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Delivery of Anti-Platelet-Endothelial Cell Adhesion Molecule Single-Chain Variable Fragment-Urokinase Fusion Protein to the Cerebral Vasculature Lyses Arterial Clots and Attenuates Postischemic Brain Edema

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ABSTRACT

Efficacy and safety of current means to prevent cerebrovascular thrombosis in patients at high risk of stroke are suboptimal. In theory, anchoring fibrinolytic plasminogen activators to the luminal surface of the cerebral endothelium might arrest formation of occlusive clots in this setting. We tested this approach using the recombinant construct antiplatelet-endothelial cell adhesion molecule (PECAM) single-chain variable fragment (scFv)-urokinase-type plasminogen activator (uPA), fusing low-molecular-weight single-chain urokinase-type plasminogen activator with a scFv of an antibody directed to the stably expressed endothelial surface determinant PECAM-1, implicated in inflammation and thrombosis. Studies in mice showed that scFv-uPA, but not unconjugated uPA 1) accumulates in the brain after intravascular injection, 2) lyses clots lodged in the cerebral arterial vasculature without hemorrhagic complications, 3) provides rapid and stable cerebral reperfusion, and 4) alleviates post-thrombotic brain edema. Effective and safe thromboprophylaxis in the cerebral arterial circulation by anti-PECAM scFv-uPA represents a prototype of a new paradigm to prevent recurrent cerebrovascular thrombosis.

Prevention of cerebrovascular thrombosis remains a major unmet need. Situations in which the risk is high, e.g., after transient ischemic attack, myocardial infarction, and post-cardiac bypass surgery, among others, have been identified (Johnston, 2002; Kang et al., 2005). However, the brain is extremely vulnerable to hemorrhage, neurotoxicity, and disruption of the blood-brain barrier (BBB), leading to cerebral edema, which narrows the therapeutic margin of existing modalities and restricts their use to a fraction of patients in need of medical intervention (Wang et al., 1998; Lo et al., 2003). To date, the possibility of using plasminogen activators (PA) for prophylaxis of cerebrovascular thrombosis has not been feasible due to their short half-life and untoward incidence of hemorrhagic and neurotoxic complications (Wang et al., 1998; Zivin, 1999; Lo et al., 2003; Kang et al., 2005).

The use of gene delivery to the endothelium to generate PA expression facilitates arterial thrombolysis in animal models (Waugh et al., 1999). This observation supports the hypothesis that if feasible, stable localization of a PA along the luminal surface of the cerebral endothelium would enhance its natural antithrombotic mechanisms (Rosenberg and Aird, 1999), helping prevent ischemic stroke. Targeted delivery of PA to endothelial luminal surface may be especially helpful in settings where the propensity for recurrent thrombosis is high (Johnston, 2002) and the acuity of the risk makes gene therapy unsuitable.

Vascular immunotargeting of PA fused with antibody to platelet-endothelial cell adhesion molecule-1 single-chain variable fragment (anti-PECAM scFv-PA) has been shown to

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ABBREVIATIONS: BBB, blood-brain barrier; PA, plasminogen activator(s); PECAM, platelet-endothelial cell adhesion molecule; scFv, single-chain variable fragment; uPA, urokinase-type plasminogen activator(s); MCA, middle cerebral artery; PBS, phosphate-buffered saline; CBF, cerebral blood flow.

provide thromboprophylaxis in the pulmonary circulation (Ding et al., 2005), but analogous approaches to deliver PA to the cerebral endothelium have not been reported. PECAM is stably expressed on endothelial surface in all blood vessels, including cerebral arteries (Giri et al., 2000). The endothelium neither internalizes PECAM nor anti-PECAM scFv-PA (Muzykantov et al., 1999; Ding et al., 2005), thereby maintaining intravascular activity of PECAM-anchored drugs.

To determine whether we could use this approach to protect the cerebral vasculature from thrombotic occlusion, we tested an anti-PECAM scFv fused with low-molecular-weight single-chain urokinase-type PA (scFv-uPA), a prodrug that expresses essentially no activity until cleaved by plasmin (Pannell and Gurewich, 1987; Ding et al., 2005). We tested whether PECAM-directed targeting 1) delivers uPA to the cerebral vasculature; 2) facilitates lysis of cerebral arterial clots without causing intracerebral hemorrhage; and 3) accelerates reperfusion, thereby alleviating postischemic cerebral edema.

