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Fibrin Affinity of Erythrocyte-Coupled Tissue-Type Plasminogen Activators Endures Hemodynamic Forces and Enhances Fibrinolysis in Vivo

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ABSTRACT

Plasminogen activators (PAs; e.g., tissue-type, tPA) coupled to red blood cells (RBCs) display in vivo features useful for thromboprophylaxis: prolonged circulation, minimal extravasation, and preferential lysis of nascent versus preexisting clots. Yet, factors controlling the activity of RBC-bound PAs in vivo are not defined and may not mirror the profile of soluble PAs. We tested the role of RBC/PA binding to fibrin in fibrinolysis. RBC/tPA and RBC/tPA variant with low fibrin affinity (rPA) bound to and lysed plasminogen-containing fibrin clots in vitro comparably. In contrast, when coinjected in mice with fibrin emboli lodging in pulmonary vasculature, only RBC/tPA accumulated in lungs, which resulted in a more extensive fibrinolysis versus RBC/rPA ($p < 0.01$). Reconciling this apparent divergence between in vitro and in vivo behaviors, RBC/tPA, but not RBC/rPA perfused over fibrin in vitro at physiological shear stress bound to fibrin clots and caused greater fibrinolysis versus RBC/rPA ($p < 0.001$). These results indicate that because of high fibrin affinity, RBC/tPA binding to clots endures hemodynamic stress, which enhances fibrinolysis. Behavior of RBC/PAs under hemodynamic pressure is an important predictor of their performance in vivo.

Ineffective delivery and permeation of plasminogen activators (PAs) into clots limit the thrombolytic utility (Holvoet et al., 1993; Sakharov and Rijken, 1995). This problem cannot be solved by escalating the dose because of an untoward risk of collateral damage (e.g., bleeding). Current means to modify PAs have had relatively limited impact on their clinical pharmacokinetics (typically <1-h circulation in the blood) or their propensity to extravasate, especially into damaged tissues such as the central nervous system, which causes morbidity (Tsirka et al., 1995; Wang et al., 1998).

The use of red blood cells (RBCs) as drug carriers might help solve this problem by converting PAs into thromboprophylactic agents. RBC carriage 1) markedly prolongs the intravascular circulation of PAs, permitting their inclusion into nascent clots followed by rapid fibrinolysis from within ("Trojan horse lysis") (Murciano et al., 2003; Ouriel, 2003); and 2) precludes the penetration of PAs into existing hemostatic clots and limits extravasation, thus minimizing bleeding and collateral damage in the central nervous system and probably other tissues. RBC-coupled tPA (RBC/tPA) circulates in mice and rats for many hours without harm to the carrier RBC or without causing bleeding, while selectively dissolving nascent clots resistant to even higher doses of soluble tPA (Murciano et al., 2003).

Given this potential to change how PAs act and can be used, it is important to determine how biochemical properties and in vivo behavior of PAs are altered when they are linked to RBCs and how to predict for such changes. Previous in vivo studies show that RBC carriage overrides differences in pharmacokinetics among soluble PAs (Ganguly et al., 2005). Thus, soluble tPA is cleared from the blood more rapidly than

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ABBREVIATIONS: PA, plasminogen activator; RBC, red blood cell; tPA, tissue-type plasminogen activator; rPA, Retavase; Pg, plasminogen; NC-Pg, noncleavable plasminogen; FDP, fibrin degradation product; PPACK, D-Phe-Pro-Arg-CMK, HCl; PBS, phosphate-buffered saline; ME, microemboli; RT, room temperature; PAI-1, plasminogen activator inhibitor-1.

rPA (Retavase), a truncated tPA variant lacking the growth factor (G), finger (F), and first kringle (K1) domains (Kohnert et al., 1992). Yet, both RBC/tPA and RBC/rPA circulate for at least many hours in animals (Murciano et al., 2003; Ganguly et al., 2005). Previous studies also revealed that coupling to RBCs attenuates tPA inhibition by PAI-1 (Ganguly et al., 2005).

Another important property of tPA is its high affinity for fibrin, mediated primarily through binding of its F domain to cryptic binding sites on the fibrin γ-chain (D region; γ312–324, exposed after fibrinogen cleavage by thrombin) in a *lys*-independent manner (Medved and Nieuwenhuizen, 2003). Binding is reinforced by interactions between the K domains of fibrinolytic proteins and lysine residues that are exposed upon fibrin polymerization. Plasminogen (via K1 and four domains) and tPA compete for binding to the Aα residues (Aα148–160) in the D region of fibrin and also bind to αC residues (Aα392–610) in a noncompetitive manner (Kaczmarek et al., 1993; Medved and Nieuwenhuizen, 2003). Partial proteolysis of αC domain of fibrin (nicking) by plasmin exposes abundant -COOH terminal lysine residues on fibrin that increase the *lys*-dependent K2-mediated tPA binding (Kaczmarek et al., 1993; Yakovlev et al., 2000; Medved and Nieuwenhuizen, 2003). Retavase (rPA) has the residual *lys*-dependent binding site conferred by K2, but it lacks *lys*-independent binding provided by the F domain (Kohnert et al., 1992).

