▨) was determined by using H5V mouse endothelioma cells (E) and human REN/PECAM-1 cells (F). Basal levels of uPA antigen (■) and amidolytic activity (▩) 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.

**Endothelial targeting of anti-PECAM scFv-uPA facilitates lysis of pulmonary emboli**

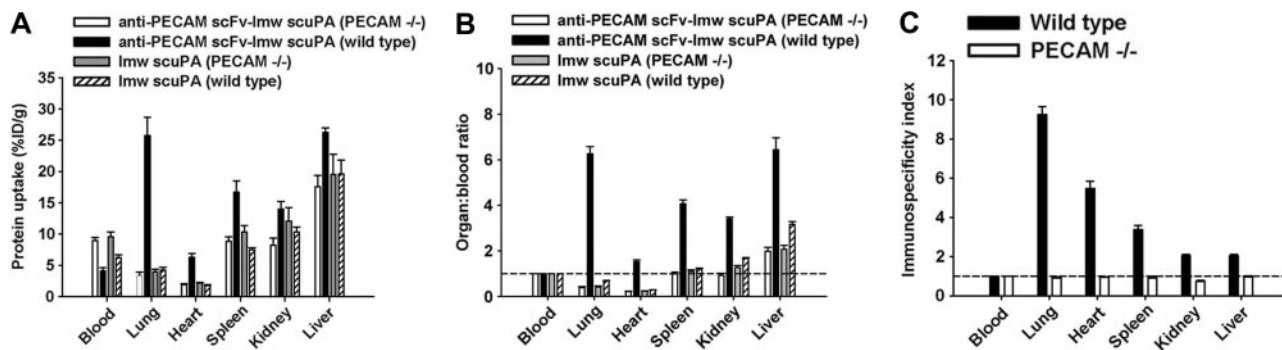
Given these favorable targeting and pharmacokinetic characteristics, we tested the effect of scFv-uPA delivery to endothelial PECAM in a mouse model of acute pulmonary thrombosis induced by injecting radiolabeled fibrin emboli (3-5 µm in diameter). After tail vein injection, these emboli form larger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

To model prophylactic fibrinolysis, we injected various doses of scFv-uPA and the same amount of nontargeted 1mw-scuPA prior to injecting <sup>125</sup>I-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

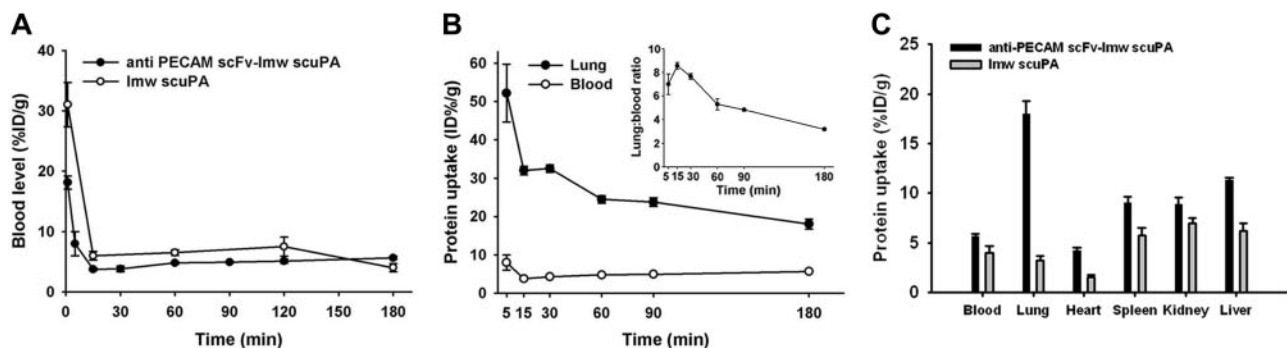
nontargeted 1mw-scuPA ( $P < .025$ ) (Figure 6A). This effect could not be attributed to the potential benefit of blocking of PECAM-1, because fibrinolysis by a mixture of 1mw-scuPA and anti-PECAM did not exceed that produced by 1mw-scuPA alone.