Materials and Methods

Reagents. Chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. The design, synthesis, pharmacokinetics, and enzymatic activity of anti-PECAM scFv-uPA have been described previously (Ding et al., 2005). Proteins [scFv-uPA, nonmodified single-chain low-molecular-weight urokinase (hereafter uPA) and fibrinogen] were radiolabeled with ^{125}I-Na (PerkinElmer Inc., Wellesley, MA) using iodogen (Pierce Chemical, Rockford, IL).

Tracing of Cerebral Accumulation of Anti-PECAM scFv-uPA versus uPA. Male C57BL/B6 mice (6–8 weeks old) were studied following protocols compliant with National Institutes of Health guidelines and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Radiolabeled scFv-uPA or uPA was injected via the carotid artery (i.a.) or jugular vein (i.v.) in anesthetized mice. One hour later, mice were sacrificed, and the ^{125}I content in the brain was measured in a gamma counter (Ding et al., 2005).

Quantitative Measurement of Cerebral Embolism. We studied the effect of scFv-uPA in a mouse model of cerebrovascular thrombosis induced by injecting ^{125}I-labeled fibrin emboli (~3 μm in diameter, prepared as described previously; Atochin et al., 2004; Ding et al., 2005), into the middle cerebral artery (MCA). Immediately after intravascular injection, the fibrin microemboli form aggregates invested with blood elements, which lodge in the downstream vasculature (Murciano et al., 2002). Previous studies showed that within 5 min after injection, the chosen dose of emboli (~1.4 × 10^6 particles) causes ~80% cessation of blood flow in the MCA, leading to an extensive ipsilateral cerebral infarction, similar to that caused by 20 h of mechanical MCA occlusion in the standard filament model (Atochin et al., 2004).

Studies in anesthetized mice and rats followed protocols compliant with Institutional Animal Care and Use Committee and Environmental Health and Radiation Safety policies. Drugs (uPA, scFv-uPA, or PBS placebo) were injected in a standard 120-μl volume (PBS) via polyethylene catheters inserted into the right femoral vein. Ten minutes after injection of fibrinolytics in anesthetized mice, a suspension of ^{125}I-fibrin emboli was injected via the right MCA, as described previously (Atochin et al., 2004). One hour later, mice were sacrificed, and the ^{125}I content of the brain was measured to determine extent of cerebrovascular thrombolysis based on the amount of residual radiolabeled clots residing in the brain (Atochin et al., 2004).

Monitoring of Cerebral Blood Flow in Mice. After injection of fibrinolytics and microemboli injection via the MCA, cerebral blood flow (CBF) in the ipsilateral hemisphere was monitored for 60 min by laser Doppler (Transonic Systems Inc., Ithaca, NY) (Atochin et al., 2004). The probe, approximately 2 mm in diameter (Transonic Systems Inc.), was placed 2 mm posterior and 5 mm lateral to bregma along the surface of the skull. The head of the animal, as well as the probe, was fixed in a stereotaxic frame (Harvard Apparatus Inc., Holliston, MA) to permit stable monitoring of the CBF over the ensuing hour after injection of emboli into the right (ipsilateral) MCA.

Injection of microemboli via the MCA causes a drop in ipsilateral CBF to 15 to 20% of normal, and it results in severe ischemic brain injury manifested by an extent of infarction and neurological deficit comparable with the damage induced by filamentous occlusion of the MCA for 18 h (Atochin et al., 2004).