It is not known, however, whether 1) RBC-coupled tPA retains its affinity and access to fibrin; 2) RBC/PA binds stably to clots; and 3) the high affinity for fibrin, present in tPA and deleted in rPA, is an important factor in RBC/PA localization and/or fibrinolytic activity in vivo. Last, it is not clear how to use in vitro assays to model the factors that govern RBC/PA activity in vivo.

We studied fibrin binding and dissolution by RBC/tPA versus RBC/rPA in a mouse model of pulmonary embolism and in vitro models and found that the fibrin affinity of tPA 1) is retained when it is bound to RBC; 2) suffices to anchor RBC/tPA to clots; and 3) facilitates fibrinolysis by RBC/tPA. Hemodynamic forces control the binding of RBC/PA to clots, an issue of importance in designing formulations of fibrinolytic agents.

Materials and Methods

Reagents. Human recombinant tPA (Alteplase) was from Genentech (South San Francisco, CA), rPA (Reteplase) was from Centocor, Inc. (Horsham, PA), and plasminogen (Pg) was from American Diagnostica (Greenwich, CT). cDNA encoding a plasmin cleavage site-resistant mutant of plasminogen, referred as noncleavable plasminogen (NC-Pg [$R^{561}A$]; hereafter NC-Pg) was a kind gift from Dr. Castellino (University of Notre Dame, Notre Dame, IN) (Grella and Castellino, 1997). Fibronectin was from Roche Palo Alto LLC (Palo Alto, CA). Calf skin type III collagen, plasmin, plasmin inhibitor (H-D-Val-Phe-Lys-CMK), plasminogen activator inhibitor (D-Phe-Pro-Arg-CMK, HCl), and streptavidin were from Calbiochem (San Diego, CA). Thrombin, fibrinogen, and components of buffer solutions were from Sigma-Aldrich (St. Louis, MO). Annexin II was from Biodesign International (Kennebunk, ME).

RBC/PA Binding to Fibrin Clots, Immobilized Plasma Components, and Vascular Proteins. Approximately 6×10^4 tPA or rPA molecules were bound per RBC without loss of enzymatic activity and RBC biocompatibility, as described previously (Murciano et al., 2003; Ganguly et al., 2005). Suspensions of RBC/PA were incubated for 20 min at 4°C in plastic wells containing Pg-depleted fibrin clots prepared by coagulating a 0.5-ml solution of 3 mg/ml fibrinogen. RBC/PA binding was determined by adding water and measuring hemoglobin absorbance at 405 nm in lysates. In some wells, fibrin clots were supplemented with Pg (0.2 μM) or NC-Pg (0.2 μM) before addition of RBCs. To detect fibrin nicking, we measured fibrin degradation products (FDPs) released into the supernatant fluid from ^{125}I-fibrin clots as described previously (Murciano et al., 2003; Ganguly et al., 2005). In a separate series of experiments, RBC/PA was preincubated with 10 M excess of the PA inhibitor D-Phe-Pro-Arg-CMK, HCl (PPACK) and then added to Pg-supplemented fibrin clots.

Fibrinogen, fibronectin, type III collagen, Annexin II, and albumin were immobilized on 24-well polystyrene plates (overnight incubation at 4°C at protein concentration 0.2 μM/well). Protein-coated wells were blocked with PBS-bovine serum albumin (3%) and incubated with RBC/PA for 20 min on a horizontal shaker at 2 g at room temperature. Binding of RBCs was measured by measuring hemoglobin at 405 nm in water lysate as described above.

Biodistribution of Free versus RBC-Conjugated PA in Mice. ^{125}I-tPA, ^{125}I-rPA, ^{51}Cr-RBC, or dual-labeled ^{51}Cr-RBC/^{125}I-PA complexes (1–2 μg of PA/animal) were injected i.v. in anesthetized male mice (C57 black). One hour later, animals were sacrificed, and the radioactivity in the blood and organs was measured.

Fibrin Affinity and Fibrinolytic Activity of RBC/PA in a Mouse Model of Pulmonary Embolism. We used a mouse model of thrombosis using suspensions of fibrin microemboli (ME; mean diameter 3–5 μm), which embolize into the pulmonary vessels immediately after i.v. injection (Murciano et al., 2002). To test the dissolution of ME lodged in the pulmonary vasculature, unlabeled RBC/tPA or RBC/rPA (0.08 or 0.4 mg/kg PA/mouse) were incubated with ^{125}I-ME suspensions 20 min on ice before injections. Residual ^{125}I-ME radioactivity in the lungs was measured 1 h postinjection. To test the adhesion of RBC/PA to ME in vivo, ^{51}Cr-RBC/tPA, ^{51}Cr-RBC/rPA, or control ^{51}Cr-RBC was incubated with unlabeled ME for 20 min on ice before injections via the jugular vein, and ^{51}Cr was measured in the lungs 10 min postinjection (to minimize emboli lysis and account for most RBC "targeted" to lungs by clots).

