Endogenous fibrinolysis.

Although endogenous fibrinolysis by uPA and tPA are each stimulated dramatically by fibrin\(^1\), the mechanisms by which their activities are enhanced differ. tPA exerts only modest plasminogen activation activity in the absence of fibrin\(^2\) (Ms. Figure 2D). Fibrinolysis is initiated when tPA and the pro-enzyme plasminogen bind to the surface of fibrin\(^2\) (Figure S1-A), which increases the local concentrations of the reactants\(^2,3\). tPA cleaves the Arg\(^{560}\)-Val\(^{561}\) bond in plasminogen on the surface of the fibrin clot to generate fibrin-bound plasmin, which then cleaves fibrin into diverse degradation products (FDPs) (Figure S1-A). uPA also cleaves plasminogen to plasmin on the fibrin surface (Figure S1-B) but without binding to fibrin\(^4,6\). Plasminogen binds to N-terminal lysine residues on fibrin through its lysine binding sites (LBS) in kringles 1-3. Plasminogen then undergoes a conformational change, which makes it a better substrate for uPA\(^7\) (Figure S1 B). The conformational changes in plasminogen induced by fibrin do not affect its activation by tPA. It is worth noting that in the absence of fibrin, uPA exerts greater plasminogen activation than tPA (Ms. Figure 2D), which increases its potential to generate unbound plasmin in the circulation and in other biologic fluids. Free plasmin activates, inactivates and promotes the clearance of activated coagulation factors\(^8,9\) and platelets\(^10\).

Lysine analogs such as tranexamic acid (TA) and \(\varepsilon\)-aminocaproic acid (EACA) have been used extensively as anti-fibrinolytics to prevent bleeding based on the assumption that tPA is the principal plasminogen activator in the circulation. Tranexamic acid and \(\varepsilon\)-aminocaproic acid bind to the LBS of plasminogen and thereby inhibit its binding to fibrin and its activation by tPA (Figure S1-C)\(^11\). However, uPA compensates for genetic deletion of tPA\(^12,13\) and contributes to plasma fibrinolysis. Binding of lysine analogs to plasminogen mimics the effect of fibrin and enhances its susceptibility to be activated by uPA\(^11,14,15\) (Figure S1-D). Based on these principles, it would be expected that lysine analogs would enhance generation of plasmin in a fibrin-independent mode (Figure S1-D). Furthermore, plasmin generated in presence of lysine analogs (Figure S1-D) is protected from inactivation by \(\alpha_2\)-antiplasmin\(^16,17\), which would increase potential undesired effects on coagulation factors and platelets.
The enhanced activation of plasminogen-lysine analogs by uPA leading to the formation of soluble plasmin that does not bind to fibrin, but remains enzymatically active and causes fibrinolysis, which could also contribute to the development of ICH. In support of this, Collen at al. showed that microplasmin is an effective thrombolytic agent although it cannot bind to fibrin18-20.

Our data show that tPA-S\(^{481}\)A binds to fibrin (Ms., Figure 1A) and inhibits the binding of WT-tPA (Ms., Figure 1B). In contrast to WT-tPA\(^2\), the interaction of tPA-S\(^{481}\)A with plasminogen is enhanced by fibrin (Ms., Figure 1C) or lysine analogs (Ms., Figure 2D), which inhibits uPA-mediated as well as tPA-mediated fibrinolysis (Ms., Figures 2A-D). We posit that by virtue of its two-step interaction with fibrin and with plasminogen bound to fibrin (Suppl., Figures 2 A-B), tPA-S\(^{481}\)A is a more fibrin-targeted and potent inhibitor of fibrinolysis than either aprotonin, tranexamic acid or ε-aminocaproic acid.

**Paradoxical effect of tranexamic acid of bleeding**

The results of the CRASH-2 trial showed that tranexamic acid had a paradoxical effect on bleeding in trauma patients\(^21\). Treatment within three hours post trauma reduced the risk of death due to bleeding; treatment given to the same groups of patients three hours after trauma increased the risk of death due to bleeding\(^21\). Tranexamic acid was ineffective in patients with TBI\(^22\). Based on these outcomes, the US Department of Defense Hemorrhage and Resuscitation Research and Development Steering Committee called for more research efforts to better understand the mechanism of action of tranexamic acid\(^23\), which was supported by the Australian Defense Force and Burns Trauma and Critical Care Research Centre\(^24\).

Based on the data presented in this manuscript, we hypothesize that the paradoxical effect of tranexamic acid on uPA-mediated fibrinolysis could explain the results of CRASH-2. Our hypotheses is supported by the findings presented in Figure 6A of the manuscript showing tranexamic acid given post TBI increased intracerebral bleeding assessed by measuring brain hemoglobin in tPA\(^{-/-}\) mice, increased d-Dimers and caused thrombocytopenia but these outcomes did not occur in uPA\(^{-/-}\) mice (MS Figures 6C and 6D).
To examine this hypothesis we measured time-dependent changes in the concentrations of tPA and uPA in the CSF after CHI. tPA increases immediately after TBI, and falls to 50% of its maximal concentration by 4 hours post TBI, whereas the increase in uPA occurs later and uPA levels remain high for a longer period of time (Ms Figure 7A). These findings may help to explain why tranexamic acid fails to prevent persistent intracerebral bleeding 8 hours after TBI (Ms, Figure 7B), consistent with the adverse effect on bleeding seen with delayed administration in CRASH-2.