**Discussion**

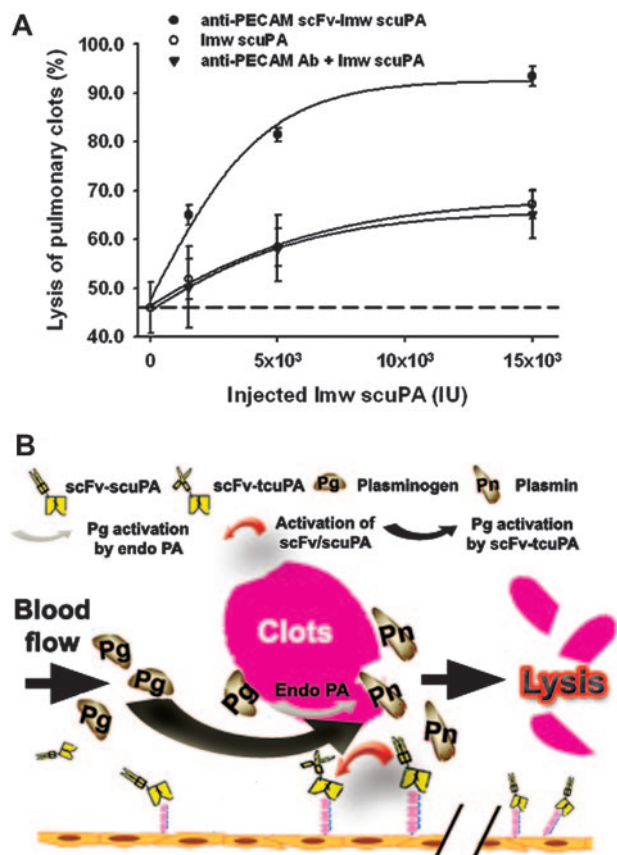
Recent studies using in vivo fluorescence microscopy suggest that mural fibrin contributes to explosive formation of occlusive intravascular thrombi via complex hemodynamic-dependent pathways involving the generation of thrombin and platelet activation.<sup>34</sup> Anchoring a fibrinolytic agent to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



**Figure 4. Biodistribution of anti-PECAM scFv-1mw scuPA and 1mw-scuPA in vivo.** Ten micrograms fusion protein or equal molar 1mw-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 blood level; ratio equal to 1.0. (C) Immunospecificity index (ISI), calculated as ratio of organ-to-blood ratios of targeted and untargeted counterpart. The interrupted line shows an ISI of 1.0, reflecting equal tissue levels of targeted and untargeted counterparts. Error bars indicate SEM.



**Figure 5. Blood clearance of anti-PECAM scFv-sc uPA and kinetics of in vivo pulmonary targeting.** (A) Blood clearance of targeted fusion construct (●) and nontargeted scuPA (○) in %ID/g (percentage of injected dose per gram tissue). (B) Kinetics of the targeted fusion protein levels in lungs (●) and blood (○). Fusion protein exhibited a rapid and prolonged accumulation in lung tissues. Lung-to-blood ratios at indicated time points were calculated (inset). (C) In vivo biodistribution of <sup>125</sup>I-scFv-uPA (■) and free uPA (□) 3 hours after intravenous injection in wild-type mice. Error bars indicate SEM.