RBC/PA Binding to Fibrin under Flow. A fibrin surface was formed by adding thrombin (2 U/ml) to the rectangular glass capillaries flow chamber (Goel and Diamond, 2002) for 2 h at RT, followed by perfusion of a fibrinogen solution (3 mg/ml) for 5 min at 200 s^{-1}. A layer of nicked fibrin was formed by perfusing plasmin (0.01 U/ml) over the fibrin for 5 min and then treating the partially degraded fibrin surface with the plasmin inhibitor H-D-Val-Phe-Lys-CMK (100 μM). RBC-PA or normal RBCs were then perfused through the flow chamber over layer of fibrin or nicked fibrin at flow rates of 50 and 250 μl/min, corresponding to shear stresses of 0.625 and 3.125 dyne/cm^2, and wall shear stress of 62.5 and 312.5 s^{-1}, respectively.

RBC/PA Fibrinolysis under Flow. Calf skin collagen (100 μg/ml)-coated capillary flow chambers were incubated with citrated platelet-rich plasma for 30 min at RT. Recalcified citrated platelet-free plasma containing RBC-tPA, RBC/rPA, and/or normal RBCs and corn trypsin inhibitor (50 μg/ml; inhibitor of factor XIIa) was perfused over collagen-adherent platelets at a wall shear rate of 62.5 s^{-1} until a prominent RBC-containing fibrin clot was deposited. The chamber was immediately perfused with Hanks' balanced salt solution at a controlled flow rate of 50 or 250 μl/min for 60 min or until the fibrin completely dissolved. Images of adherent RBCs and fibrin undergoing lysis were acquired using a charge-coupled device camera (Hamamatsu, Bridgewater, NJ). After image acquisition, bound RBCs per unit area were counted in multiple fields of view ($n > 10$).

Lysis of Fibrin Clots after Continuous and Transient Exposure to Free PA versus RBC-PA. Soluble PA or RBC/PA (2.5 nM) was applied to the surface of Pg-containing ^{125}I-fibrin clots (500 μl). Clots were exposed to PA either continuously for 2 h at 37°C or transiently for 5 min followed by washing, addition of 200 μl of PBS to the top of the clot, and then a 2-h incubation at 37°C. Fibrinolysis

was assessed by measuring the release of radioisotope into the supernatants.

Results

RBC/PA Binding to Fibrin Clots. We incubated suspensions of RBCs or RBC/PA in wells containing clots prepared from Pg-depleted fibrin for 20 min at 4°C. Binding of naive RBCs in clot-containing wells (Fig. 1A, dashed line) exceeded binding to albumin-coated wells ($\sim 6 \pm 0.9 \times 10^4$ versus $2 \pm 0.6 \times 10^3$ RBCs/well; $p < 0.01$), probably because of mechanical trapping of RBCs between the clots and the chamber walls. However, we observed 10-fold greater ($p < 0.01$) binding of RBC/tPA to clots (designated 100%), whereas RBC/rPA binding did not differ from control. RBC/tPA binding in wells containing fibrin clots ($8.1 \pm 0.9 \times 10^5$ RBC/well) was ~50% of the theoretical maximum number of RBC that could bind as a monolayer in this size well (Samokhin et al., 1983).

Supplementation of fibrin clots with Pg enhanced the binding of RBC/tPA (but not control RBCs) by ~25% above binding to Pg-depleted fibrin clots (Fig. 1A). RBC/rPA bound to Pg-supplemented fibrin clots to the same extent as RBC/tPA. In contrast, Pg replacement by its noncleavable mutant, NC-Pg, reduced the binding of RBC/tPA by 50% and eliminated binding of RBC/rPA. Preincubation of RBC/tPA and RBC/rPA with the PA inhibitor PPACK suppressed their binding to Pg-supplemented fibrin clots by ~25 and ~75%, respectively. Thus, RBC/PA-mediated Pg conversion into plasmin enhances the RBC/tPA binding to fibrin and permits the binding of RBC/rPA, probably because of fibrin nicking.

Plasminogen-Dependent Fibrin Nicking by RBC/PA. Released FDPs from ^{125}I-labeled fibrin clots was measured after incubation with RBC/PA for 20 min at 4°C to test whether plasmin-mediated fibrin nicking initiated by RBC/PA indeed occurred (Fig. 1B). Naive RBCs did not release FDP even in the presence of Pg (dashed line). In contrast, both RBC/tPA and RBC/rPA caused significant FDP release from Pg-treated (~5%; $p < 0.001$), but not from Pg-depleted fibrin clots (Fig. 1B). Substitution of Pg by NC-Pg and inhibition of PA activity by PPACK blocked FDP release induced by RBC/PA. Thus, Fig. 1 shows that in this in situ model, RBC/PA interaction with Pg generates plasmin, which nicks fibrin, exposing abundant -COOH terminal lysine residues and thus enhanced RBC/PA binding.

