Camouflaged and Thrombin-triggered delivery of tissue plasminogen activator for targeted thrombolysis

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ABSTRACT SUMMARY

In this study, we developed a thrombin-cleavable delivery system for tissue plasminogen activator (tPA) to attenuate bleeding complications associated with conventional thrombolytic therapy. tPA was conjugated with human serum albumin (HSA) via a thrombin-cleavable peptide (GFPRGFPAGGCtPA) (Fig. 1). The resulting camouflaged tPA construct has been designed to mask the enzymatic activity of tPA in the circulation and lead the construct to concentrate over thrombus by means of a homing peptide, CQQHHLGAKQAGDV that binds with GPIIb/IIIa expressed on activated platelets. Upon accumulation of the construct over thrombus, tPA’s activity would be regenerated by thrombin, present in excess in the clot microenvironment (Fig. 2). The construct demonstrated a thrombin-triggered release of tPA in vitro as determined by chromogenic and fibrin agar plate assays. Stability study suggests that the camouflaged structure remained intact in human plasma and blood environment for at least 30 minutes. The camouflaged tPA binds with activated platelets as confirmed by microscopic study. Furthermore, pharmacological efficacy study in rat thrombosis model suggests that camouflaged tPA retained the same thrombolytic activity as that of native tPA with 2.5 fold reduced depletion of circulating fibrinogen, indicating less risk of systemic tPA activity.

INTRODUCTION

Tissue Plasminogen Activator (tPA) is the most widely used thrombolytic agent for the treatment of acute thrombotic events such as myocardial infarction and stroke. It converts plasminogen to plasmin, which in turn degrades the fibrin mesh and dissolves thrombus. However, tPA potentiates bleeding complications by acting upon circulating plasminogen that causes depletion of other clotting factors including fibrinogen and α-2 antiplasmin, leading the body to a ‘lytic state’. tPA induced life-threatening bleeding complications can be attenuated by delivering the thrombolytic agent at the thrombus site and minimizing its exposure to circulating clotting factors. Since acute thrombotic events require rapid therapeutic intervention, a drug should be released promptly to produce its thrombolytic effects. To address these problems, we propose a construct wherein tPA will be shielded by human serum albumin (HSA) to protect its fibrinolytic activity in the systemic circulation. A homing peptide will guide the camouflaged tPA towards the occlusive site and upon reaching thrombus, the clot-lytic activity of tPA will be regenerated by thrombin by cleaving the linkage between HSA and tPA.

EXPERIMENTAL METHODS

Carboxyl group of HSA was conjugated to the N-terminus amine group of the cleavable peptide by EDC chemistry, while the primary amines of tPA were conjugated with the C-terminus cysteine of the peptide using thiolation (SPDP) chemistry. To prevent non-specific conjugation, the Cys-34 of HSA was blocked by iodoacetamide and the primary amines were reversibly blocked by citraconylation which were later unblocked to conjugate the homing peptide. All subsequent purification was performed by FPLC (fast protein liquid chromatography) and/or centrifugal filtration.

The thrombin-triggered activity was determined by plasmin-specific chromogenic substrate S-2251. The fibrinolytic activity was monitored by agar plate assay. To test the effect of thrombin-stimulation, the camouflaged-tPA construct was treated with 25 nM thrombin for an hour prior to measuring the activity. To test the stability, human plasma and blood were collected from Coffee Memorial Blood Center (Amarillo, TX). The samples (with or without thrombin treatment) were incubated for 30 minutes at 37°C and the activity of tPA was then measured by a chromogenic assay.

To test the binding characteristic of the camouflaged-tPA with activated platelets, human platelet rich plasma was collected and activated using a published procedure and binding was tested under a fluorescence microscope using fluorescent labeled constructs.

The efficacy of camouflaged-tPA was tested in an inferior vena cava (IVC) thrombosis model.
Briefly, IVC of rats was surgically exposed and platelet-rich thrombus was induced using 35% FeCl₃ solution. Camouflaged-tPA, native tPA or saline was administered via the penile vein to different groups (n=4) of animals. One hour after administration, the thrombus was meticulously collected and weighed. Blood sample was also collected to test fibrinogen level using ELISA assay.

RESULTS AND DISCUSSION
Preparation and characterization of thrombin-cleavable camouflaged-tPA: The free-thiol group of HSA (Cys-34) was successfully blocked by 5-fold excess of iodoacetamide (Fig. 3A). A 50-fold excess of citraconic anhydride effectively blocked the free amines of HSA (Fig. 3B) which was subsequently unblocked at pH 5.0, as confirmed by TNBS assay and FPLC (Fig. 3C, 3D). Conjugation of the homing peptide was confirmed by monitoring the release of pyridyl-2-thione from SPDP-activated HSA. An average of two albumin molecules/tPA was found in the camouflaged-construct.

Albumin-masked tPA demonstrates thrombin mediated triggered release in vitro: Camouflaged tPA possessed only 25% enzymatic activity compared to that of native tPA (Fig. 4). Importantly, this activity was regenerated following incubation with thrombin, suggesting that thrombin might have cleaved the peptide and facilitated regeneration of tPA activity. The observation was further confirmed by the agar plate assay in which the fibrinolytic activity of the camouflaged-tPA was significantly less than that of native tPA, which was regenerated by incubation with thrombin (Fig. 5). Both in vitro experiments revealed that camouflaged tPA had a suppressed enzymatic activity that was subsequently regained in the presence of thrombin.

The construct binds with GPIIb/IIIa present on activated platelets: The peptide decorated construct demonstrated preferential binding affinity to the activated platelets (Fig. 6), the presence of which was confirmed by staining with anti-GPIIb/IIIa antibody. This outcome is consistent with the hypothesis that the homing peptide facilitates accumulation of the construct on the thrombus by binding with the integrin GPIIb/IIIa expressed on the activated platelets during clot formation.

The camouflaged tPA remained stable in human plasma and demonstrated efficient clot-lysis with minimal systemic effect in rat model of thrombosis: A critical concern towards success of this construct is its stability in plasma condition. Following 30 minutes incubation in human plasma and blood, camouflaged tPA retained its integrity (not shown). When the pharmacological efficacy was tested in rat thrombosis model, clot-lytic activity was similar to that of native tPA (Fig. 7), suggesting that tPA activity was regenerated. Moreover, the activity of tPA was suppressed in the systemic circulation, as demonstrated by 2.5 fold reduced depletion of the fibrinogen level compared to that of native tPA.

CONCLUSION
It is feasible to prepare a thrombin-cleavable albumin-camouflaged delivery system for tPA in which the enzymatic activity can be significantly suppressed in the circulation and regenerated by thrombin to preferentially at the clot-site.