

## Forum Minireview

# Novel Situations of Endothelial Injury in Stroke — Mechanisms of Stroke and Strategy of Drug Development:

## Novel Mechanism of the Expression and Amplification of Cell Surface–Associated Fibrinolytic Activity Demonstrated by Real-Time Imaging Analysis

Yuko Suzuki<sup>1,\*</sup> and Tetsumei Urano<sup>1</sup><sup>1</sup>*Department of Physiology, Hamamatsu University School of Medicine,  
1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan**Received September 29, 2010; Accepted November 17, 2010*

**Abstract.** Vascular endothelial cells (VECs) secrete tissue plasminogen activator (tPA) in an active form and thus its facilitated secretion directly enhances fibrinolytic activity. We have recently demonstrated its unique secretory dynamics in GFP-tagged tPA expressing VECs using total internal reflection fluorescence microscopy. tPA-GFP appeared to remain on the cell surface after secretion. Studies using a domain-deleted mutant of tPA-GFP suggested that its binding to the cell surface was heavy-chain dependent. PA inhibitor-1 (PAI-1) facilitated dissociation of tPA-GFP by forming a high molecular weight complex. Lack of dissociation from the cell surface of catalytically inactive mutant tPA-GFP, which does not complex with PAI-1, supported PAI-1 dependence of the disappearance of tPA from the VEC surface. To confirm the possibility that retained active tPA modified cell surface fibrinolytic activity, we analyzed binding of Alexa Fluor 568–labeled plasminogen (plg-568) in tPA-GFP expressing cells. Plg-568 appeared to accumulate at tPA-GFP–retained spots as well as in pericellular/matrix adhesive areas. Either modification of the active site or deletion of the tPA-GFP heavy chain resulted in decreased accumulation of plg-568. Prolonged retention appeared essential for tPA to effectively express and amplify fibrinolytic activity on VECs, which may also be responsible for development of deleterious effects in the case of stroke.

**Keywords:** tissue plasminogen activator, vascular endothelial cell, real-time imaging, fibrinolysis, plasmin, endothelial injury

### 1. Introduction

Tissue plasminogen activator (tPA) is a 68 kDa serine protease that cleaves a single peptide bond in plasminogen to generate plasmin, which subsequently dissolves fibrin clots in the vasculature (1). Intravenous infusion of tPA for up to 3 h from the onset of symptoms is currently the standard protocol for treatment of acute ischemic stroke patients. There is a growing body of evidence, however, indicating that tPA also has deleterious effects in the ischemic brain such as induction of cerebral hem-

orrhage (2), edema, and ischemic cell death (3). Disruption of the neurovascular unit (NVU), also known as the blood-brain barrier (BBB), which consists of endothelial cells, astrocytes, adhesive proteins, basement membrane, and so on, is a critical event in the pathogenesis of acute ischemic stroke (4). We therefore believe it important to understand how proteases such as matrix metalloproteinases (MMPs) and plasmin disrupt NVU and how tPA exerts its harmful effects which are distinct from its beneficial thrombolytic effects. Recently, we succeeded in visualizing the secretory dynamics of GFP-tagged tPA (tPA-GFP) expressed in VECs using total internal reflection fluorescence (TIRF) microscopy (5). This technique employs an evanescent wave to excite the fluorophore present only in the immediate vicinity of the plasma

\*Corresponding author. seigan@hama-med.ac.jp  
Published online in J-STAGE on April 16, 2011 (in advance)  
doi: 10.1254/jphs.10R23FM

membrane (evanescent field) (6–8), which is advantageous for analysis of events taking place near the plasma membrane.

Here, we review the unique secretory dynamics of tPA in vascular endothelial cells (VECs) and the means by which fibrinolytic activity is expressed and amplified on the cell surface.

## 2. Visualization of secretory dynamics of tPA-GFP from its secretory granule in VECs

We used the human VEC line EA.hy926 (9), which has been shown to retain EC-specific functions including their fibrinolytic characteristics (10). To visualize the process of tPA-GFP release from its secretory granules, we performed time-lapse analysis using TIRF microscopy. TIRF illuminates only the localized area to a depth  $\leq 100$  nm from the glass coverslip into the specimen because the wave energy drops off exponentially with distance from the coverslip-specimen interface. This has the advantage that molecular events taking place only near the plasma membrane can be analyzed. Using this technique, several sequential steps leading to single granular exocytosis of tPA-GFP were visualized as changes of fluorescence intensities.

