Cerebrovascular Thromboprophylaxis in Mice by Erythrocyte-Coupled Tissue-Type Plasminogen Activator

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Background—Cerebrovascular thrombosis is a major source of morbidity and mortality after surgery, but thromboprophylaxis in this setting is limited because of the formidable risk of perioperative bleeding. Thrombolytics (eg, tissue-type plasminogen activator [tPA]) cannot be used prophylactically in this high-risk setting because of their short duration of action and risk of causing hemorrhage and central nervous system damage. We found that coupling tPA to carrier red blood cells (RBCs) prolongs and localizes tPA activity within the bloodstream and converts it into a thromboprophylactic agent, RBC/tPA. To evaluate the utility of this new approach for preventing cerebrovascular thrombosis, we examined the effect of RBC/tPA in animal models of cerebrovascular thromboembolism and ischemia.

Methods and Results—Preformed fibrin microemboli were injected into the middle carotid artery of mice, occluding downstream perfusion and causing severe infarction and 50% mortality within 48 hours. Preinjected RBC/tPA rapidly lysed nascent cerebral thromboemboli, providing rapid, durable reperfusion and reducing morbidity and mortality. These beneficial effects were not achieved by preinjection of tPA, even at a 10-fold higher dose, which increased mortality from 50% to 90% by 10 hours after embolization. RBC/tPA injected 10 minutes after tail amputation to simulate postsurgical hemostasis did not cause bleeding from the wound, whereas soluble tPA caused profuse bleeding. RBC/tPA neither aggravated brain damage caused by focal ischemia in a filament model of middle carotid artery occlusion nor caused postthrombotic hemorrhage in hypertensive rats.

Conclusions—These results suggest a potential RBC/tPA utility as thromboprophylaxis in patients who are at risk for acute cerebrovascular thromboembolism. (Circulation. 2008;118:1442-1449.)

Key Words: erythrocytes ■ fibrinolysis ■ plasminogen activators ■ stroke

A need exists for safer and more effective short-term thromboprophylaxis in settings in which the risks of cerebral thromboembolism and hemorrhage are known to be high.1 The risk is greatest particularly in the first hours to days after major vascular surgeries because of inflammation, activation of clotting, and immobilization, among other causes.2 Approximately 45% of perioperative strokes occur within 24 hours of surgery.1 For patients suffering perioperative ischemic stroke, intravenous tissue-type plasminogen activator (tPA; the only drug approved for ischemic stroke treatment3) is contraindicated because of the high risk of lysing hemostatic plugs and bleeding into the surgical bed.1,4 Local intraarterial injection of tPA or mechanical thrombolysis is not widely available5 and is limited by bleeding. Furthermore, tPA also poses a risk of central nervous system (CNS) toxicity.6,7 Perioperative thromboprophylaxis by platelet and thrombin inhibitors, providing some reduction in risk,8 is often withheld for many hours or days before and after surgery,2,9 leaving patients unprotected during the most vulnerable period.10

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Hemostasis is attained within an hour after uncomplicated surgeries, whereas the highest risk of thrombosis persists for 24 to 48 hours.1,11 In theory, prophylactic administration of tPA shortly after surgery could expedite thrombolysis of nascent clots. However, short circulation time (<5 minutes) precludes prophylactic use of fibrinolytics. Furthermore, no fibrinolytic, including newer agents designed to enhance potency and affinity for thrombi,12,13 distinguishes between preformed hemostatic and nascent pathological thrombi, leaving no margin of safety for a patient with a perioperative stroke.

Coupling to red blood cells (RBCs) converts tPA into a prophylactic agent, RBC/tPA, that effectively lyses nascent...
thrombi that otherwise may cause sustained vascular occlusion. The large size of RBC/tPA precludes it from entering and lysing preexisting clots and prevents extravasation, thereby limiting CNS toxicity. Studies in animals have shown that tPA carriage by RBCs prolongs its circulation by orders of magnitude, permitting prophylactic administration; permits tPA access to the interior of nascent intravascular clots, which are then rapidly lysed from within; and blocks tPA penetration into hemostatic clots. We sought to characterize the effectiveness and toxicity of prophylactic administration of RBC/tPA in animal models of cerebral thromboembolism and ischemia.