**Figure 6. Accumulation of fusion protein in pulmonary vasculature facilitates local fibrinolysis.** (A) Dose-response curve of pulmonary thrombolysis. Dissolution of <sup>125</sup>I-labeled microemboli lodged in mouse pulmonary vasculature by bolus injection of 300, 100, and 30  $\mu$ g fusion protein, the equivalent amounts of lmw-sc uPA (150, 50, and 15  $\mu$ g) versus a mixture of lmw-sc uPA and parental antibody, respectively. Thrombolytic potency was expressed as the percentage of lysis versus the dose administered. Interrupted line indicates spontaneous lysis. Error bars indicate SEM. (B) Simplified model of a proposed strategy for thromboprophylaxis using vascular immunotargeting of genetically engineered anti-PECAM scFv-uPA fusion protein. Anti-PECAM scFv-uPA circulates in a form of a prodrug, single-chain uPA, binds to PECAM-1, and remains anchored on the luminal surface of endothelium for at least several hours. In situ thrombosis or embolism induces initial local conversion of plasminogen (Pg) into plasmin (Pn) by endogenous plasminogen activators (Endo-PA). Plasmin (and perhaps other enzymes) formed in the vicinity of the clot converts the endothelium-bound scFv-sc uPA into enzymatically active tcuPA (Figure 3A), which in turn amplifies local formation of plasmin, reinforcing local thrombolysis, preventing clot extension and reocclusion.

thereby inhibiting clot propagation, local recurrence, and ischemia. A strategy using prophylactic immunotargeting of plasminogen activators may, therefore, improve the clinical outcome of thrombolysis.

In this study, we report the efficacy of a recombinant fusion protein consisting of scFv of an anti-PECAM antibody linked to lmw-sc uPA. 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)<sup>12,13,31,35,36</sup> 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-sc uPA 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 sequences<sup>37</sup> 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.<sup>38-45</sup> 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.<sup>33</sup>

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.<sup>16,19</sup> Indeed, the kinetics studies (Figures 3 and 5) showed that (1) endothelial targeting, assessed by elevated pulmonary <sup>125</sup>I-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 future 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 to prevent extension/recurrence of pulmonary thromboembolism<sup>32,46</sup> and attenuate acute lung graft rejection because of thrombotic occlusion of the pulmonary vasculature.<sup>47</sup> Because of the pan-endothelial nature of PECAM expression and the opportunity to enrich local delivery using catheters,<sup>17</sup> 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.”<sup>12,48</sup> Antibody-directed targeting of drugs to specific vascular areas has been reported.<sup>49,50</sup> 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.