**Supplement, figure legends**

**Supplement Figure 1S: Fibrinolytic Cascade:**  
**Panel A. Activation of the fibrinolytic cascade by tPA.** Activation of the fibrinolytic cascade by tPA begins when tPA (1) and plasminogen (2) bind to fibrin. High local concentrations of both reactants on the clot surface accelerate cleavage of plasminogen to plasmin (3), which degrades fibrin to fibrin degradation products (4) (FDP).  
**Panel B. Activation of the fibrinolytic cascade by uPA.** Activation of the fibrinolytic cascade by uPA begins by binding of plasminogen to fibrin (1). Plasminogen binds to fibrin through its lysine binding sites (LBS) to N-terminal lysine residues on fibrin. This induces conformational changes in plasminogen (Plasminogen*), which makes it a better substrate for uPA (2) and accelerates cleavage of plasminogen to plasmin (3), which degrades fibrin to fibrin degradation products (4) (FDP).  
**Panel C. Inhibition of tPA mediated fibrinolysis by tranexamic acid.** Tranexamic acid (TA) binds to circulating plasminogen (5) through its lysine binding sites (LBSs). This, in turn, inhibits binding of plasminogen to fibrin (6) and thereby inhibits its activation by tPA. Tranexamic acid (TA) must be present at high concentrations to fully occupy LBSs in plasminogen (plasma concentration 2-3 µM).  
**Panel D. Inhibition of uPA mediated fibrinolysis by tranexamic acid.** Binding of tranexamic acid (TA) (1) to free plasminogen inhibits binding of plasminogen to fibrin (2), but changes its conformation (Plasminogen*) similar to the changes induced by fibrin (see Panel B) and thereby stimulates plasminogen activation by uPA in a fibrin independent fashion (3). Thus, tranexamic acid enhances plasmin generation in the circulation (3) and, we posit, within the pericontusional regions in the brain, which partially offsets its anti-fibrinolytic effect on tPA. Free plasmin (not bound to fibrin) has greater opportunities to act on coagulation factors and platelets.
Supplement, Figure 2S: Inhibition of fibrinolysis by tPA$^{5481A}$: Panel A. tPA mediated fibrinolysis. tPA$^{5481A}$ binds to fibrin (1) tPA-S$^{481}$A thereby inhibits the binding of WT-tPA (2), its resultant plasminogen activator activity (3), generation of plasmin (4) and fibrinolysis (5). tPA-S$^{481}$A also binds to fibrin bound plasminogen (Plasminogen*) (6) and thereby competes with WT-tPA binding and subsequent plasminogen activation, in that way exerting a second level of inhibition of tPA activity. Panel B. uPA mediated fibrinolysis. tPA-S$^{481}$A binds to fibrin-bound plasminogen (Plasminogen*) (1), and thereby inhibits its activation by uPA (2), generation of plasmin (3), and fibrinolysis (4).

Supplement, Figure 3. Intracerebral hemorrhage following closed head injury (CHI): Appearance of brain extracted 2, 8 and 24 hours after CHI.

Supplement, Figure 4. Assessment of intracerebral hemorrhage: At the indicated times post CHI, both the ipsilateral and contralateral hemispheres were extracted from mice after extensive transcardial perfusion. Distilled water (300 µl) was added to each hemisphere, followed by homogenization for 30 seconds, sonication on ice for 1 minute, and centrifugation at 5000 g for 30 minutes. Drabkin reagent (80 µL; Sigma) was added to a 20 µL aliquot of hemoglobin-containing supernatant, which was allowed to stand for 15 minutes at room temperature and the optical density was measured at 540 nm to determine hemoglobin content. Hemoglobin concentration in mg% were determined using a standard curve (Figure S4) generated by adding blood obtained by cardiac puncture from anesthetized control mice and added at incremental volumes (0, 0.5, 1.0, 2.0, 4.0, and 8.0 µL) to 300 µL of lysate from naïve hemispheres. The hemoglobin content was then measured as above.

References


Figure S1 A

Fibrinolysis by tPA

1. tPA
2. Plasminogen
3. Plasminogen* → Plasmin
4. FDP
Figure S1 B

Fibrinolysis by uPA

Plasminogen

1

uPA

2

+ 

Plasminogen*

3

Plasmin

4

Plasmin

FDP
Figure S1 C

Inhibition of tPA mediated fibrinolysis by TA
Figure S1 D

*Inhibition of uPA mediated fibrinolysis by TA*

![Diagram showing the inhibition process]

1. Plasminogen + TA → Plasminogen*-TA
2. uPA → Plasminogen* → Plasmin
3. Plasminogen*-TA + FDP → Plasmin-TA
4. Plasmin-TA
Inhibition of tPA mediated fibrinolysis by tPA$^{S481A}$

Figure S2 A

- tPA-S$^{481A}$
- tPA
- Plasminogen
- Plasminogen$^*$
- Plasmin
- FDP
Inhibition of uPA mediated fibrinolysis by tPA\textsuperscript{S481A}

**Figure S2 B**

- **1.** tPA-S\textsuperscript{481A} binds to Plasminogen.
- **2.** uPA is inhibited by tPA-S\textsuperscript{481A}.
- **3.** Plasmin is activated from Plasminogen.
- **4.** Plasmin cleaves fibrinogen to FDP.
Figure S3

ICH induced by CHI

2 hrs                                     8 hrs                                          24 hrs
Figure S4

**Standard curve of blood extravasation**

![Graph showing standard curve of blood extravasation with units of Hemoglobin (mg%) on the y-axis and Blood in 300 µl of brain lysate on the x-axis. The graph displays a linear relationship between the two variables.]