First, the recruitment of tPA-GFP-containing granules to the evanescent field and subsequent docking to the plasma membrane were imaged as the appearance of a static fluorescent spot. Second, granular opening was documented as a sudden increase in fluorescence due mainly to neutralization of the acidic pH in tPA-GFP-containing granules by culture medium at neutral pH. Indeed a spatial shift of tPA-GFP closer to the coverslip during granule opening also contributes to the increase in fluorescence. The third stage of the tPA-GFP secretory process, that is, material release according to this definition, was also detected as a decrease in fluorescence intensity at the granular opening spot. The third stage was more heterogeneous than the other two steps. We calculated the time required for the fluorescence intensity to decline to half of its peak value during the exponential reduction period following the sudden brightening due to granule opening and designated this as  $T_{F1/2}$ . Individual  $T_{F1/2}$  values during the tPA-GFP releasing process varied widely from a maximum of 266 s to a minimum of 1.13 s. Notably, almost all opened granules, even those with the shortest  $T_{F1/2}$ , remained detectable as fluorescent spots during the entire recording period. Similar heterogeneity in the dynamics of fluorescent protein-labeled tPA release has also been described previously in bovine chromaffin cells (11) and in insulin-producing MIN6 cells (12). However, the underlying mechanisms and reasons for this have not yet been determined.

## 3. Retention of exocytosed tPA on the cell surface via its heavy chain

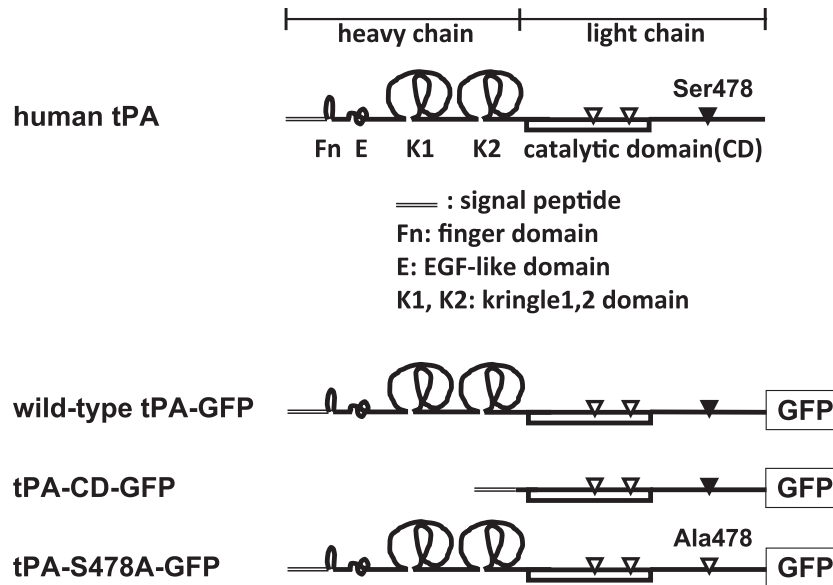
Exocytosed tPA-GFP was retained at the membrane surface without rapid retrieval and then diffused gradually from the site of the opened granule membrane into the fluid phase and/or plasma membrane in a VEC-specific manner. To investigate the tPA domain responsible for its slow release from the opened granule, we constructed a mutant tPA lacking the heavy chain and possessing only the signal peptides and catalytic domain and fused this with GFP (Fig. 1: tPA-CD-GFP). The heavy chain of tPA is composed of finger, epidermal growth factor-like, kringle 1, and kringle 2 (FEK1K2) domains and plays an important role in its binding to fibrin clots.

After opening, the fluorescence of tPA-CD-GFP disappeared immediately, in the manner of a flashlight being turned off and did not leave any fluorescent spots. Therefore, FEK1K2 domains appeared responsible for the binding of exocytosed tPA-GFP to the membrane surface as well as for its slow release. Retention of the active form of tPA on the cell surface without retrieval may be beneficial for maintaining high fibrinolytic activity at the vascular wall.

## 4. Plasminogen activator inhibitor-1 (PAI-1) accelerates the dissociation of membrane surface-retained tPA

Intravascular fibrinolytic potential is finely tuned by the balance between the amounts of tPA and its specific inhibitor, PAI-1. The latter is present in molar excess over tPA in human plasma. Not only an elevation of PAI-1 plasma concentration, but also an impairment of tPA release results in the decrease in fibrinolytic activity regarded as a risk factor for ischemic cardiovascular events (13). Although interactions between these two molecules in the fluid phase have been extensively studied, interactions on the surface of solid-phase structures such as fibrin or the cell surface, where functional fibrinolysis actually takes place, have not been fully elucidated (14).