Analysis of the Post-Thrombotic Cerebrovascular Permeability. The integrity of the BBB and extent of brain edema were tested by extravasation of Evans blue dye (2% in saline; 4 ml/kg; injected i.v. 2 h before sacrifice). Intravenous injection of drugs (scFv-uPA versus uPA) or saline followed 15 min later by MCA injection of emboli was performed as described above. Evans blue dye (100 μl of 2% solution in PBS) was injected i.v. 3 h after emboli. Two hours later, the chest was opened under anesthesia, and saline perfusion was done through the left ventricle until colorless perfusion fluid came from the right atrium. The times chosen to inject Evans blue dye and to measure accumulation of dye in the parenchyma were based on findings in control mice injected with emboli in the absence of drug (data not shown). After decapitation, the brain was weighed and placed in 50% trichloroacetic acid solution. After homogenization and centrifugation, the Evans blue dye was extracted from tissue with ethanol (1:3), and the optical density at A_{550} in the homogenate extracts was measured in a Carry spectrophotometer (Varian, Inc., Palo Alto, CA).

Data Analysis. We analyzed the experimental data using *t* test or analysis of variance (for multivariant comparison). and the results are presented as the mean ± S.E.M.

Results

Vascular Delivery of Anti-PECAM scFv-uPA in the Brain. The cerebral vasculature expresses high levels of PECAM-1 constitutively (Giri et al., 2000). Consistent with this observation, 1 h after injection of ^{125}I-labeled anti-PECAM scFv-uPA via the jugular vein, the radioactivity in the brain was increased 2-fold above the level measured after injection of ^{125}I-labeled uPA (Fig. 1). Administration of scFv-

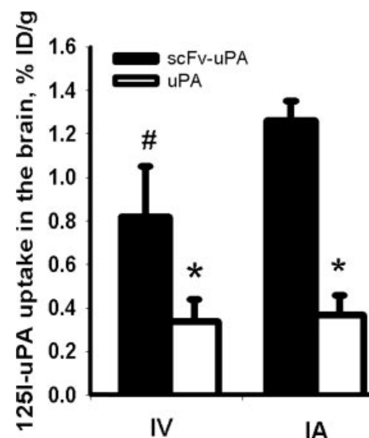

Fig. 1. Accumulation of anti-PECAM scFv-uPA in the cerebral vasculature in mice. Brain uptake 1 h after injection of ^{125}I-labeled scFv-uPA (closed bars) or equimolar amounts of ^{125}I-uPA (open bars) in anesthetized mice via the carotid artery (i.a.) or jugular vein (i.v.). Unless specified otherwise, the data are shown as mean ± S.E.M. $n = 3$ mice per group; ∗, $p < 0.05$ scFv-uPA versus uPA; #, $p < 0.05$ i.a. versus i.v. for scFv-uPA.

uPA via the carotid artery, which avoids an initial depletion of injected anti-PECAM in the extracerebral vasculature (Muzykantov et al., 1999), further increased cerebral accumulation of the fusion protein by ~30% (difference between arterial versus venous routes was significant; $p < 0.05$). These data indicate that local administration of drugs conjugated to anti-PECAM via the carotid artery augments binding by the cerebral vasculature. Accumulation of scFv-uPA in the brain after arterial injection was 3-fold higher compared with nontargeted uPA; therefore, this route was used in the subsequent studies.

Anti-PECAM scFv-uPA Delivery in the Brain Augments Cerebrovascular Fibrinolysis. We then tested whether the intra-arterial delivery of scFv-PA to the cerebral vasculature stimulates local fibrinolysis. To do so, we injected ^{125}I-fibrin thrombi into the MCA, which we have shown previously lodge in the ipsilateral hemisphere (Atochin et al., 2004). In mice injected with PBS or uPA 15 min before embolization, ~30% of injected radioactivity remained in the brain at 1 h (Fig. 2A). In contrast, prophylactic injection of scFv-uPA caused an ~5-fold reduction in brain radioactivity to less than 5% of the injected dose, demonstrating marked augmentation of clot dissolution (Fig. 2A).

We analyzed the distribution of residual radiolabeled emboli within the brain. Nontargeted uPA caused thrombolysis (although inferior to scFv-uPA) in the anterior lobes and cerebellum, i.e., areas that received a minor fraction of emboli injected via the MCA (Fig. 2B). In contrast, scFv-uPA, but not free uPA, lysed ^{125}I-labeled emboli lodged in the ipsilateral temporal lobe, which is within the distribution of the MCA (Fig. 2B). This heterogeneity in cerebral thrombolysis may reflect regional differences in perfusion, extent of vascular occlusion due to different thrombotic burden, and the balance between pro- and antifibrinolytic mechanisms.

