Vascular Medicine

Plasmin Cleavage of von Willebrand Factor as an Emergency Bypass for ADAMTS13 Deficiency in Thrombotic Microangiopathy

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Background—Von Willebrand factor (VWF) multimer size is controlled through continuous proteolysis by ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13). This prevents spontaneous platelet agglutination and microvascular obstructions. ADAMTS13 deficiency is associated with thrombotic thrombocytopenic purpura, in which life-threatening episodes of microangiopathy damage kidneys, heart, and brain. Enigmatically, a complete ADAMTS13 deficiency does not lead to continuous microangiopathy. We hypothesized that plasmin, the key enzyme of the fibrinolytic system, serves as a physiological backup enzyme for ADAMTS13 in the degradation of pathological platelet–VWF complexes.

Methods and Results—Using real-time microscopy, we determined that plasmin rapidly degrades platelet–VWF complexes on endothelial cells in absence of ADAMTS13, after activation by urokinase-type plasminogen activator or the thrombolytic agent streptokinase. Similarly, plasmin degrades platelet–VWF complexes in platelet agglutination studies. Plasminogen directly binds to VWF and its A1 domain in a lysine-dependent manner, as determined by enzyme-linked immunosorbent assay. Plasma levels of plasmin–α2-antiplasmin complexes increase with the extent of thrombocytopenia in patients with acute episodes of thrombotic thrombocytopenic purpura, independent of ADAMTS13 activity. This indicates that plasminogen activation takes place during microangiopathy. Finally, we show that the thrombolytic agent streptokinase has therapeutic value for Adamts13−/− mice in a model of thrombotic thrombocytopenic purpura.

Conclusions—We propose that plasminogen activation on endothelial cells acts as a natural backup for ADAMTS13 to degrade obstructive platelet–VWF complexes. Our findings indicate that thrombolytic agents may have therapeutic value in the treatment of microangiopathies and may be useful to bypass inhibitory antibodies against ADAMTS13 that cause thrombotic thrombocytopenic purpura. (Circulation. 2014;129:1320-1331.)

Key Words: fibrinolysis ▪ plasminogen ▪ platelets ▪ streptokinase ▪ urokinase ▪ von Willebrand factor

von Willebrand factor (VWF) circulates in plasma as multimers after release from endothelial cells or activated platelets. VWF circulates in a globular form, but it has an unfolded conformation during its release.1 In this state, VWF can directly bind platelets via its A1 domain2 and passively bridge multiple platelets together without inducing their activation (agglutination). The metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13) cleaves VWF in its A2 domain,3 preferably when VWF is unfolded.4 This proteolysis is essential to prevent the formation of ultralarge VWF multimers and pathological platelet–VWF complexes that obstruct the microvasculature, as occurs in patients with thrombotic thrombocytopenic purpura (TTP).5 TTP patients characteristically experience recurrent episodes of microangiopathy, intermitted by prolonged remission periods. This is thought to be a result of fluctuating ADAMTS13 activity levels, triggered by inhibitory antibodies. Remarkably, patients and mice that are completely and persistently deficient in ADAMTS13 activity can achieve remission and do not present with continuous symptoms of microangiopathy.5 This suggests that additional factors beyond ADAMTS13 activity modulate the presentation of TTP attacks.

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We here propose a hypothesis that may help to explain the unpredictable nature of TTP pathology: A second proteolytic mechanism exists that is capable of degrading dangerously large VWF-platelet complexes in the absence of ADAMTS13. One candidate enzyme that can substitute for ADAMTS13 and degrade platelet–VWF complexes is plasmin. Active plasmin is able to cleave VWF and alters its platelet-binding capacity under purified conditions.7,8 Plasminogen can be activated by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Whereas tPA has a high affinity for tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Whereas tPA has a high affinity for fibrin,9 uPA has little affinity for fibrin before activation.10 As a result, tPA is considered the most important plasminogen activator in fibrinolysis.11 In contrast, uPA becomes an efficient plasminogen activator on its receptor uPAR, which is among others presented by activated endothelial cells during hypoxia.12,13

In the present study, we investigated whether plasmin could substitute for ADAMTS13 to degrade platelet–VWF complexes. We found evidence that plasmin can destruct platelet agglutinates and is triggered during microangiopathy. Furthermore, we found that induction of plasminogen activation by thrombolytic agents can be used to controllably degrade platelet agglutinates in vitro and in vivo, which may offer therapeutic opportunities for the complicated clinical management of TTP.