## Acknowledgments

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

## References

1. Topol EJ, Morris D, Smalling R, et al. A multicenter, randomized, placebo-controlled trial of a new form of intravenous recombinant tissue-type plasminogen activator (Activase) in acute myocardial infarction. *J Am Coll Cardiol*. 1987;9:1205-1213.
2. Marler JR, Goldstein LB. Stroke: tPA and the clinic. *Science*. 2003;301:1677.
3. Collen D. Towards improved thrombolytic therapy. *Lancet*. 1993;342:34-36.
4. Sakharov DV, Rijken DC. Superficial accumulation of plasminogen during plasma clot lysis. *Circulation*. 1995;92:1883-1890.
5. Zhu Y, Carmeliet P, Fay WP. Plasminogen activator inhibitor-1 is a major determinant of arterial thrombolysis resistance. *Circulation*. 1999;99:3050-3055.
6. Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nat Med*. 1998;4:228-231.
7. Vassalli G, Dichek DA. Gene therapy for arterial thrombosis. *Cardiovasc Res*. 1997;35:459-469.
8. Waugh JM, Kattash M, Li J, et al. Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. *Proc Natl Acad Sci U S A*. 1999;96:1065-1070.
9. Falkenberg M, Tom C, DeYoung MB, Wen S, Linemann R, Dichek DA. Increased expression of urokinase during atherosclerotic lesion development causes arterial constriction and lumen loss, and accelerates lesion growth. *Proc Natl Acad Sci U S A*. 2002;99:10665-10670.
10. Murciano JC, Medina S, Eslin D, Atochina E, Cines DB, Muzykantor VR. Prophylactic fibrinolysis through selective dissolution of nascent clots by tPA-carrying erythrocytes. *Nat Biotechnol*. 2003;21:891-896.
11. Ding BS, Zhou YJ, Chen XY, et al. Lung endothelium targeting for pulmonary embolism thrombolysis. *Circulation*. 2003;108:2892-2898.
12. Oh P, Li Y, Yu J, et al. Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature*. 2004;429:629-635.
13. Muzykantor VR. Targeting pulmonary endothelium. In: Muzykantor V and Torchilin V, eds. *Biomedical Aspects of Drug Targeting*. Boston, MA: Kluwer Academic Publishers; 2003:129-148.
14. Muzykantor VR, Christofidou-Solomidou M, Balyasnikova I, et al. Streptavidin facilitates internalization and pulmonary targeting of an anti-endothelial cell antibody (PECAM): a strategy for intraendothelial drug delivery. *Proc Natl Acad Sci U S A*. 1999;96:2379-2384.
15. Kozover BD, Christofidou-Solomidou M, Sweitzer TD, et al. Immunotargeting of catalase to the pulmonary endothelium alleviates oxidative stress and reduces acute transplantation lung injury. *Nat Biotechnol*. 2003;21:392-398.
16. Muro S, Koval M, Muzykantor VR. Endothelial endocytic pathways: gates for vascular drug delivery. *Curr Vasc Pharmacol*. 2004;2:281-299.
17. Scherpereel A, Rome JJ, Wiewrodt R, et al. Platelet-endothelial cell adhesion molecule-1-directed immunotargeting to cardiopulmonary vasculature. *J Pharmacol Exp Ther*. 2002;300:777-786.
18. Murciano JC, Muro S, Koniari L, et al. ICAM-directed vascular immunotargeting of anti-thrombotic agents to the endothelial luminal surface. *Blood*. 2003;101:3977-3984.
19. Muro S, Wiewrodt R, Thomas A, et al. A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J Cell Sci*. 2003;116:1599-1609.
20. Muzykantor VR, Barnathan E, Atochina E, Danilov S, Fisher AB. Targeting of antibody-conjugated plasminogen activators to the pulmonary vasculature. *J Pharmacol Exp Ther*. 1996;279:1026-1034.
21. Pannell R, Gurewich V. Activation of plasminogen by single-chain urokinase or by two-chain urokinase: a demonstration that single-chain urokinase has a low catalytic activity (pro-urokinase). *Blood*. 1987;69:22-26.
22. Stump DC, Lijnen HR, Collen D. Purification and characterization of a novel low molecular weight form of single-chain urokinase-type plasminogen activator. *J Biol Chem*. 1986;261:17120-17126.
23. Blasi F, Carmeliet P. UPAR: a versatile signaling orchestrator. *Nat Rev Mol Cell Biol*. 2002;3:932-943.
24. Bdeir K, Kuo A, Sachais BS, et al. The kringle stabilizes urokinase binding to the urokinase receptor. *Blood*. 2003;102:3600-3608.
25. Smythe WR, Hwang HC, Amin KM, et al. Use of