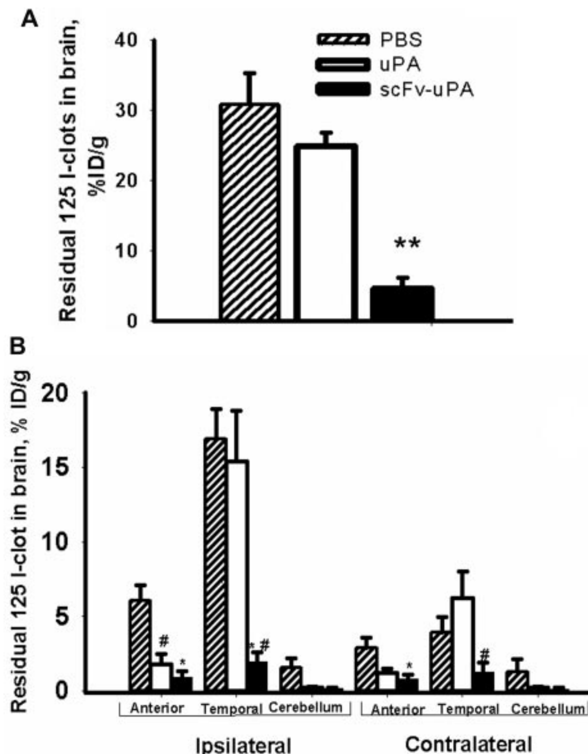

Fig. 2. Fibrinolytic effect of anti-PECAM scFv-uPA in the cerebral vasculature. A, residual ^{125}I-labeled fibrin emboli in the brain 1 h after injection of PBS (hatched bar), 4 mg/kg uPA (open bar), or 4 mg/kg scFv-uPA (closed bar) into the right MCA 10 min before embolization. Asterisks indicate $p < 0.01$ for scFv-uPA versus PBS and uPA ($n = 10$, 5, and 4 mice per group, respectively). B, distribution of residual ^{125}I-labeled fibrin emboli in the indicated areas of ipsilateral (right) and contralateral (left) hemispheres. *, $p < 0.05$ scFv-uPA versus PBS and uPA; **, $p < 0.01$ scFv-uPA versus PBS; and #, $p < 0.05$ uPA versus PBS.

Cerebrovascular Fibrinolysis by Anti-PECAM scFv-uPA Augments Reperfusion. We next determined whether this fibrinolytic activity translated into improved cerebral blood flow. Laser Doppler revealed near total occlusion of the MCA in the ipsilateral hemisphere 5 min after injection of fibrin thrombi (Fig. 3). Perfusion was not reestablished over the ensuing hour in mice injected with PBS or uPA 15 min before embolization. In contrast, rapid, complete, and persistent reperfusion was seen in mice pretreated with the same dose of scFv-uPA (Fig. 3).

Cerebrovascular Fibrinolysis by Anti-PECAM scFv-uPA Does Not Aggravate Brain Edema. Disruption of the BBB leading to cerebral edema is a common sequel of thrombosis and ischemia that may be exacerbated by cerebrovascular fibrinolysis (Pluskota et al., 2003). Therefore, we followed the extravasation of Evans blue dye into the brain as a marker of BBB disruption and brain edema after administration of scFv-uPA. Accumulation of dye was readily apparent in the brains 5 h after embolism in all groups, especially in the ipsilateral hemispheres (Fig. 4A). Neither agent increased dye uptake in the brain of naive mice (Fig. 4A).

Spectrophotometer analysis showed that injection of emboli caused 4-fold elevation of the dye uptake by the brain (Fig. 4B). Pretreatment with uPA exacerbated post-thrombotic extravasation Evans blue dye, whereas extravasation was reduced significantly ($p < 0.05$) in animals that had been pretreated with scFv-uPA compared with uPA and PBS controls (Fig. 4B). No hemorrhages were detected in the brains at post-mortem examination by gross inspection or by light microscopic analysis of tissue sections in animals treated with scFv-uPA.

