Regulation of plasminogen activation on cell surfaces and fibrin

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Summary. The fibrinolytic system dissolves fibrin and maintains vascular patency. Recent advances in imaging analyses allowed visualization of the spatiotemporal regulatory mechanism of fibrinolysis, as well as its regulation by other plasma hemostasis cofactors. Vascular endothelial cells (VECs) retain tissue-type plasminogen activator (tPA) after secretion and maintain high plasminogen (plg) activation potential on their surfaces. As in plasma, the serpin, plasminogen activator inhibitor type 1 (PAI-1), regulates fibrinolytic potential via inhibition of the VEC surface-bound plg activator, tPA. Once fibrin is formed, plg activation by tPA is initiated and effectively amplified on the surface of fibrin, and fibrin is rapidly degraded. The specific binding of plg and tPA to lytic edges of partly degraded fibrin via newly generated C-terminal lysine residues, which amplifies fibrin digestion, is a central aspect of this pathophysiological mechanism. Thrombomodulin (TM) plays a role in the attenuation of plg binding on fibrin and the associated fibrinolysis, which is reversed by a carboxypeptidase B inhibitor. This suggests that the plasma procarboxypeptidase B thrombin-activatable fibrinolysis inhibitor (TAFI), which is activated by thrombin bound to TM on VECs, is a critical aspect of the regulation of plg activation on VECs and subsequent fibrinolysis. Platelets also contain PAI-1, TAFI, TM, and the fibrin cross-linking enzyme, factor (F) XIIIa, and either secrete or expose these agents upon activation in order to regulate fibrinolysis. In this review, the native machinery of plg activation and fibrinolysis, as well as their spatiotemporal regulatory mechanisms, as revealed by imaging analyses, are discussed.

Keywords: fibrinolysis; plasminogen; plasminogen inactivators; thrombin-activatable fibrinolysis inhibitor; tissue-type plasminogen activator.

Introduction

The primary role of the fibrinolytic system is to dissolve fibrin-containing thrombi by the major extracellular protease plasmin (plm), in order to maintain vascular patency. The activation of the zymogen, plasminogen (plg), to plm is catalyzed by the physiological plg activators (PAs), viz., urokinase-type PA (uPA) and tissue-type PA (tPA) [1,2]. In the vasculature, tPA, the major PA, is secreted from vascular endothelial cells (VECs) as an active enzyme. Because PA inhibitor type 1 (PAI-1) exists in molar excess of tPA in plasma, the plg activation potential is mainly determined by the molar balance of tPA and PAI-1 [3,4]. Plg activation efficiently takes place when both tPA and plg are bound to insoluble materials (e.g., fibrin, denatured proteins, and specific receptors of these proteins expressed on cell surfaces) [2,5,6]. The binding of plg to its receptors is mediated by plg lysine binding sites (LBSs) contained in its kringle domains, primarily to C-terminal lysines, or secondarily to internal lysine isosteres, in the corresponding proteins [7,8]. These interactions generate a lysine binding-associated conformational change, which is a key event that triggers fibrinolysis [8]. Several distinct mechanisms exist to regulate fibrinolysis under physiological conditions, for example, removal of C-terminal lysine residues by carboxypeptidase B, which are essential to protect hemostatic thrombi from premature lysis and to prevent bleeding [9]. The concept of these sophisticated spatiotemporal regulatory mechanisms in fibrinolysis was established based upon earlier indirect studies, mainly on structure-function investigations of the involved proteins [7,10–13]. However, recent advances in imaging analyses have made it possible to directly examine the dynamics of single molecules and intermolecular interactions (Table 1). The secretory dynamics of tPA from VECs [14,15] and the substantial
Plg activation potential in plasma and on the surface of VECs

tPA is the main PA of the vasculature and expresses its specific activity when solid materials with C-terminal residues, mainly fibrin, are present. The potential for tPA to express such activity is finely regulated by PAI-1, both in plasma and on the surface of VECs.