Methods

Materials

Human recombinant tPA was from Genentech (South San Francisco, Calif). Thrombin, fibrinogen, and components of buffer solutions were purchased from Sigma-Aldrich (St Louis, Mo). Proteins were radiolabeled with Na251CrO4 (Perkin Elmer, Wellesley, Mass) using Iodogen (Pierce, Rockford, Ill).

Coupling of tPA to Carrier RBCs

RBCs were isolated from fresh anticoagulated animal blood and radiolabeled in PBS (pH 7.4) with Na251CrO4 (Perkin Elmer) as described. Biotinylated tPA was coupled to biotinylated RBCs via streptavidin, producing RBC/tPA possessing 5×104 tPA molecules per RBC as described.

Studies in Mice

Drugs (tPA, RBC/tPA, or PBS placebo) in 120 μL PBS were injected via the right femoral vein into anesthetized C57/BL6 male mice (25 to 30 g; The Jackson Laboratory, Bar Harbor, Me). Temperature probes were inserted into the rectum and the left temporalis muscle. Heating lamps and a warm thermostat “blanket” (Omega, model 410; Harvard Apparatus, Norwell, Mass) were used to maintain physiological rectal and cranial temperature (36.9±0.5°C and 36.5±0.5°C, respectively). Mean arterial pressure measured with an indwelling femoral arterial catheter connected to the Gould TA240 Easy-Graf Blood Pressure Monitor 15 minutes before occlusion and at the end of the surgery was within the normal values in all groups and was not affected by injection of emboli or intervention with tPA or RBC/tPA (not shown).

Mouse Model of Cerebral Thrombosis Caused by Fibrin Emboli

Fibrin microemboli were prepared as described. Briefly, fibrinogen (10 mg/mL) was added to citrated human plasma and coagulated by adding 20 mmol/L CaCl2 and 0.2 U/mL thrombin. Clots were homogenized in a PT-3100 Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Microemboli (>98% with a mean diameter of 1.5 to 5 μm) were isolated by centrifugation. Microemboli suspension (100 μL; 1.4×106 particles) was injected via the internal carotid artery into the middle cerebral artery (MCA). As we described, microemboli form aggregates invested with blood elements, occluding downstream vessels, causing >80% cessation of blood flow, and leading to an extensive ipsilateral cerebral infarction comparable to that caused by 20 hours of standard filament occlusion.

Transient MCA Occlusion by Intraluminal Suture in Mice

The right common carotid artery was exposed through the neck incision; the occipital branches of the external carotid artery were coagulated; and the posterior gastric artery was ligated. A monofilament 7-0 suture (Harvard Apparatus) coated with silicon was placed via the proximal external carotid artery into the internal carotid artery, occluding the MCA for 1 hour as described.

Evans Blue Uptake in the Brain

Evans blue (2% in PBS, 4 mL/kg) was injected intravenously in mice 3 hours after the injection of emboli or after transient occlusion of the MCA with a nylon suture. Two hours later, the chest was opened; animals were perfused with PBS/citrate through the left ventricle and decapitated; and blue color was visualized in 2-mm-thick coronal brain sections. Evans blue was extracted from the brain as described, and dye content per 1 g tissue was measured in a spectrophotometer (A550).

Model of Hemorrhagic Transformation

A single 3-cm clot prepared by coagulating blood of donor rats in a 50 PE catheter was injected into the MCAs of anesthetized male 250- to 300-g spontaneously hypertensive rats (SHRs; Taconic, Germantown, NY). Six hours later, 0.05 mg/kg RBC/tPA, 10 mg/kg soluble tPA, or PBS vehicle was injected intravenously, together with a suspension of 51Cr-labeled rat RBCs, over 20 minutes via an infusion pump (PHD 2000, Harvard Apparatus). Eighteen hours later, the rats were anesthetized, blood samples were collected, and animals were perfused with PBS/citrate and killed.

Analysis of Neurological Deficit

Neurological impairment was assessed 48 hours after stroke using a 4-tier score as described (1, normal spontaneous movements; 2, the animal circles clockwise; 3, the animal spins clockwise longitudinally; 4, the animal is unresponsive to noxious stimuli).