We investigated the effects of PAI-1 on the dynamics of tPA release using the following three different approaches: i) Monitoring of tPA-GFP exocytosis after supplementation with recombinant PAI-1, ii) Monitoring of tPA-GFP exocytosis after suppression of PAI-1 synthesis by siRNA, iii) Monitoring of the exocytosis of a GFP tagged mutant tPA, tPA-S478A-GFP (Fig. 1). In the first approach, we analyzed the effect of recombinant PAI-1 (rPAI-1) in tPA-GFP expressing VECs.  $T_{F1/2}$  was significantly reduced on addition of 40 nM rPAI-1. When the cells were incubated with rPAI-1 for 60 min, tPA



**Fig. 1.** Molecular structure of wild-type and mutant forms of tPA-GFP.

increased in a dose-dependent manner in the supernatant only, in the form of a complex with rPAI-1, as detected by fibrin autography. In the second approach, in order to more clearly demonstrate the effect of exogenous rPAI-1 on membrane surface-retained tPA, the cells were treated with PAI-1 siRNA to negate the effects of intrinsically synthesized PAI-1. Transient transfection with two different sequences (5' GUC ACA UUG CCA UCA CUC UTT, 5' AAG CAC AAC UCC CUU AAG GUC TT) of PAI-1 siRNA reduced the amounts of free PAI-1 in the culture medium after 18 h, although the amounts of complex form with tPA did not change a great deal. Under such conditions,  $T_{F1/2}$  was prolonged significantly in comparison with control siRNA-transfected cells. These effects of PAI-1 siRNA were reversed by the addition of 40 nM rPAI-1. Therefore, PAI-1 appeared to regulate the amounts of cell surface-associated tPA on VECs by facilitating the dissociation of tPA from the cell surface. To confirm this, in the third approach, we constructed a GFP-tagged mutant form of tPA, tPA-S478A (Fig. 1), in which Ser 478 was substituted by Ala so as to be catalytically inactive and unable to form covalent complexes with PAI-1; Then we analyzed its exocytosis dynamics. Although the efficacy of expression of tPA-S478A-GFP and wild-type tPA-GFP was similar, the former did not appear in the culture medium after 24 h, either complexed with PAI-1 or in the free form. The intracellular distributions of tPA-S478A-GFP and tPA-GFP were indistinguishable, as judged by epifluorescence images. In contrast, much stronger fluorescence of tPA-S478A-GFP than of wild-type tPA-GFP was demon-

strated by TIRF microscopy, suggesting that tPA-S478A-GFP is present at higher concentrations near the plasma membrane. The secretory dynamics of tPA-S478A-GFP also differed from those of the wild-type, and it remained attached to the membrane surface for a longer period of time after granule opening. These results suggest that PAI-1 accelerates the dissociation of tPA from the VEC surface by forming a high-molecular weight complex (Fig. 2). A decrease in the amount of tPA retained at the VEC surface because of an excess of PAI-1 in the plasma appears to be one possible mechanism of hypo-fibrinolysis under pathological conditions. This notion is in agreement with a well-established concept that elevated plasma PAI-1 levels, mainly due to obesity and insulin resistance, are risk factors for cardiovascular events (15).

### 5. Generation of plasmin efficiently amplifies plasminogen accumulation on the tPA-GFP expressing cell surface

Single-chain tPA is enzymatically active (16), in contrast to most of the other serine proteases of which the single chain forms are inactive zymogens. VECs are the main sources of secretion of active tPA and importantly, fibrinolytic components assemble and express their functions on their cell surface (14). It is believed that the catalytic efficiency of tPA for activation of cell bound plasminogen is about tenfold higher than that in solution; this is possibly due to tri-molecular complex formation as well as conformational alteration of Glu-plasminogen to