PAs

tPA is synthesized and secreted from VEC via constitutive or regulated mechanisms [21] as a single-chain form [22] having endogenous activity due to a non-optimally oriented regulatory triad of Asp-His-Ser in the zymogen [23]. After either a conversion to its two-chain form catalyzed by plm [23] or a binding to fibrinogen [24], tPA acquires an approximately 10-fold higher activity, and plays a pivotal role in intravascular fibrinolysis [25]. uPA is expressed in an inducible manner in a variety of cells, including inflammatory cells and cancer cells [26]. It is synthesized as a single-chain zymogen, which matures to a two-chain protease upon proteolytic activation, and functions mainly in extravascular spaces (e.g., tissue remodeling, angiogenesis and tumor invasion), together with its specific receptor, uPAR [1,27,28]. Although the function of uPA in intravascular fibrinolysis remains controversial [29], the indispensable roles of both tPA and uPA in thrombolysis and other physiological phenomena, including growth, fertility and survival, were clearly shown using double inactivations of these two genes in mice [30].

Our analyses of the secretory dynamics of green fluorescent protein (GFP)-tagged tPA (tPA-GFP) from VECs using total internal reflection fluorescence (TIRF) microscopy revealed that tPA-GFP has unique secretory dynamics and is retained on the surface of VECs after its release from the granules [14]. This binding to VECs is dependent on the finger-domain and the kringle 2-domain of tPA, similar to its binding to fibrin [31], and also on a positively charged region consisting of K296, H297, R298, and R299 [32]. The secretion and the retention of tPA contribute to the effective activation of plg bound on the surface of VECs, and to effective fibrinolysis when fibrin is generated [15].

PA inhibitors

PAI-1 and PA inhibitor type 2 (PAI-2), regulators of PA activity, play important roles in a variety of pathophysiological events [20,33,34]. PAI-1 was first identified in the culture medium of VECs [35] as a member of the serine protease inhibitor (SERPIN) family [36]. The indispensable role of PAI-1 in hemostasis was demonstrated by three genetically confirmed PAI-1-deficient patients in unrelated families [37–39], each of which showed life-threatening bleeding after trauma and surgery, or during menstruation and pregnancy [38,39]. These symptoms are not observed in PAI-1 gene inactivated mice [40], which indicates the limitation of animal models of these maladies [41]. PAI-2 was first identified in placenta as a member of the SERPIN family [42] and was shown to inhibit both uPA and tPA [43]. Its plasma levels are barely detectable, however, except during late pregnancy [44], its efficacy in inhibiting tPA is weaker than that of PAI-1 [45], and its role in hemostasis is questionable [43]. Other biological roles in inflammation, immunity and tumor growth have been suggested [43,44].

Plg activation potential

Because PAI-1 has high affinity for tPA [33], and tPA possesses endogenous activity even in its single-chain form [23], the amounts of free tPA, which correlate well with plasma tPA activity, are determined by the balance of plasma concentrations of tPA and PAI-1 [4,46]. These values are also inversely correlated with the euglobulin clot lysis time, which measures the tPA-generated contribution of the surfaces of VECs [15], platelets [16,17], and fibrin [18,19] in amplifying plg activation and in triggering subsequent fibrinolysis have been also confirmed. These cells are also actively involved in the negative regulation of fibrinolysis, as mediated by other plasma and cellular components (e.g., thrombin-activatable fibrinolysis inhibitor [TAFI] [9,16] and PAI-1 and FXIII-dependent inhibition of fibrinolysis [17]).

On fibrin surfaces

- Accumulation of plg at the lytic edge of the fibrin net [15,16,18,19].
- Accumulation of plg in microthrombi in mouse models [68]
Factors that modify both fibrin structure and the efficacy of fibrinolysis
- Fibrin thickness [19]
- Red blood cells (RBCs) [95]
- Clot contraction and RBCs [94]
- DNA, neutrophil extracellular traps (NETs) and histones [103,104].

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proteolytic activity of plasma in the absence of plm inhibitors. tPA fully expresses its activity for plg activation only in the presence of fibrin, suggesting that the amount of free tPA determined by the balance of tPA and PAI-1 is an indicator of the plg activation potential in plasma.