Fibrinogen Consumption In Vivo

One hour after intravenous injection of soluble tPA (2 mg/kg) or RBC/tPA (0.2 mg/kg) into mice, blood was drawn into citrate and centrifuged at 10 000g for 10 minutes to obtain plasma that was diluted (1:7500) and incubated for 1 hour at 37°C in ELISA wells coated with anti-fibrinogen antibody (BD PharMingen, San Diego, Calif). After washing with PBS, bound fibrinogen was detected with a biotinylated anti-fibrinogen antibody (1 μg/mL, BD PharMingen) and streptavidin–horseradish peroxide ELISA kit (Calbiochem).

Disruption of Postsurgical Hemostatic Thrombi by tPA Versus RBC/tPA

The assay was performed as described previously. Segments of the tail of immobilized anesthetized mice were amputated. Ten minutes later, the tails were immersed in warm (37°C) saline containing 0.01 mmol/L trisodium citrate. RBC/tPA (0.2 mg/kg) or tPA (2 mg/kg) was injected intravenously. Aliquots were taken from the buffer solution over the ensuing 60 minutes; a lytic buffer was added to lyse the RBCs, and the hemoglobin released was determined by measuring the outer diameter at 405 nm.

Statistical Analysis

Data are expressed as mean±SEM. Differences in the volume of infarcts between groups were analyzed using the 2-tailed Student t test. Significance between multiple groups was measured with ANOVA. The type I error rate for each outcome was controlled with the omnibus test for heterogeneity across all included groups was statistically significant. Survival differences between the 2 groups were analyzed with the log-rank test. Statistical significance of neurological deficits was analyzed with the Mann-Whitney test. All statistical analyses were performed with Sigma Stat 3.1 software throughout. Differences between groups were considered significant at values of P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscripts as written.
Results

Pretreatment With RBC/tPA Prevents Occlusive Cerebrovascular Thromboembolism and Restores Cerebral Blood Flow

Soluble tPA (2 mg/kg, the dose used throughout the study unless specified otherwise) injected 5 minutes before MCA occlusion by 125I-labeled emboli caused thrombolysis. At 1 hour, the residual clot burden in the brain was reduced from 31.3±4.5% in control (PBS preinjected) mice to 6.6±1.1% (Figure 1A). Therefore, tPA injected just before emboli reduced clot burden by 78.9±3.3% (ie, ~79% fibrinolysis stimulation; Figure 1B). However, when injected 30 minutes before emboli, tPA did not cause thrombolysis, whereas a 10-fold lower dose (0.2 mg/kg) of RBC/tPA injected 5, 30, or 60 minutes before emboli caused ~50% fibrinolysis stimulation (Figure 1A and 1B). As a result, RBC/tPA, but not tPA, injected 30 minutes before emboli rapidly restored cerebral blood flow to 60% of the initial level, where it remained (Figure 1C). Injection of control RBCs 30 minutes before microemboli did not affect cerebral blood flow and therefore did not account for the alleviation of cerebral ischemia by RBC/tPA (not shown).

RBC/tPA Thromboprophylaxis Alleviates Postembolic Loss of Blood-Brain Barrier Function

Evans blue accumulation in the brain was evident in the ipsilateral hemisphere 5 hours after thromboembolism (Figure 2A). Pretreatment with RBC/tPA, but not with tPA, 30 minutes before emboli attenuated the intensity of staining (Figure 2A) and amount of the dye extracted from the brain tissue (Figure 2B).

RBC/tPA Thromboprophylaxis Reduces Postembolic Mortality and Morbidity

This model of thromboembolism in mice is characterized by severe brain damage20 and >50% mortality within 24 hours
Pretreatment with tPA 30 minutes before embolism increased mortality to 90% within 10 hours (Figure 3A); hence, it was not possible to analyze subsequent outcomes in this cohort. In contrast, pretreatment with RBC/tPA 30 minutes before embolism prevented mortality (Figure 3A).