Methods

Methods of blood collection, isolation and culture of human umbilical vein endothelial cells (HUVECs), immunofluorescence microscopy, determination of VWF release by ELISA, biochemical interaction studies, platelet-aggregation and platelet-agglutination studies, Western blotting, and substrate conversion assays are provided in the online-only Data Supplement.

Real-Time Microscopy of Platelet–VWF Complex Formation on HUVECs

Confluent HUVECs were cultured on glass coverslips and preactivated with 100 mmol/L phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Zwijndrecht, Netherlands) for 60 minutes. Coverslips were placed into a laminar-flow perfusion chamber under an inverted microscope (Zeiss observer Z.1, Carl Zeiss, Sliedrecht, Netherlands). Lyophilized platelets (BioData Corporation, Horsham, PA) were resuspended in serum-free M199 culture medium (Invitrogen/Life Technologies, Carlsbad, CA) and perfused at a shear rate of 300 s⁻¹, triggering platelet–VWF string formation. String stability was ensured during 5 minutes of perfusion. Next, serum-free M199 medium containing 100 mmol/L PMA, with 216 μg/mL plasminogen, 10 ng/mL uPA, 10 U/mL streptokinase, 2 μg/mL anti-uPAR (R&D Systems, Abingdon, United Kingdom; clone 62022), or 200 mmol/L ε-aminocaproic acid (εACA) or in combinations was perfused over platelet–VWF strings. Similarly, perfusions were performed in the presence of 5 μg/mL ADAMTS13 (R&D Systems), 20 mmol/L elastase (from human leukocytes, Sigma-Aldrich), and 300 mmol/L granzyme B and M (generously provided by Dr Niels Bovenschen).9,14

Patient Studies

Plasma samples from 19 healthy individuals, 26 samples from patients with acute TTP, and 32 samples from TTP patients in remission were collected in trisodium citrate (3.2% wt/vol). Baseline characteristics of these 3 groups are provided in Table I in the online-only Data Supplement. All samples were studied in all assays described below. Plasma samples of TTP patients in remission without clinical signs of microangiopathy were obtained between 1 month and 6 years after they experienced their last episode. Samples from patients with acute TTP were obtained before plasma transfusion and on following days during the acute disease state. All donors and patients gave written informed consent. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht. Platelet-poor plasma was stored at −80°C. Control samples were obtained from healthy volunteers and were matched by age and sex to the TTP patients. Plasminogen-antiplasmin (PAP) complexes were determined by ELISA according to the manufacturer’s protocol (Technoclone, Vienna, Austria). ADAMTS13 activity was determined by use of a fluorescence resonance energy transfer (FRETs) assay for ADAMTS13 activity (Peptides International, Lexington, KY). ADAMTS13 activity of normal pooled plasma was set at 100%, and the values obtained in individual plasma samples were expressed as percentages. Similarly, α₂-antiplasmin activity was determined by incubating 10 μL of purified plasmin (13 nanokatal per milliliter; Roche, Woerden, Netherlands) for 30 seconds with 10 μL of 5x prediluted citrated patient plasma in HBS. After incubation for 30 seconds, 40 μL of plasmin substrate was added (6 mmol/L; MM-ε-Arg-pNA, Roche), and conversion was measured at 405 nm for 300 seconds. Slopes were determined and related to the capacity of normal pooled plasma (defined as 100%) to inhibit plasmin activity.

In Vivo Studies

Animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of KU Leuven (Belgium). Male Adamts13−/− mice (CASA/Rk-C57BL/J-O-129X1/SvJ background) with a body weight between 16 and 20 g were anesthetized with isoflurane and intravenously injected with 2000 U/kg recombinant human VWF (rhVWF; Baxter, Vienna, Austria) and 20 mg/kg human plasminogen via the right retro-orbital plexus to induce symptoms of TTP15 (n=9). After 5 or 15 minutes, 10 U/mL streptokinase (which reacts specifically with human plasminogen)16 was administered intravenously via the left retro-orbital plexus (5 minutes, n=5; 15 minutes, n=10). As controls, completely untreated mice (n=8) and mice that were treated with thrombolytic treatment without being challenged with rhVWF (n=5) were used. After 24 hours, venous blood was drawn into trisodium citrate (resulting in a final concentration of 0.61% wt/vol), and the mice were exanguinated under deep anesthesia with isoflurane. Cell counts were determined within 30 minutes with a Hemavet hematolysis system (Drew Scientific, Dallas, TX). Heart, kidneys, and liver were collected from all mice, rinsed in PBS, and fixed in 4% paraformaldehyde overnight, then embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin-eosin for morphological analysis. Pictures were taken with an Olympus DP71 camera with a 40x objective and Cell^P imaging software (Olympus, Zoeterwoude, Netherlands).