- recombinant adenovirus to transfer the herpes simplex virus thymidine kinase (HSVtk) gene to thoracic neoplasms: an effective in vitro drug sensitization system. *Cancer Res.* 1994;54:2055-2059.
26. Scherpereel A, Wiewrodt R, Christofidou-Solomidou M, et al. Cell-selective intracellular delivery of a foreign enzyme to endothelium in vivo using vascular immunotargeting. *FASEB J.* 2001;15:416-426.
  27. Yan HC, Baldwin HS, Sun J, Buck CA, Albelda SM, DeLisser HM. Alternative splicing of a specific cytoplasmic exon alters the binding characteristics of murine PECAM-1. *J Biol Chem.* 1995;270:23672-23680.
  28. Derbyshire EJ, Gottstein C, Thorpe PE. Immunotoxins. In: Turner M, Johnstone A, eds. *Immunochemistry 1: A Practical Approach.* New York, NY: Oxford University Press; 1997:239-273.
  29. Gottstein C, Wels W, Ober B, Thorpe PE. Generation and characterization of recombinant vascular targeting agents from hybridoma cell lines. *Biotechniques.* 2001;30:190-200.
  30. Duncan GS, Andrew DP, Takimoto H, et al. Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. *J Immunol.* 1999;162:3022-3030.
  31. Danilov SM, Gavriluyk VD, Franke FE, et al. Lung uptake of antibodies to endothelial antigens: key determinants of vascular immunotargeting. *Am J Physiol Lung Cell Mol Physiol.* 2001;280:L1335-L1347.
  32. Murciano JC, Harshaw D, Neschis DG, et al. Platelets inhibit the lysis of pulmonary microemboli. *Am J Physiol Lung Cell Mol Physiol.* 2002;282:L529-L539.
  33. Vaporciyan AA, DeLisser HM, Yan HC, et al. Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. *Science.* 1993;262:1580-1582.
  34. Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med.* 2002;8:1175-1181.
  35. Kiely JM, Cybulsky MI, Luscinskas FW, Gimbrone MA Jr. Immunoselective targeting of an anti-thrombin agent to the surface of cytokine-activated vascular endothelial cells. *Arterioscler Thromb Vasc Biol.* 1995;15:1211-1218.
  36. Ogawara K, Rots MG, Kok RJ, et al. A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells in vitro and in vivo. *Hum Gene Ther.* 2004;15:433-443.
  37. Yang WP, Goldstein J, Procyk R, Matsueda GR, Shaw SY. Design and evaluation of a thrombin-activable plasminogen activator. *Biochemistry.* 1994;33:606-612.
  38. Bode C, Matseuda GR, Hui KY, Haber E. Antibody-directed urokinase: a specific fibrinolytic agent. *Science.* 1985;229:765-767.
  39. Hagemeyer CE, Tomic I, Weirich U, et al. Construction and characterization of a recombinant plasminogen activator composed of an anti-fibrin single-chain antibody and low-molecular-weight urokinase. *J Thromb Haemost.* 2004;2:797-803.
  40. Holvoet P, Laroche Y, Lijnen HR, et al. Characterization of a chimeric plasminogen activator consisting of a single-chain Fv fragment derived from fibrin fragment D-dimer-specific antibody and a truncated single-chain urokinase. *J Biol Chem.* 1991;266:19717-19724.
  41. Peter K, Graeber J, Kipriyanov S, et al. Construction and functional evaluation of a single-chain antibody fusion protein with fibrin targeting and thrombin inhibition after activation by factor Xa. *Circulation.* 2000;101:1158-1164.
  42. Runge MS, Quertermous T, Zavodny PJ, et al. A recombinant chimeric plasminogen activator with high affinity for fibrin has increased thrombolytic potency in vitro and in vivo. *Proc Natl Acad Sci U S A.* 1991;88:10337-10341.
  43. Schnee JM, Runge MS, Matsueda GR, et al. Construction and expression of a recombinant antibody-targeted plasminogen activator. *Proc Natl Acad Sci U S A.* 1987;84:6904-6908.
  44. Fujise K, Revelle BM, Stacy L, et al. A tissue plasminogen activator/P-selectin fusion protein is an effective thrombolytic agent. *Circulation.* 1997;95:715-722.
  45. Dong N, Da Cunha V, Citkovicz A, et al. P-selectin-targeting of the fibrin selective thrombolytic Desmodus rotundus salivary plasminogen activator alpha1. *Thromb Haemost.* 2004;92:956-965.
  46. Goldhaber SZ. Prevention of recurrent idiopathic venous thromboembolism. *Circulation.* 2004;110:IV20-IV24.
  47. Fujita T, Toda K, Karimova A, et al. Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nat Med.* 2001;7:598-604.
  48. Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E. Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. *J Clin Invest.* 1998;102:430-437.
  49. Dienst A, Grunow A, Unruh M, et al. Specific occlusion of murine and human tumor vasculature by VCAM-1 targeted recombinant fusion proteins. *J Natl Cancer Inst.* 2005;97:433-447.
  50. Huang X, Molema G, King S, Watkins L, Edgington TS, Thorpe PE. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Science.* 1997;275:547-550.