Infarct volume, measured 48 hours after embolism, was reduced 10-fold in animals pretreated with RBC/tPA compared with the survivors in the PBS-pretreated group (Figure 3B). RBC/tPA markedly attenuated the severity of the neurological deficit; the score was reduced from 2.92±0.21 in PBS-pretreated mice to 1.46±0.18 in RBC/tPA-pretreated mice (P<0.001), close to the normal performance score of 1 (Figure 3C).

Figure 4. RBC/tPA safety profile. A, RBC/tPA does not cause fibrinogen (Fg) consumption from plasma. Fibrinogen was measured in plasma obtained 1 hour after injection of 2 mg/kg tPA (hatched bar) or 0.2 mg/kg RBC/tPA (closed bar) via the jugular vein (n=4 per group). The difference between naïve mice (dash line) and mice injected with tPA is significant (*P<0.01). B, RBC/tPA does not cause postsurgical bleeding. Profound bleeding from the hemostatic clot formed in surgical wound in amputated tail was visualized within 10 minutes after injection of tPA but not RBC/tPA or PBS (open, hatched, and closed bars, respectively) (n=3 per group). tPA injection caused significantly greater bleeding than PBS and RBC/tPA (*P<0.001 vs PBS; ##P<0.001 vs RBC/tPA). C, RBC carriage restrains tPA uptake in the brain. 125I-tPA (hatched bar) or RBC/125I-tPA (closed bar) was injected into the tail vein of rats. Radioactivity in the brain was measured 1 hour later and corrected for differences in the rate of blood clearance of tPA and RBC/tPA. The brain uptake of 125I-tPA was ~10 times higher than RBC/125I-tPA (n=3 rats per group). The difference between RBC/tPA and tPA groups is significant (##P<0.001).
fibrin stimulation than tPA\textsuperscript{16–18} and with the fact that the effective dose of RBC/tPA is lower (Figure 1).

RBC/tPA injected 10 minutes after the bleeding caused by tail clipping stopped did not cause lysis and rebleeding, whereas tPA caused profuse bleeding (Figure 4B), likely because of the 10-fold lower effective dose of RBC/tPA and the intravascular confinement of RBC/tPA, which restricted its penetration into postsurgical mural clots and extravascular tissues. In support of the latter feature, the level of RBC/tPA does not cause postthrombotic hemorrhage in naive rats was \textasciitilde 10-fold less than after injection of the same dose of soluble \textsuperscript{125}I-tPA (Figure 4C).

**RBC/tPA Does Not Cause Postthrombotic Hemorrhagic Transformation in SHRs**

RBC/tPA, but not RBC/tPA, injected 6 hours after injection of an occlusive clot in the MCA of SHRs aggravated postthrombotic intracerebral hemorrhage compared with rats given PBS after thrombosis. Massive hemorrhages were visible in brain sections of the ipsilateral hemisphere of tPA-treated mice but not in animals given RBC/tPA (Figure 5A). To quantify the extent of hemorrhage, we measured extravasation of \textsuperscript{51}Cr-labeled RBCs (Figure 5B). After the basal level of \textsuperscript{51}Cr-RBC retention in the brain of naive SHRs was subtracted (0.2%; dash line in Figure 5B), tPA-treated postthrombotic animals showed 4-fold more blood loss into the brain compared with mice in the PBS control group (1.5\pm0.4\% versus 0.4\pm0.1\%; \(P<0.05\)). In contrast, an \textasciitilde 50\% reduction was found in postthrombotic extravasation of \textsuperscript{51}Cr-RBC in the brain of SHRs treated with RBC/tPA compared with the same control population, with borderline significance (Figure 5B; 0.2\pm0.1\% versus 0.4\pm0.1\%; \(P=0.056\), NS).