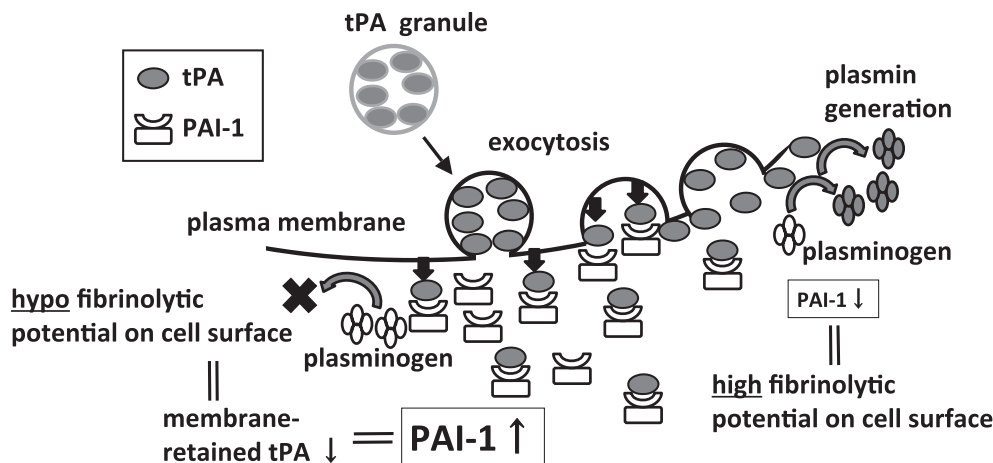


Fig. 2. Proposed function of retained tPA on the VEC surface and its modification by PAI-1.

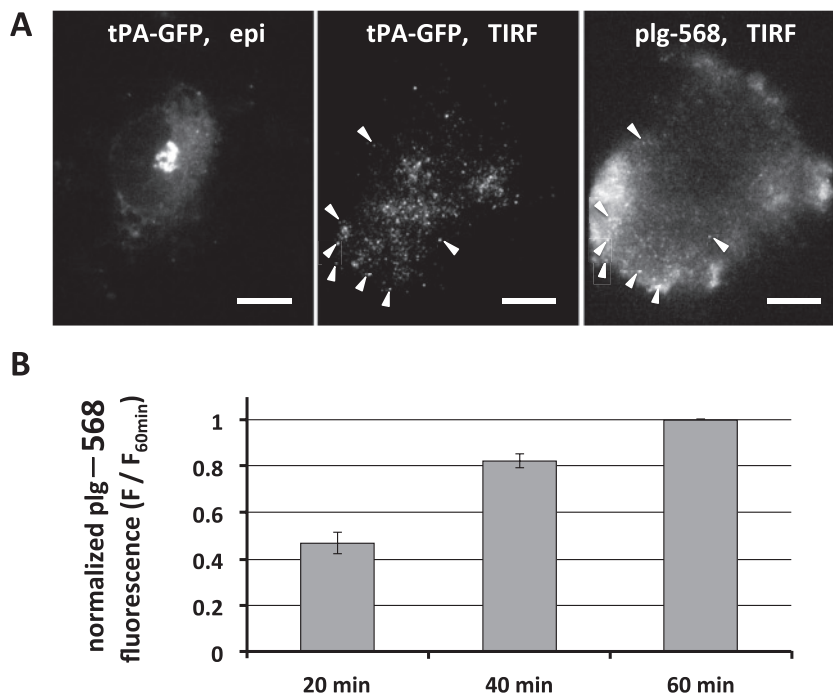


Fig. 3. Accumulation of plg-568 in tPA-GFP expressing VECs. A) Localization of tPA-GFP (middle panel) and plg-568 (right panel) in a single cell. Fluorescence was visualized by epifluorescence (epi) and TIRF microscopy. The arrowheads indicate colocalized tPA-GFP and plg-568 spots. The bars represent 10  $\mu$ m. B) Time-dependent increase in plg-568 fluorescence. The red fluorescence in tPA-GFP expressing single cell was normalized by the red fluorescence in 60 min after application of plg-568.

a more readily activatable structure (17). These facts prompted us to investigate the potential role of cell-surface retained active tPA in the expression of fibrinolytic activity.

To assess the functional potential for exocytosed tPA-GFP to express plasminogen activator activity, we analyzed the distribution and accumulation of both tPA-GFP and its substrate plasminogen on the cell surface. After application of Alexa Fluor 568-labeled plasminogen, which had been purified from fresh frozen human plasma by lysine Sepharose (plg-568), to tPA-GFP expressing VECs, plg-568 appeared to accumulate on the cell sur-

face at spots where tPA-GFP was retained, as well as in pericellular/matrix adhesive areas (Fig. 3). The amounts of plg-568 accumulated on the cell surface increased with time; this suggests that C-terminal lysine, newly exposed on the cell surface due probably to plasmin-dependent proteolysis, seems to function as a *de novo* generated cell-surface plasminogen binding site (Fig. 4).