Discussion

Current approaches to thromboprophylaxis provide incomplete protection (Topol et al., 1999; Jackson et al., 2000;

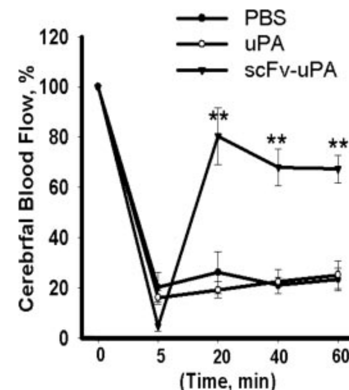

Fig. 3. Prophylactic cerebrovascular immunotargeting of anti-PECAM scFv-uPA facilitates post-thrombotic reperfusion. Mice were given an i.a. injection of PBS (closed circles), 4 mg/kg uPA (open circles), or 4 mg/kg scFv-uPA (closed triangles) followed 10 min later by an i.a. injection of fibrin thrombi. Cerebral blood flow in the MCA distribution was measured by Doppler ultrasound prior (time 0) and at the indicated times after injection of thrombi. **, $p < 0.01$ for scFv-uPA versus PBS and uPA ($n = 4$, 10, and 4 per group, respectively).

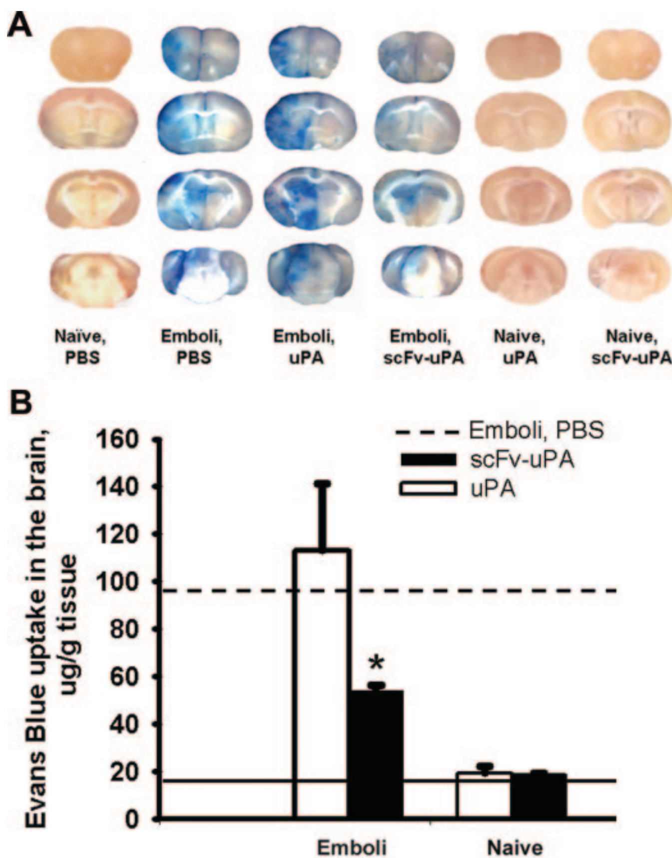

Fig. 4. Effect of targeted anti-PECAM scFv-uPA versus nontargeted uPA on post-thrombotic brain edema. Mice were given an i.a. injection of PBS, 4 mg/kg uPA, or 4 mg/kg scFv-uPA followed 10 min later by an i.a. injection of fibrin emboli. In control groups, emboli injection after drugs was omitted (naive mice). Evans blue dye was extracted 5 h after injection of drugs. A, characteristic sections of the brain of animals from each experimental group: naive animals (injected with PBS), control (emboli and PBS), animals injected with uPA followed by emboli (uPA; emboli), scFv-uPA injected (mentioned as scFv-uPA, emboli), naive animals (uPA, naive and scFv-uPA, naive). B, amount of Evans blue dye extracted from the brain tissue homogenates. n = 4, 6, 4, 4, 4, and 4 mice per group, respectively; $*$, $p < 0.05$ scFv-uPA versus saline and uPA-treated groups. Solid line shows basal level of the dye uptake in the brain of naive mice, and dashed line shows the level of dye accumulation in the brain of emboli-injected animals without pretreatment by either uPA or scFv-uPA.