**RBC/tPA Does Not Aggravate Ischemic Brain Injury**

The RBC/tPA toxicity could be more evident when the integrity of the blood-brain barrier is compromised by ischemia. To avoid potentially overriding salutary effects of prophylactic thrombolysis (Figures 1 through 3), we examined the RBC/tPA toxicity in a model of ischemia induced by filamentous occlusion of the MCA. The reduction in blood flow by the filament and the extent of reperfusion were identical in all groups at 1 hour (Figure 6A). Immediately after the filament was removed, mice were injected with PBS, RBC/tPA, or tPA as a positive control (at a dose of 10 mg/kg, used commonly in this species because mouse plasminogen is relatively resistant to human tPA).\textsuperscript{26} Mice were injected 3 hours later with \textsuperscript{51}Cr-labeled RBCs and Evans blue and killed after an additional 2 hours to measure dye and \textsuperscript{51}Cr-RBC extravasation (Figure 6B and 6C). Separate cohorts of mice were killed 24 hours after ischemia to measure infarct size (Figure 6D). RBC/tPA at the dose that protected against thromboembolism (Figures 1 through 3) did not aggravate postfilament/ischemic leakage of Evans blue (Figure 6C), extravasation of blood into the brain (Figure 6B), or infarct size (Figure 6D). In contrast, tPA exacerbated ischemic CNS damage assessed by the last 2 parameters.

**Discussion**

tPA doses required to overcome its limited bioavailability have a high propensity to cause hemorrhage in the ischemic brain,\textsuperscript{21} disruption of the blood-brain barrier, and damage to ischemic parenchyma.\textsuperscript{6,7,27,28} Attempts to alleviate these adverse effects of tPA have shown little clinical utility to date.\textsuperscript{6,29–32} In this study, tPA worsened outcomes in a filament occlusion model of brain ischemia (Figure 6) and aggravated the deleterious effect of cerebrovascular thrombosis in mice when given as prophylaxis (Figure 3A). In contrast, prophylactic injection of a 10-fold lower dose of RBC/tPA rapidly lysed cerebrovascular emboli, markedly alleviating brain edema, injury, and neurological impairment (Figures 2 and 3), all of which contributed to eliminating mortality (Figure 3A). Of note, outcomes at 48 hours could be determined only among the survivors in the PBS-treated cohort. Undoubtedly, these outcomes were far more severe in mice that succumbed to cerebral injury within the first 10 hours (Figure 3A). Therefore, the extent of protection against cerebrovascular thromboembolism provided by RBC/tPA prophylaxis is likely underestimated. RBC/tPA caused no detectable adverse effects in models of brain ischemia in mice and postthrombotic intracerebral hemorrhage in SHRs (Figures 5 and 6).
Thus, RBC/tPA provides effective and safe cerebrovascular thromboprophylaxis, whereas tPA was ineffective even at a 10-fold higher dose. Undoubtedly, much of the reduction in toxicity compared with tPA is attributable to the lower dose of RBC/tPA required for thrombolysis (Figure 1). RBC carriage also prevents uptake of tPA by the brain (Figure 4C). The reduction in adverse effects in the CNS also is attributable to diffusional and steric limitations imposed by carrier RBCs on coupled tPA. In theory, even if RBC/tPA enters the CNS as a result of cerebral hemorrhage, diffusion of tPA into the parenchyma will be restricted, and the RBC glycocalyx and steric constraints will inhibit interactions with tissue targets and increase the requirement for fibrin for enzymatic effects.16

Regardless of the mechanism, tPA caused profound bleeding from surgical wounds, was deleterious if given as soon as 1 hour after induction of cerebral ischemia by mechanical occlusion of the MCA, and was ineffective and deleterious if given >5 minutes before thrombotic occlusion. In contrast, RBC/tPA prophylaxis showed no detectable toxicity and protected against thromboembolism. Prior studies in mice and rats revealed that RBC/tPA neither prolongs the blood clotting time nor causes bleeding during surgical access for vascular catheterization.14,15 It caused no bleeding in the tail amputation test (Figure 4B). These data indicate that hemostatic mural clots become impervious to RBC/tPA within minutes and justify further assessment of RBC/tPA in more clinically relevant hemorrhage-prone settings. RBC/tPA, but not tPA, retained its fibrinolytic activity for hours after injection in mice and rats because of prolonged pharmacokinetics14,16,17 and protection against plasma inhibitors by the RBC glycocalyx.18 Therefore, RBC/tPA can be used relatively early in the postoperative period to prevent occlusive cerebrovascular thromboembolism.