PAI-1 also regulates tPA activity on the surface of VECs. The retention time of exocytosed tPA-GFP on VEC surfaces is widely variable and is significantly prolonged when PAI-1 synthesis is suppressed by siRNA. Supplementation of recombinant PAI-1 reversed this prolongation and increased the amounts of tPA/PAI-1 complexes in the supernatant. These results suggest that PAI-1 facilitates the dissociation of the membrane-bound tPA on the surface of VECs and regulates plg activation potential (upper left panel). Reproduced from [108]. [Color figure can be viewed at wileyonlinelibrary.com]

Fig. 1. Native machinery of plasminogen (plg) activation and fibrinolysis and its modification by vascular endothelial cells (VECs) and platelets. (1) The upper right panel shows native machinery of plg activation and fibrinolysis. VECs secrete and retain tissue-type plasminogen activator (tPA) and maintain adequate fibrinolytic potential on their surfaces. When fibrin is generated, tPA and plg accumulate on fibrin surfaces through C-terminal lysine residues bound to the lysine binding sites (LBSs) of plg, which trigger plg activation and fibrinolysis. (2) The lower right panel illustrates the inhibitory function of VECs on fibrinolysis. When thrombin is generated adjacent to thrombomodulin (TM)-expressing VECs, TM-bound thrombin effectively generates the carboxypeptidase B, thrombin-activatable fibrinolysis inhibitor (TAFIa), which specifically cleaves C-terminal lysine residues and inhibits fibrinolysis. (3) The lower left panel shows the inhibitory function of platelets on fibrinolysis. Activated platelets secrete plasminogen activator inhibitor type 1 (PAI-1) and the FXIIIa catalytic A subunit, the latter of which cross-links α2AP to fibrin to stabilize thrombus. The mechanisms shown in (2) and (3) are coordinated to protect hemostatic thrombi from immature lysis. (4) In plasma, free tPA and free plasmin are effectively inhibited by PAI-1 and α2AP, respectively. PAI-1 also inhibits tPA activity on VECs by facilitating the dissociation of the membrane-bound tPA on the surface of VECs and regulates plg activation potential (upper left panel). Reproduced from [108]. [Color figure can be viewed at wileyonlinelibrary.com]

Native machinery of plg activation

All PAs catalyze the limited cleavage of a single peptide bond at Arg561-Val562 of Glu1-plg to generate Glu1-plm, which has an N-terminal 561-amino acid-containing heavy chain and a C-terminal 230-amino acid light chain linked by two disulfide bonds. High plasma concentrations of the tPA/PAI-1 complex reflect the decrease in the retained tPA on the surface of VECs as a result of release of this complex into the circulation. These observations probably explain the findings that elevated levels of tPA antigen in plasma, mostly existing as tPA/PAI-1 complexes, were associated with increased risk of thrombosis [49,50]. PAI-1 also exists in platelet α-granules and is secreted upon platelet activation [51–53], especially under shear [54,55]. Secreted PAI-1 not only inhibits the lysis of platelet-rich thrombi [56,57], but also contributes to the growth of thrombi [55] by inhibiting ongoing thrombolysis. In addition to plasma and VEC surfaces, the surface of activated platelets was also suggested to be involved in similar regulation of plg activation under shear [17,53]. These results may account for the positive relationship of elevated PAI-1 and the risk of arterial thrombosis and other coronary artery diseases [58,59], although there still remains controversy concerning these latter points [60].
less efficacy of activation in the plasma milieu where \( \text{Cl}^- \) is present [61], whereas it adopts the R-conformation after the binding to either C-terminal lysine residues of fibrin or its specific receptors on cell surface, through the LBSs of its kringle domains [8]. The mutual transitions in these two conformations under different environments regulate the activation efficacy of Glu1-plg [8].

**Plg**

Glu1-plg consists sequentially of a N-terminal Pan-apple domain followed consecutively by, five kringle domains, which are triple-disulfide linked peptides of approximately 80 amino acids, and a carboxy-terminal serine protease domain [8]. The kringle domains contain the LBS, which specifically binds \( \omega \)-amino acids (e.g., lysine and its analogue, \( \varepsilon \)-amino caproic acid [EACA]), and thus C-terminal lysines of proteins and peptides (e.g., enolase [62], annexin [63,64] and glycer-aldehyde-3-phosphate dehydrogenase) [65].

The X-ray crystal structure of Glu1-plg revealed a closed, activation-resistant conformation (T-conformation), in which Lys50, Arg68 and Arg70 from the Pan-apple domain (viz., the 77-residue activation peptide) [8] bind to the LBS of kringle 5 [66] (Fig. 2). This interaction forces the linker region between kringle 3 and kringle 4 to shield the activation sequence, and thus protects this proenzyme from unnatural activation. The existence of \( \text{Cl}^- \) at this interface, and other locations, contributes to retention of the T-conformation [66]. When this intramolecular binding is dissociated after occupancy of the LBS of kringle 5 by \( \omega \)-amino acids, Glu1-plg adopts a more relaxed conformation (R-conformation) [8] in which the kringle 3-kringle 4 linker is moved away from the activation sequence [66].