Hennan et al., 2002). Timely (within 3 h from the onset of cerebral ischemia) therapeutic use of fibrinolytic plasminogen activators improves the outcome of carotid arterial thrombosis; yet, the risk of intracranial hemorrhage and neurotoxicity remains unacceptably high (Thomas et al., 1994; Wang et al., 1998; Zivin, 1999; Liberatore et al., 2003; Lo et al., 2003). Rapid elimination from blood, inadequate delivery to the interior of clots, and serious side effects in the central nervous system restrict the therapeutic utility and preclude prophylactic use of these proteases and their existing derivatives (Verstraete et al., 1985; Wang et al., 1998; Rijken et al., 2004; Melchor and Strickland, 2005). In theory, anchoring fibrinolytics to the luminal surface of the cerebral vasculature could prolong their antithrombotic potential and restrict their diffusion into the parenchyma.

Drug delivery to the cerebral vasculature is an important and challenging goal (Zhang and Pardridge, 2005). Some endothelial antigens potentially useful for targeting, such as angiotensin-converting enzyme (Muzykantov et al., 1996b), are readily internalized (Muzykantov et al., 1996a), a downside from the standpoint of localizing drugs intended to act within the vascular lumen. In addition, binding of drugs conjugated to targeting antibodies or other affinity moieties may block, cross-link, and otherwise affect the functionality of important endothelial determinants to the detriment of the host. For example, inhibition of endothelial thrombomodulin by immunotargeting may exacerbate thrombosis (Christofidou-Solomidou et al., 2002).

Alternatively, blocking endothelial cell adhesion molecules, including selectins and PECAM-1, may inhibit leukocyte adhesion and transmigration, thereby providing a secondary beneficial effect in the context of cerebrovascular thrombosis and inflammation (Muro and Muzykantov, 2005). Occupancy of PECAM-1 might also promote endothelial survival, while inhibiting platelet adhesion and leukocyte transmigration as mentioned above (Maas et al., 2005; Falati et al., 2006). Thus, scFv-uPA may secondarily provide pro bono benefits by attenuating thrombosis, inflammation, and reperfusion injury.

Another advantage of PECAM-1 for targeting antithrombotic agents to the endothelial lumen is that it is constitutively and stably expressed by endothelium at high levels (millions of copies per cell), affording the opportunity for robust targeting. The expression of selectins is both transient and 10-fold lower, even at its peak. In addition, endothelial cells internalize selectins via clathrin-mediated pits, leading to the disappearance of targeted drugs from the luminal surface and their accumulation in lysosomes (Everts et al., 2002).

In contrast, monoclonal antibodies to PECAM-1 are not internalized unless they are deliberately conjugated to form large multimolecular complexes (Muzykantov et al., 1999; Muro et al., 2003). As a result, the fusion protein used in this work has a half-life on the endothelial surface of ~12 h (Ding et al., 2005). Thus, use of a monovalent anti-PECAM scFv fragment avoids both stimulation of endocytosis caused by antigen cross-linking and potential adverse effects resulting from Fc-receptor-mediated activation of leukocytes, platelets, and complement (Holvoet et al., 1991).

We hypothesized that targeting PA to stable endothelial determinants (e.g., PECAM-1) in at-risk vasculature would provide more effective prophylaxis than using determinants that emerge as a result of thrombosis, such as fibrin (Fujise et al., 1997; Peter et al., 2000), because clots rapidly become impermeable to plasma proteins (Sakharov and Rijken, 1995).

PECAM-1 is a pan-endothelial determinant. Anti-PECAM conjugates and fusion constructs bind to endothelium throughout the vasculature after systemic injection. Previous studies demonstrated that local infusion of anti-PECAM conjugates via conduit arteries markedly augments binding to the downstream vasculature of target organs, including the heart (Scherpereel et al., 2002) and lungs (Danilov et al., 2001). This study extends this paradigm to cerebrovascular drug delivery.