Cerebrovascular embolization is a common complication of cardiopulmonary bypass surgery, coronary artery bypass grafting, carotid artery endarterectomy, and stenting. Perioperative thromboembolism is a catastrophic complication that occurs primarily with the first 48 hours of surgery when the risk of postoperative bleeding is maximal. Postsurgical microemboli cause local or diffuse cerebral ischemia identified on transcranial Doppler ultrasound and diffusion-weighted magnetic resonance imaging. Initial manifestations of cerebral embolization range from severe
stroke to more subtle but still potentially disabling postoperative cognitive dysfunction. Some symptoms may improve, but in many cases, postoperative cognitive dysfunction persists or progresses to dementia.

RBC/tPA may be of use in some patients with cerebrovascular ischemia caused by acute or recurrent thromboembolism rather than by atheroembolism, vasculitis, vasospasm, or other pathological mechanisms, but the incidence and therapeutic window for thromboprophylaxis in most settings have not been established.

Acute myocardial infarction and non-ST-elevation myocardial infarction, transient ischemic attack, and stroke are associated with considerable short-term risk of acute secondary thrombosis and stroke. For example, ≈20% of ischemic stroke patients with an initially favorable clinical response to intravenous tPA develop symptomatic recanalization, whereas up to 15% of patients with acute myocardial infarction, transient ischemic attack, or stroke develop secondary cerebrovascular occlusion within the first few days of a sentinel event. Such patients are generally hospitalized to facilitate timely thrombolysis if needed. Therefore, a number of additional clinical situations commonly complicated by cerebrovascular thromboembolism are known, and subpopulations at high risk have been identified. Such patients would be amenable to prophylactic intervention, which currently is insufficient.

Neither anticoagulants nor antiplatelet agents provide complete thromboprophylaxis, even in the majority of eligible patients, because of the redundancy of hemostatic pathways. In the early postoperative period, these agents are not used because of the risk of bleeding. In contrast, the mechanism of action of RBC/tPA suggests that it will not affect hemostatic clots formed within the first hours after surgery, whereas lysing clots formed subsequent to its administration, many of which lead to ischemic tissue injury.

More practical means for loading RBCs with tPA, including injection of tPA derivatives directly targeted to RBC determinants in vivo, are being developed. Incorporation of RBC/tPA into clots that have begun to form despite the use of platelet agents and anticoagulants will reinforce thromboprophylaxis by inhibiting sequentially engaged and mechanistically distinct (e.g., fibrin-mediated) aspects of thrombosis. As studies of RBC/tPA are extended to larger and more complex animal models, we will learn the limits of this approach and the clinical scenarios in which it might provide benefit to patients with imminent or recurrent cerebrovascular thromboembolism.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Cerebrovascular thrombosis is a major cause of mortality and morbidity, including after surgery when the use of thromboprophylaxis is limited by the formidable risk of perioperative bleeding. Nor can thrombolytics (eg, tissue-type plasminogen activator [tPA]) be used prophylactically in this and other high-risk settings because of their short duration of action and risk of causing hemorrhage. Coupling tPA to red blood cells (RBCs) prolongs and confines activity within the bloodstream and enhances resistance to plasma inhibitors, thereby providing a novel thromboprophylactic agent, RBC/tPA. Our results indicate that injection of RBC/tPA into mice before thromboembolic occlusion of the middle carotid artery facilitates clot lysis, assists in rapid and stable cerebrovascular reperfusion, alleviates ischemic brain damage, and eliminates mortality, whereas pretreatment with tPA is not protective even at a 10-fold higher dose at which mortality is aggravated. At protective doses, RBC/tPA did not consume plasma fibrinogen or cause postbypassing or hemorrhage and toxicity in the central nervous system in rodent models of brain ischemia and thrombosis. This animal studies indicate that RBC/tPA might provide thromboprophylaxis to patients at risk for cerebrovascular thromboembolism in the postoperative period; after transient ischemic attack, myocardial infarction, and stroke; or in the setting of non–ST-elevation acute myocardial infarction, which is characterized by multiple cycles of intravascular rethrombosis and incomplete thrombolysis. Nascent and growing occlusive clots represent the preferable target for RBC/tPA. Use of injectable antithrombotic prodrugs conjugated with RBC binding peptides may further enhance the clinical application of this new approach toward prophylactic thrombolysis within the cerebral and other vasculatures.
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