**Plg activation**

Plg activation by tPA is ineffective in the fluid phase. For more efficient activation, fibrin, cell surfaces, or misfolded proteins containing a cross-beta structure [6] are required. These solid phases function as templates for tPA and plg binding. In addition, the binding of plg to C-terminal lysines of cell surface receptors, or partially digested fibrin, evokes the T to R conformational change of plg and triggers its activation. The surfaces of VECs show similar properties, wherein the secreted tPA is retained and several plg receptors containing C-terminal lysine residues, including annexin II [63,64] and \( \varepsilon \)-enolase, are expressed [62]. Once plm is formed, this protease cleaves cell surface

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*Fig. 2. Conformation of Glu1-plg from X-ray crystallography and its sequential changes after binding to fibrin through the lysine binding sites (LBSs). Glu1-plg consists of the Pan-apple domain (PA), five kringle (K) domains and a serine protease domain. Glu1-plg adopts the tight (T) conformation, in which Lys50 from the Pan-apple domain binds to the LBS of K5 (A). In this case, the activation sequence is shielded by the linker region between K3 and K4 (A). After binding to partially digested fibrin through the LBS of K1 (B), followed by that of K5 (C), Glu1-plg adopts a more relaxed (R) conformation, in which the activation sequence is exposed to be easily cleaved by PAs to generate plasmin (D). Modified from [66] with permission. [Color figure can be viewed at wileyonlinelibrary.com]*
proteins at peptide bonds C-terminal of either lysine or arginine, which amplifies plg accumulation at the newly generated C-terminal lysine residues on the surface of VECs [15] (Fig. 3A). The LBS-dependent plg binding to partially digested fibrin was first demonstrated as a continuous binding of fluorescein-labeled plg to the surface of dissolving plasma clots stimulated by either tPA or uPA in an in vitro model that mimics thrombolytic therapy [18]. The importance of this mechanism was also demonstrated in more physiological environments. When fibrin fibers are generated on the surface of VECs, their rapid lysis is observed, which originates from the surface of tPA-GFP-expressing cells. In the lytic process, plg is observed at the lytic edge of partially digested fibrin nets [15,16,19] (Fig. 3B,C). As speculated, fibrin fiber generation itself triggered fibrinolysis by supplying a solid binding surface for both tPA and plg, which was further amplified by plm-dependent generation of C-terminal lysine residues [15,67]. Although a difference in affinity between fibrin and the VEC surfaces is likely to be a driving force for tPA to relocate, the mechanism whereby tPA is transferred from VEC surfaces to fibrin remains to be elucidated.

Triggering fibrinolysis at the early phase of thrombus formation and plm-activity-dependent accumulation of plg, are also demonstrated in an in vitro mouse model. Plg accumulated at the early phase of thrombus formation at the core of thrombi where platelet-exposed phosphatidyl serine (PS) and fibrin were identified [68]. When tPA was administered to this model, plg accumulated further in the thrombi, and eventually the thrombi dissolved [68]. Initiation of fibrinolysis after accumulation of plg was also demonstrated on the surfaces of activated platelets in an in vitro system under either shear [53] or a static condition [16]. Plg was bound to the surface of platelets through glycoprotein IIb/IIIa, especially when platelets were activated to express PS on their surfaces [53,68–71]. As a result, PS exposed on platelet surfaces contributes to thrombin generation and subsequent fibrin formation, and also triggers plg activation by assembling plg and tPA on their surfaces [16]. Because these processes require fibrin bound on the surface of activated platelets through glycoprotein IIb/IIIa [70,71], fibrin generation is essential to triggering and amplifying fibrinolysis. Activated platelet surfaces appear to be one of the more important regions for the native machinery of plg activation to take place and subsequently to evoke effective fibrinolysis.