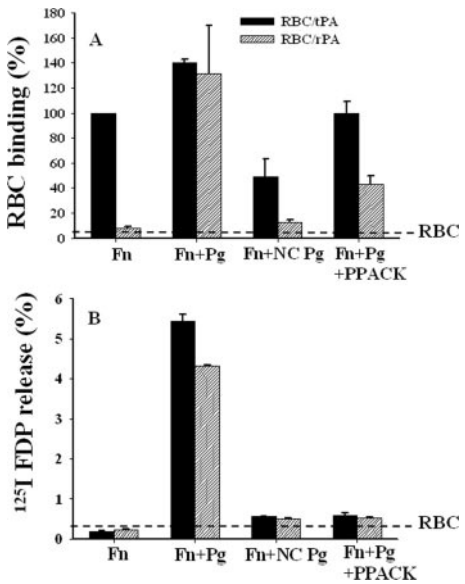

Fig. 1. Binding of RBC/tPA versus RBC/rPA to static fibrin clots. RBC/tPA (black columns) versus RBC/rPA (hatched columns) was incubated for 20 min at 4°C in wells with fibrin clots (Fn). A, RBC binding to clots. RBC/tPA binding to fibrin clots ($8.1 \pm 0.9 \times 10^5$ RBCs/well; left black column) is designated 100% relative to other columns showing effects of Pg, NC-Pg, or PA inhibitor PPACK. Dashed line, binding of naive RBCs. B, release of ^{125}I-labeled FDP. Dashed line, basal effect of naive RBCs. The data in this and other figures show the mean ± S.E.M., with $n = 5$, unless specified otherwise.

Binding of RBC/PA to Immobilized Plasma and Vascular Proteins. Binding of RBC/rPA to wells coated with immobilized fibrinogen, fibronectin, type III collagen, or Annexin II did not exceed binding of either RBC/rPA or naive RBCs ($4.9 \pm 0.2 \times 10^3$ RBCs/well) to albumin-coated wells. In contrast, RBC/tPA displayed significantly greater binding to immobilized fibrinogen ($85 \pm 22 \times 10^3$ RBCs/well) ($p < 0.001$). However, RBC/tPA binding to Annexin II did not differ from background binding of naive RBCs to albumin-coated wells (Fig. 2).

Distribution of Injected RBC/tPA versus RBC/rPA in Mice. In vivo binding of both RBC/tPA and RBC/rPA to fibrin would be expected because of fibrin nicking by plasma Pg. To test this assumption, we traced dual isotope-labeled RBC/tPA and RBC/rPA injected i.v. in mice.

First, we traced the tissue distribution of RBC/tPA versus RBC/rPA 1 h after i.v. injection in naive mice. ^{51}Cr was found primarily in the blood at levels similar to control ^{51}Cr-RBC, excluding adverse effects of PA on RBC survival (Fig. 3A). Neither RBC/tPA nor RBC/rPA showed substantial renal or cardiac uptake (<0.5% ID) and only modestly elevated (10–15% ID) splenic uptake, the site of elimination of senescent RBCs. Neither RBC/tPA nor RBC/rPA accumulated in the lungs to a significant extent compared with naive RBCs, excluding aggregation and mechanical retention in the alveolar capillaries.

The blood level of free ^{125}I-tPA and ^{125}I-rPA was <10 and ~25% ID 1 h post-i.v. injection, respectively (Fig. 3B). Because of rapid clearance and metabolism, residual ^{125}I-PA was found only in liver and kidney at this time point. In contrast, the organ distribution of RBC-bound tPA and rPA followed that of the carrier RBCs: 50 to 70% of injected

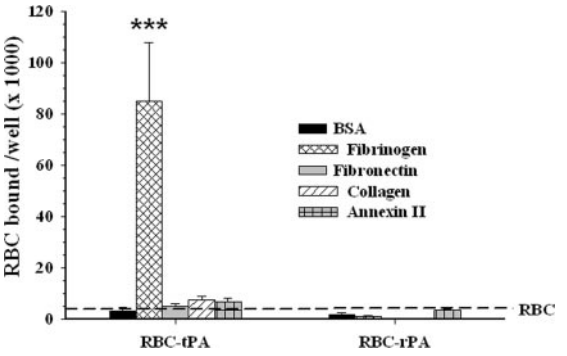

Fig. 2. Binding of RBC/PA to immobilized plasma components and vascular proteins. RBC, RBC/tPA, and RBC/rPA were incubated with albumin (black columns), fibrinogen (hatched columns), fibronectin (gray columns), collagen (stripped columns), and Annexin II (gray checkerboard columns) for 20 min at RT on a slow-speed horizontal shaker. Binding of RBCs was measured by hemoglobin analysis in water lysate at 405 nm. RBC/tPA showed significant binding with fibrinogen (***, $p <$ 0.001 versus RBC binding to bovine serum albumin and fibrinogen).