Targeting a suitably designed fibrinolytic agent to the cerebrovascular endothelium might be useful in patients experiencing recurrent transient ischemic attacks, stroke in evolution, or other high-risk settings (Zivin, 1999; Mohr et al., 2001; Johnston, 2002). This may be accomplished using a prodrug with enhanced and prolonged specific binding to

cerebral arterial endothelium, positioning the drug where it will be activated by plasmin formed at the site of thrombosis. Based on this concept, we hypothesized that fusing a genetically modified urokinase prodrug to an scFv directed at an endothelial cell adhesion molecule would offer a combination of features that could be exploited to prevent recurrent cerebrovascular thrombosis.

In support of this concept, we found that anchoring pro-uPA to the lumen of the cerebral vasculature led to plasmin-mediated activation of thrombolysis and more rapid reperfusion than soluble pro-uPA in a mouse model of cerebrovascular thromboembolism (Figs. 2 and 3). Anti-PECAM scFv-uPA mediated reperfusion without exacerbating the characteristic side effects of cerebrovascular fibrinolysis, disruption of the BBB, and intracerebral hemorrhage. At therapeutic doses needed to compensate for rapid elimination and lack of targeting (1–2 mg/kg in humans and up to 10 mg/kg in rodents), uPA disrupts the BBB barrier via plasmin-mediated proteolysis as well as nonproteolytic intracellular signaling mediated in part through the interaction of uPA with its cognate receptor (uPAR/CD87) expressed on endothelium and other vascular cells (Pluskota et al., 2003). Lack of BBB disruption by scFv-uPA (Fig. 4) is at least partly attributable to the fact that this construct lacks uPAR-binding growth factor domain, supporting the importance of nonproteolytic pathways in the development of cerebral edema (Yepes et al., 2003; Armstead et al., 2006). Furthermore, the alleviation of brain edema in scFv-uPA-treated animals (Fig. 4) and the absence of intracerebral hemorrhage suggest that both sequelae of cerebrovascular ischemia can be ameliorated if reperfusion is rapidly restored (Fig. 3).

Although scFv-uPA caused almost complete clot lysis, essentially restored cerebral perfusion and decreased brain edema compared with the free uPA, the prevention of brain edema was incomplete. Neither uPA nor scFv-uPA caused Evans blue dye extravasation in control mice in the absence of injury induced by cerebral thrombosis (Fig. 4), indicating that neither agent per se provokes brain edema. Therefore, the incomplete protection against edema is unlikely to be due to the BBB injury caused by the drug. Rather, it is more likely that BBB injury is attributable to the complex nature of vascular injury in this model that is associated with ~50% mortality within the first 20 h in control mice (Atochin et al., 2004). For example, lysis of labeled microemboli and reperfusion monitored by Doppler within the first hour may not account for 1) rethrombosis or delayed secondary emboli, which, in contrast to injected ^{125}I-emboli, are unlabeled and thus they are not detected by isotope analysis in the brain; 2) regional diversity in cerebral thrombolysis (Fig. 2B) and perfusion changes in the downstream vasculature (the Doppler methodology is not suitable to detect perfusion in the cerebral microvasculature and in the deep subcortical areas of the brain); and 3) endothelial and tissue injury caused by activated leukocytes or complement. Each of these factors might limit protection by the fusion protein drug, especially when the dose is suboptimal. Systematic studies are in progress to assess the effect of scFv-uPA on several key parameters of cerebrovascular thrombosis (including the extent of the brain tissue injury, animal survival, and neurological deficit) as well as the optimal dose, time, and duration of fusion administration. The results of these studies will provide insight into the importance of maintaining BBB integrity in this model.

The results of this study support the concept that immunotargeting using stable endothelial determinants expressed on the cerebral vasculature may represent a promising approach to prevent acute cerebrovascular insults. Animal models that simulate human cerebrovascular pathologies more closely and a more extensive analysis of the risk of bleeding are needed before this approach can be translated into the clinical domain. However, the modular nature of the scFv fusion technology described in this article may prove applicable to the delivery of antithrombotic, anti-inflammatory, and other protective interventions within the cerebral vasculature.

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