Regulation of plg activation by TAFI

Because C-terminal lysine residues play essential roles in the native machinery of plg activation and fibrinolysis, an inhibitory mechanism of fibrinolysis exists via the removal of C-terminal lysines [72,73,74] (Fig. 1, right lower panel). TAFI was isolated by two distinct groups and initially named as carboxypeptidase unstable (CPU) [75] and carboxypeptidase arginine (CPA) [76], respectively. This carboxypeptidase was also identified as a plg binding protein and named procarboxypeptidase B [77]. Accordingly, TAFI is now recognized as a proenzyme of carboxypeptidase B encoded by the CPB2 gene [78]. TAFI is produced mainly in the liver and circulates in plasma, but this protein is also present in platelets and is released upon platelet activation [79]. After limited cleavage at the Arg92-Ala93 peptide bond of TAFI, catalyzed by thrombomodulin (TM)-bound thrombin [13,80], and less effectively by plm and thrombin alone [72,81], TAFI is activated to TAFIa, which cleaves C-terminal lysine or arginine residues of a variety of functional peptides. These include C3a, C5a, bradykinin and partially digested fibrin [74,82]. TAFIa loses its specific activity with a very short half-life (<10 min at 37 °C) [74] by either conformational changes [83] or proteolytic cleavages [84].

TAFI-deficient mice do not show an apparent spontaneous phenotype, and the results obtained employing these mice confound the physiological function of TAFI [85,86]. However, studies with a variant TM in humans were suggestive of the physiological relevance of TAFI in normal hemostasis [87]. In this variant, p.Cys537Stop [88], the soluble TM levels in plasma were elevated and the plasma clot lysis time was delayed. Because this delay in plasma clot lysis was reversed by inhibiting TM/thrombin-dependent activation of TAFI [87], the increased soluble TM appeared to have successfully functioned as a cofactor for thrombin to activate TAFI in plasma and to prolong plasma clot lysis. Thus, when TAFI is present at a site where both TM and thrombin exist, TAFI is effectively generated and suppresses fibrinolysis.

Our in vitro experiments also suggest a relevant role for TAFI in hemostasis. When platelet-rich plasma was treated with tissue factor, together with Ca\(^{2+}\), fibrin mesh formation was successfully observed that originated from the surface of the activated and PS-exposed platelets and spread to the periphery. When tPA was added, fibrinolysis was clearly observed originating from the coagulation initiation site (i.e., the surface of activated platelets), which then spread to the periphery (Fig. 4A). Fibrin lysis was always preceded by the binding of fluorescent-labeled tPA, as well as plg, to the lytic edge. When soluble TM was supplemented to mimic the function of TM expressed by VECs, the accumulation of plg was abolished and clot lysis was highly prolonged (Fig. 4B), all of which was strongly inhibited by a carboxypeptidase B inhibitor [16]. However, the effect of TM on the accumulation of tPA was limited, suggesting that binding sites on fibrin, other than C-terminal lysines, are also responsible for the tPA accumulation [29,31,89].

Available evidence clearly indicates that the existence of TM in proximity to thrombin is required for TAFI to be activated and to successfully suppress fibrinolysis. Because platelets express TAFI [79], as well as TM [90], and provide PS as a coagulation initiation site after activation, this environment is a site where generated thrombin can co-exist...
with TM. Although much attention has been paid to TM from the viewpoints of protein C activation and anticoagulation, the physiological role of TM in platelets needs to be considered from the viewpoint of fibrinolysis.

Fibrinolysis and its regulation

Plm cleaves and digests both fibrinogen and fibrin into similar fragments, except for D-dimer, which is generated only from fibrin cross-linked by the transglutaminase, FXIIIa [91]. Because fibrin, but not fibrinogen, accelerates tPA-catalyzed plg activation as a template, the generated plm effectively digests the fibrin bound to plm to fibrin degradation products. Thus fibrin, including soluble fibrin, but not fibrinogen, is considered the physiological target of plm in vivo.

Fibrin structure

The structure of fibrin itself modulates the efficacy of fibrinolysis by changing both the rate of plg activation and clot permeability, both of which are modified by clot retraction [92]. Clot retraction, which itself is facilitated by fibrinolysis [93], renders the clot resistant to lysis by modifying the overall structure of the blood clot and the shape and function of the involved platelets and red blood cells (RBCs) [94]. In addition, the binding of RBCs to fibrin through a specific receptor also modifies fibrin structure to become more resistant to fibrinolysis [95]. Most of these studies suggest that a tight structure of the fibrin clot makes it resistant to lysis. Possible differences in the contents of the cross-beta structures to which tPA adheres with high affinity [6], or in the thickness of the fiber [19], could also modulate fibrin susceptibility to its lysis [73].