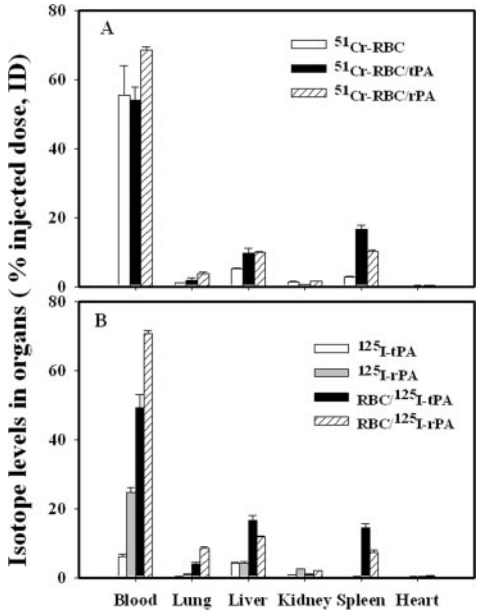

Fig. 3. Distribution of dual-labeled RBC/PA injected in mice. A, distribution of ^{51}Cr-labeled RBC (white columns), RBC/tPA (black columns) and RBC/rPA (hatched columns). B, distribution of ^{125}I-labeled soluble tPA (white columns), rPA (gray columns), RBC-coupled tPA (black columns), and RBC-coupled rPA (hatched columns). The data are shown as percentage of injected dose (% ID) of labeled RBCs or PA found in blood and major visceral organs 1 h after i.v. injection.

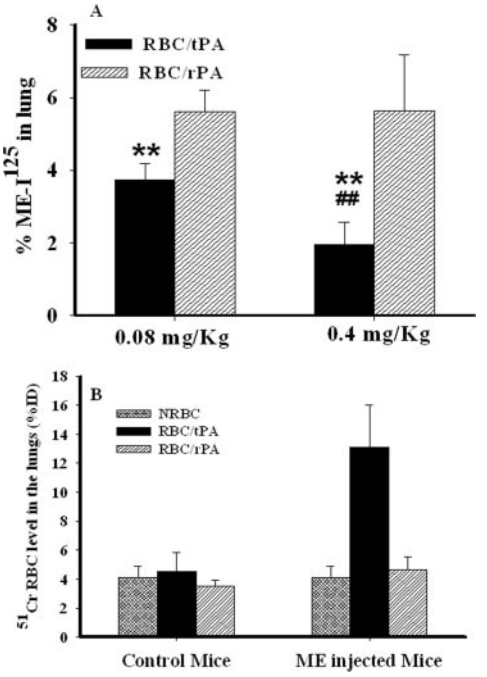

Fig. 4. RBC/tPA binds to and lyses pulmonary emboli more effectively versus RBC/rPA. A, residual pulmonary level of ^{125}I-fibrin ME, preincubated for 20 min on ice with either RBC/tPA (black columns) or RBC/rPA (hatched columns) at 0.08 or 0.4 mg/kg, 1 h after i.v. injection of mixtures through the jugular vein. Differences between tPA versus rPA groups (**) and 0.08 versus 0.4 mg/kg groups (##) were statistically significant at $p < 0.01$. B, pulmonary uptake of ^{51}Cr-labeled RBC, ^{51}Cr-RBC/tPA, and ^{51}Cr-RBC/rPA (double hatched, black, and hatched columns) injected alone (left three columns) or preincubated with unlabeled ME (right three columns). The data are shown as % ID of ^{51}Cr-labeled RBC recovered in the lungs 10 min after i.v. injection.

RBC/^{125}I-tPA and RBC/^{125}I-rPA was found in the blood (Fig. 3B). Importantly for the results that follow, the amount of RBC/tPA found in the lungs of naive mice did not exceed that of RBC/rPA, as assessed by measuring either ^{51}Cr (Fig. 3A) or ^{125}I (Fig. 3B).

RBC/tPA, but Not RBC/rPA, Binding to Fibrin Clots Facilitates Lysis of Pulmonary Microemboli. To test the adhesion of RBC/PA to fibrin clots and their fibrinolytic effect in vivo, we injected Pg-containing fibrin microemboli into mice i.v. ME rapidly become invested with blood elements, enlarge, and deposit in precapillary pulmonary arterioles (Murciano et al., 2002).

Equal doses of unlabeled RBC/tPA and RBC/rPA were incubated with ^{125}I-labeled microemboli on ice for 20 min, the mixtures were injected i.v., and the residual radioactivity in the lungs was measured 1 h later. In agreement with our previous results, ~30% of the isotope remained in the lungs of control mice, corresponding to the fraction of emboli that had withstood spontaneous fibrinolysis (Murciano et al., 2002). Both RBC/tPA and RBC/rPA enhanced clot lysis, reducing residual ^{125}I in the lungs to <10% of baseline (i.e., at least 3-fold) compared with control animals. However, equal doses of RBC/tPA caused substantially greater dissolution of emboli versus RBC/rPA ($p < 0.01$) (Fig. 4A).

We then investigated the mechanism underlying this significant difference in the biological activity of RBC/tPA versus RBC/rPA in vivo. This disparity could not be attributed either to PA detachment from RBC or an effect of conjugation on RBC biodistribution and biocompatibility (Fig. 3). Thus, we asked whether the enhanced activity of RBC/tPA in vivo might be because of, at least in part, its greater affinity for fibrin. To test this possibility, we injected ^{51}Cr-RBC, ^{51}Cr-RBC/tPA, or ^{51}Cr-RBC/rPA that had been preincubated with unlabeled microemboli for 20 min on ice. The pulmonary level of ^{51}Cr-RBC and ^{51}Cr-RBC/rPA was no greater than that seen in control animals (Fig. 4B), whereas the pulmonary uptake of ^{51}Cr-RBC/tPA preincubated with microemboli was 3-fold greater than ^{51}Cr-RBC/tPA alone (13.1 ± 2.9 versus 4.1 ± 0.8% ID in control mice; $p < 0.05$) or ^{51}Cr-RBC or ^{51}Cr-RBC/rPA alone ($p < 0.05$ each). Therefore, high affinity to fibrin promotes RBC/tPA retention within the pulmonary clots, whereas RBC/rPA is dislodged.