To confirm the indispensability of secreted active tPA, we analyzed the effects of supplemented PAI-1, which suppresses tPA activity by accelerating dissociation of tPA from the cell surface on formation of a high molecu-

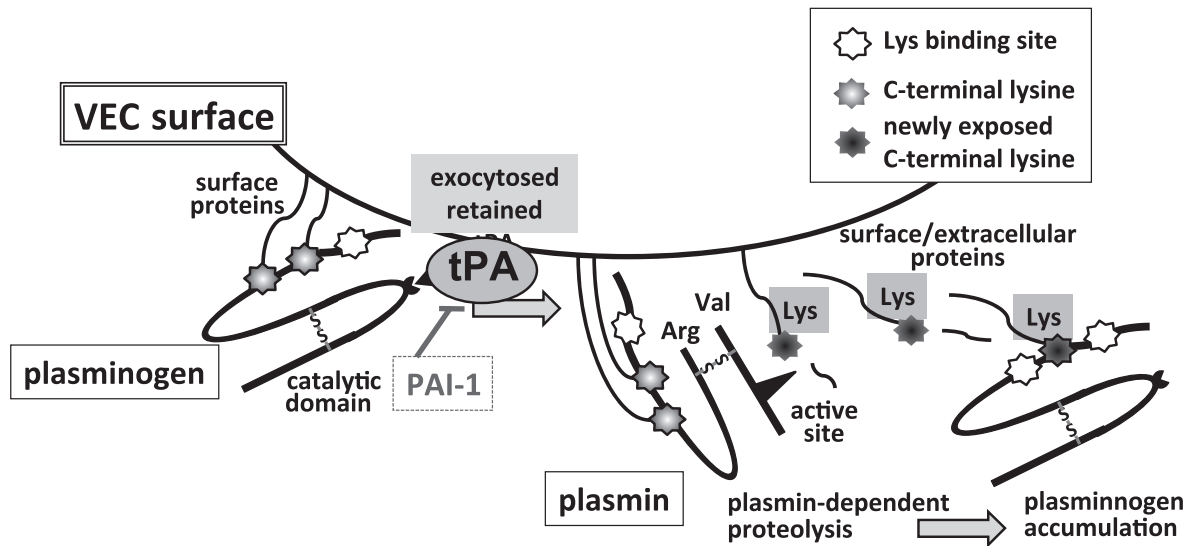


Fig. 4. Model of expression and amplification of plasminogen activation on the VEC surface.

lar weight complex. Supplementation with PAI-1 suppressed plg-568 accumulation, especially in pericellular/matrix adhesive areas. We also employed the catalytically inactivated mutant of tPA, tPA-S478A-GFP, to disable plasmin generation, and analyzed its effect on plasminogen binding. VECs expressing and secreting tPA-S478A-GFP appeared essentially not to accumulate plg-568 on their cell surfaces. Thus plasmin activity generated by exocytosed active tPA seems essential for additional exposure of carboxyl-terminal lysine residues to which plasminogen binds. This proposed positive feedback loop in plasmin generation on VECs seems similar to that on another solid structure, fibrin, where the effective expression of fibrinolytic activity is also important.

## 6. Prolonged retention of tPA on the cell surface is essential for expression and amplification of plasminogen activation

tPA binds fibrin through the second kringle domain and the finger domain in a both a Lys-dependent and -independent manner (18), which results in efficient activation of plasminogen and fibrin dissolution. We also clearly showed that the FEK1K2 domains are responsible for the retention of secreted tPA on the cell surface. To confirm the essential role for surface-retained tPA in initiating amplified plasminogen activation, we analyzed the effect of tPA-CD-GFP lacking FEK1K2 domains (which disappears quickly from the cell surface after exocytosis) on plg-568 accumulation. We found that only smaller amounts of plg-568 accumulated on the cell

surface/pericellular area compared to wild-type tPA-GFP; this indicates that the sustained retention of exocytosed tPA is essential for effective plasminogen binding to the cell surface as well as for its amplified activation on VECs. Exocytosis of active tPA and its sustained retention on the cell surface thus appeared to play a central role in maintaining high fibrinolytic potential on the cell surface.

## 7. Conclusion

VEC's appear to effectively express and propagate plasminogen activation activity on the cell surface and pericellular area through mechanisms partly similar to those at the fibrin surface. Such amplification and propagation of plasminogen activation seems indeed beneficial to the maintenance of high fibrinolytic potential on VECs. However this may be related to the observed deleterious effects of tPA that may destroy BBB function by over-expression of plasmin activity. Further analyses are required to understand the underlying mechanism, including identification of candidate plasminogen binding molecules on the surface of VECs.

## Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research to Y.S. (C: 22590826) and to T.U. (C: 21590230) from the Japan Society for the Promotion of Science (JSPS) and a grant from the Takeda Science Foundation to Y.S.



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