Anti-plasmin and FXIII

α2-antiplasmin (α2AP), a member of the SERPIN family, specifically inhibits free circulating plm by forming a stoichiometric complex [2,96]. A long C-terminal sequence, with a C-terminal lysine residue in α2AP, and LBSs located in the kringle domains on plm, function as secondary binding sites to facilitate plm inactivation (Fig. 1, upper left panel). When plm is generated from plg bound to fibrin through their LBSs, its inhibition by α2AP is no longer effective and plm remains active on the fibrin surface. Thus, plm generated on the surface of fibrin is protected from rapid inactivation by α2AP and contributes to effective fibrinolysis [2].

Activated coagulation FXIII (FXIIIa), either plasma derived or exposed on platelet surfaces [97], catalyzes cross-linkage of γ-chains of fibrin monomers [91]. This enzyme also catalyzes the cross-linking of α2AP to fibrin, where α2AP remains active [98,99]. Once α2AP is cross-linked, thrombi are protected from premature dissolution and survive as hemostatic thrombi for longer...
periods of time [20,100,101] (Fig. 1, lower left panel). The physiological importance of the cross-linking of α2AP to fibrin has been suggested as a unique ‘secondary’ hemorrhage stage after trauma or surgery that occurs with a deficiency of either α2AP or FXIII [96]. FXIIIa also catalyzes cross-linkage of TAFI to fibrin [102], although its physiological relevance has yet to be clarified [101].

**Fig. 4.** Assembly of tissue-type plasminogen activator (tPA) and plasminogen (plg) on the fibrin surface triggers fibrinolysis by forming plasmin. (A) When platelet-rich plasma was treated with tissue factor/Ca²⁺, fibrin (Alexa Fluor® 647-labeled fibrinogen; fbg-647) mesh formation was successfully observed, which originated from the surface of the activated and PS-exposed platelets and spread to the periphery. When tPA (Alexa Fluor® 488-labeled tPA; tPA-488) was added, fibrinolysis was clearly observed originating from the coagulation initiation site (i.e., the surface of the activated platelets), which also spread to the periphery. Fibrin lysis was always preceded by the binding of Alexa Fluor® 568-labeled plg (Glu-plg-568) to the lytic edge. Scale bar: 50 μm. (B) Upon addition of soluble thrombomodulin (TM) (5 nM), the accumulation of Glu-plg-568 was essentially abolished and the lysis time was significantly prolonged. This was reversed by including a carboxypeptidase inhibitor. Scale bar: 100 μm. Reproduced from [16] with permission. [Color figure can be viewed at wileyonlinelibrary.com]

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**Other fibrous components, apart from fibrin, which modify fibrinolysis**

Fibrous components of DNA, histone, neutrophil extracellular traps (NETs) [103,104], as well as polyphosphate [105], have been shown to attenuate effective tPA-dependent fibrinolysis. Although these fibrous disorganized components may enhance tPA-catalyzed plg
activation by providing a solid phase, as was shown in NETs [103], real-time imaging clearly demonstrated that DNA and histone competed with the effective binding of tPA and plg to the coexisting fibrin fibers and inhibited fibrinolysis [103]. Therefore, these inflammation-related fibrous components are proposed to inhibit the native machinery of plg activation and fibrinolysis by disrupting the assembly of tPA and plg on fibrin surfaces.

Summary

Recent advances in imaging technologies reconfirmed the physiological importance of protease-dependent exposure of C-terminal lysine residues on proteins for triggering and amplifying the native machinery of plg activation and fibrinolysis. The results obtained from this novel technology also deepened the understanding of the spatiotemporal regulation of fibrinolysis, which included the participation of VECs, platelets and Newtonian vs. non-Newtonian blood flow. The effects of newly developed drugs targeted to these regulatory factors, including PAI-1, TAFI [106], and α2AP [107], could also be adequately evaluated using these imaging techniques. The acquired knowledge is expected to provide approaches to develop novel safe and effective strategies to manage thrombotic patients.

Addendum

T. Urano, F. J. Castellino and Y. Suzuki designed the structure of the manuscript and wrote it. T. Urano and Y. Suzuki prepared the figures.

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Disclosure of Conflict of Interests

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