RBC/tPA Binding to Fibrin under Flow. One of the key differences between the in vitro (Fig. 1) and in vivo (Fig. 4) models is the presence of hemodynamic forces in the latter. To test whether fibrin affinity suffices to anchor RBC/PA to intact versus nicked fibrin under flow conditions, we perfused suspensions of RBC, RBC/tPA, and RBC/rPA for 5 min at a wall shear rate 62.5 s^{-1} across a fibrin monolayer formed from Pg-depleted fibrinogen (Fig. 5).

In this model, which allows for far less nonspecific entrapment compared with the static model, naive RBCs showed comparably low adhesion to either immobilized fibrin or albumin (Fig. 5B). Perfused RBC/tPA adhered to fibrin, but not to albumin-coated flow chambers (Fig. 5, A and B), similar to their behavior in the "static" model (Fig. 1). Nicking of the fibrin by plasmin before RBC/tPA perfusion increased the binding of RBC/tPA 4-fold ($p < 0.001$). Yet, perfused RBC/rPA did not bind to either a naive or nicked fibrin monolayer (Fig. 5). Therefore, the affinity of RBC/rPA to nicked fibrin was sufficient for adhesion to clots in a static model (Fig. 1A), but it was not enough to withstand the forces generated by physiologically relevant levels of flow (Fig. 5).

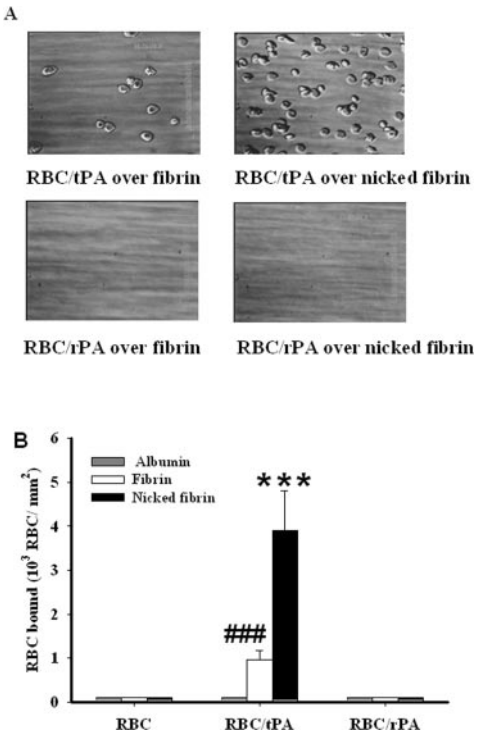

Fig. 5. Perfused RBC/tPA, but not RBC/rPA, binds to fibrin and nicked fibrin under flow conditions. RBC suspensions were perfused in flow chambers (5 min at 0.6 dyne/cm^2, followed by washing with PBS for 5 min). A, microscopic images of bound RBCs. B, quantitative analysis of RBC binding in the chambers coated with albumin (gray), fibrin (white; ###), or nicked fibrin (***) (black columns; $p < 0.001$).

RBC/tPA Binding to Fibrin Accelerates Fibrinolysis under Flow Conditions. We then tested whether RBC/tPA binding to fibrin enhances fibrinolysis under flow conditions. Recalcified citrated platelet-free plasma containing RBCs, RBC/tPA, or RBC/rPA was applied to an adherent layer of platelets in the flow chambers to form clots (Fig. 6, left, showing time 0), which were then perfused with Hanks' balanced salt solution for 0 to 60 min at a wall shear rate of 62.5 s^{-1}, which is close to the shear forces present in the lung microcirculation (Goel and Diamond, 2003).

When clots were formed in the presence of naive RBCs, the fibrin fibrils remained intact for at least 60 min (Fig. 6, top). Initial fibrin formation was not impaired in the presence of RBC/PA (Fig. 6, time 0). However, whereas RBC/tPA completely degraded the fibrin fibrils within 20 min (bottom), it took more than 40 min for RBC/rPA to initiate lysis. An increase in shear rate from 62.5 to 312.5 s^{-1} did not change fibrinolysis induced by RBC/tPA (data not shown). Therefore, RBC/tPA binding to fibrin accelerates the subsequent dissolution of nascent clots under flow.

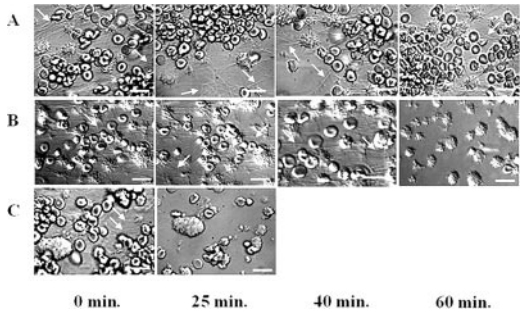

Fig. 6. RBC/tPA adhesion to fibrin clots under flow accelerates subsequent fibrinolysis. Dissolution of fibrin formed in flow chambers in the presence of perfused control RBCs (A), RBC/rPA (B), or RBC/tPA (C). Fibrin fibrils (arrows) disappeared in chambers perfused at 0.6 dyne/cm^2 with RBC/tPA versus RBC/rPA by 25 versus 40 min. Scale bar, 10 μm.

Binding to Fibrin after Transient Exposure Enhances Clot Lysis by RBC/tPA. A second in vitro model was used to examine whether RBC/tPA binding to clots facilitates fibrinolysis. Soluble or RBC-bound tPA versus rPA (2.5 nM) was added to Pg-containing fibrin clots in the absence of shear forces. When PA or RBC/PA was incubated with clots continuously for 2 h at 37°C, no significant difference in tPA- versus rPA-mediated lysis was observed, either by free or RBC-bound (Fig. 7A).

In the set of experiments, we eliminated unbound drugs after a 5-min incubation with the clots, followed by a 2-h incubation at 37°C (Fig. 7B). In this setting, imitating the in vivo condition in which lysis is produced by residual clot-associated PA (whereas unbound material is rapidly cleared), free rPA induced greater fibrinolysis than did free tPA. This result probably reflects impedance of tPA diffusion into the fibrin meshwork because of fibrin affinity, leading to its retention on the surface of clots (Fisher and Kohnert, 1997). In contrast, RBC/tPA caused 2-fold greater fibrinolysis than RBC/rPA after transient contact ($p < 0.01$), which is consistent with the notion that RBC/tPA binding to fibrin leads to greater drug retention and subsequent clot lysis.

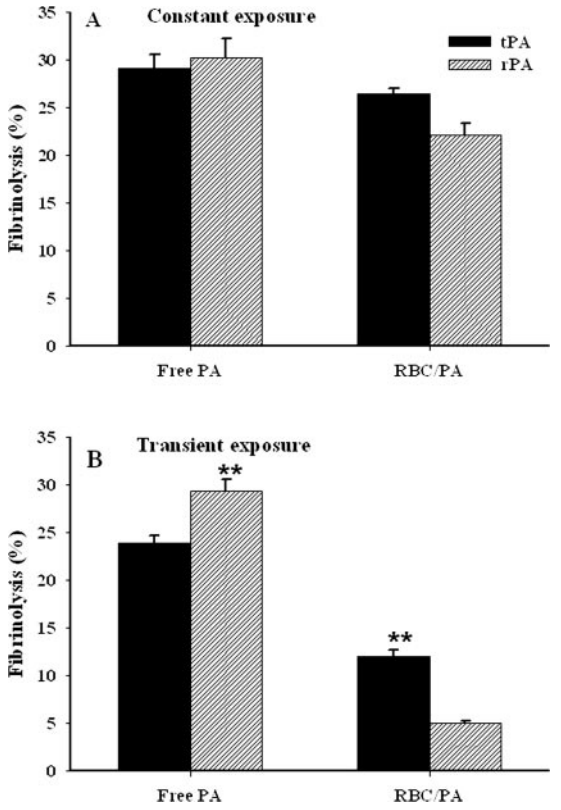

Fig. 7. In vitro dissolution of fibrin clots by soluble and RBC-coupled tPA versus rPA. tPA (black columns) or rPA (gray columns) was incubated with Pg-containing ^{125}I-fibrin clots for 2 h at 37°C (top) or for 5 min followed by washing and 2-h incubation at 37°C. The amount of released ^{125}I-labeled FDPs was measured. **, difference in tPA versus rPA was statistically significant; $p < 0.01$.

Discussion

Patients with multiple transient ischemic attacks, stroke in evolution, organ transplant rejection, poststenting crescendo angina, among others, have a high propensity for recurrent thrombosis. The vulnerable period is often of defined and of relatively limited duration (Taylor et al., 1999; Johnston, 2002), yet current means for thromboprophylaxis have limited efficacy and considerable safety concerns, which thwart dose escalation or combined therapy (Throckmorton, 2001; Lange and Hillis, 2004; Patrono et al., 2004). Fibrinolytic drugs have the potential to complement existing thromboprophylaxis (Hong et al., 2003), if their pharmacokinetic and activities can be modified to enhance drug delivery into clots and overcome safety concerns. Vascular occlusion by fibrin is the proximate morbid event in the most thrombotic disorders. Thus, prophylactic use of PAs capable of rapidly dissolving nascent, highly susceptible clots from within before vascular occlusion (Murciano et al., 2003; Ouriel, 2003) is conceptually attractive, if feasible.

Impediments to the prophylactic use of existing PAs seem insurmountable. PAs penetrate clots poorly (Rijken et al., 2004). Ironically, the advantageous property of high fibrin affinity itself impedes clot permeation by tPA (Fig. 6) (Sakharov and Rijken, 1995; Fisher and Kohnert, 1997). Administering PAs before thrombosis would alleviate this problem, if 1) their half-lives can be prolonged beyond what is currently attainable by molecular modifications (Dawson et al., 1994; Thomas et al., 1994; Liberatore et al., 2003; Moreadith and Collen, 2003; Hagemeyer et al., 2004); and 2) bleeding and collateral tissue damage are avoided (Wang et al., 1998). Thus, a drug delivery system fundamentally changing pharmacokinetics is required for prophylactic use of PAs.

Our recent results showed that coupling PAs to RBCs, an attractive prospective carrier for intravascular drug delivery (Magnani et al., 2002), may provide such a platform (Trojan horse fibrinolysis) (Murciano et al., 2003; Ouriel, 2003). Coupling to RBCs also alters some biological properties of PAs, e.g., enhances tPA resistance to PAI-1 (Ganguly et al., 2005). These encouraging data justify characterization of RBC/PA more systematically, for which in vitro methods that reliably simulate their in vivo behavior are needed.

To test relationships between fibrin affinity and fibrinolytic activity of RBC/PA, we compared in vivo fibrinolytic activity of RBC/tPA and RBC/rPA, a truncated variant with prolonged half-life in the circulation (~25 versus ~5 min for tPA) (Kohnert et al., 1992) and reduced fibrin affinity. We found that RBC/tPA retains its high affinity for fibrin clots, evident in both static and perfusion systems in vitro and in vivo. Plasmin formed from Pg by RBC/PA enhances its binding to clots (Fig. 1), probably by nicking the αC domain of fibrin, exposing -COOH terminal lysines (Medved and Nieuwenhuizen, 2003) and perhaps also via forming ternary complexes between RBC/PA, Pg, and fibrin (Yakovlev et al., 2000; Medved and Nieuwenhuizen, 2003). RBC/tPA is a more effective thrombolytic agent in vivo than is RBC/rPA (Fig. 3A), which binds only to nicked fibrin clots in a static model in vitro (Fig. 1A).

This study reveals the limitations of inferring how RBC-coupled PA will behave based on the properties of its soluble counterpart and in vitro studies. Thus, rPA has sufficient affinity for nicked fibrin to anchor RBC/rPA on Pg-containing clots in situ in static model (Fig. 1), but not in vivo (Fig. 4B). Furthermore, in the clot perfusion model only RBC/tPA, but not RBC/rPA, bound to naive and nicked fibrin (Fig. 5) and showed greater fibrinolytic activity than RBC/rPA (Fig. 6) under flow. Interestingly, adherence to exposed lysine residues in nicked fibrin, albeit insufficient to anchor perfused RBC/rPA, reinforces the RBC/tPA anchorage (Fig. 5), although again this effect was not as evident in a static model (Fig. 1A).

RBC/tPA, but not RBC/rPA, displayed plasminogen-independent binding to the cryptic tPA-binding sites that are exposed in the immobilized fibrinogen because of partial polymerization of the protein (Yakovlev et al., 2000) (Fig. 2). Of interest, RBC/tPA did not display elevated (versus naive RBC) binding to other extracellular vascular proteins, including Annexin II, which is an endothelial cell surface coreceptor for tPA and plasminogen (Hajjar et al., 1998; Kim, 2002). Furthermore, neither RBC/tPA nor RBC/rPA showed elevated (versus naive RBC) binding to endothelial cells in culture (data not shown), either control or thrombin-activated (treatment that induces exposure of Annexin II on endothelial surface) (Peterson et al., 2003). Thus, it is unlikely that Annexin II serves as an auxiliary adaptor to anchor RBC/tPA and promote its activity, unlike soluble tPA.

However, adhesion to clots is not the only reason for the enhanced activity of RBC/tPA versus RBC/rPA in vivo. For example, RBC/tPA, but not RBC/rPA, retains the ability to be activated by fibrin (Ganguly et al., 2005). Moreover, binding of tPA to RBCs renders tPA more resistant to PAI-1 than soluble tPA (Ganguly et al., 2005). It is difficult to weigh the contribution of these features of RBC/PA formulations to their performances in vivo. Yet, the correspondence between the adherence of RBC/tPA to fibrin in static systems (Figs. 1 and 7) and under hemodynamic forces (Figs. 5 and 6) and its effectiveness in vivo (Fig. 4) shows that fibrin affinity is intrinsic to RBC/tPA fibrinolytic activity.

The important role of hemodynamic forces in the RBC/PA behavior is concordant with effects of shear stress on coagulation and clot lysis, adhesion of factor VIII to collagen, platelet activation, and adhesion of blood cells to endothelium (Anand and Diamond, 1996; Fisher and Kohnert, 1997; Falati et al., 2002; Einav and Bluestein, 2004; Frenette, 2004; Leytin et al., 2004; Tomokiyo et al., 2005). Most in vitro studies of antithrombotic drugs have been performed in static models. Yet, discordance between in vivo and static in vitro models justifies the need to develop more relevant model systems in which to test the behavior of antithrombotic agents and perhaps other drugs intended to work within the vasculature.

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