Statistical Analysis

Statistical comparisons between 2 groups of samples were performed by Mann-Whitney U testing. Comparisons between multiple groups were performed by Kruskal-Wallis testing. P<0.05 was considered significant. All data analyses were performed with computer software (IBM SPSS Statistics 20.0, SPSS Inc, Chicago, IL).

Results

Plasmin Degradates Platelet–VWF Complexes on Endothelial Cells

To investigate whether plasmin formation on endothelial cells could substitute for ADAMTS13, we cultured endothelial cells and stimulated them to release VWF under flow while perfusing fixed platelets over them. Stable platelet–VWF complexes formed under flow in the absence of ADAMTS13. Subsequently, plasminogen (plasma concentration) with or without 10 ng/mL uPA was added. We found that uPA and plasminogen together immediately degraded platelet-covered strings of VWF (Figure 1A; Movie I in the online-only Data Supplement).
A monoclonal antibody that blocks the interaction of uPA with its receptor, uPAR, protected these platelet–VWF complexes from degradation (Figure 1B; Movie II in the online-only Data Supplement). The stability of these strings remained unaffected in the presence of uPA or plasminogen alone (Figure 1 and Movies III and IV in the online-only Data Supplement). String survival times (n=5 per condition) are shown in Figure 1C. These experiments indicated that...
activated endothelial cells supported sufficient uPA-dependent plasminogen activation to degrade platelet–VWF complexes in a matter of seconds. In control experiments, we confirmed that these endothelial cells externalized uPAR by immuno-fluorescence microscopy (Figure IIA in the online-only Data Supplement) and supported uPA-dependent plasmin activity after stimulation (Figure IIB in the online-only Data Supplement). It was reported previously that platelet–VWF complexes are efficiently degraded by ADAMTS13.4 We next investigated whether the binding of platelets was required for the cleavage of VWF by plasmin. In line with our previous findings, activated HUVECs retained part of their released VWF on their surface after activation (ie, not all was released into the supernatant as soluble VWF). Next, we exposed these cells (after replacing the supernatant medium) to plasminogen only or to uPA and plasminogen together. In the presence of uPA and plasminogen together, the vast majority of VWF was removed from the cell surface within 1 hour (Figure 1E) and was then present in the supernatant (Figure 1D). In comparison, we found that simultaneous incubation of activated HUVECs with tPA and plasminogen did not induce the release of VWF (Figure 1D). Moreover, platelet–VWF complexes on endothelial cells were not degraded by plasminogen in the presence of tPA under flow (not shown). These results together indicate that uPA-dependent plasmin formation can independently degrade VWF on endothelial cells in the presence and absence of platelets, although with differing efficacy.

Plasminogen Binds to and Cleaves VWF in a Lysine-Dependent Manner

ADAMTS13 requires direct binding to VWF for cleavage.17 In similar fashion, we found that plasminogen can bind directly to immobilized VWF (Figure 2A). This binding is completely inhibited by addition of the soluble lysine analog εACA (200 mmol/L),18 which indicates that this interaction is similar to the binding of plasminogen to fibrin. In correspondence, the cleavage and release of VWF by uPA-activated plasmin on endothelial cells was dependent on lysines and abrogated by the addition of εACA (Figure 2B). Similarly, εACA protected platelet-covered VWF strings against degradation in the presence of uPA and plasminogen (Figure 2C and 2D; Movie V in the online-only Data Supplement; quantification in Figure 2E). Together, these findings suggest that lysine-dependent interactions between VWF and plasmin are required for the cleavage of VWF.

ADAMTS13 recognizes VWF in its globular conformation but requires unfolding to cleave the A2 domain.17 Because the immobilization of VWF on microtiter plates expectedly led to its unfolding in our binding experiments, we investigated whether the binding of plasminogen to VWF was dependent on its conformation. In binding competition experiments, we found that the binding of plasminogen to immobilized VWF remained unaffected in the presence of a wide concentration range of soluble globular VWF (Figure IIIA in the online-only Data Supplement), whereas binding was competitively inhibited by εACA in a dose-dependent manner (Figure IIIB in the online-only Data Supplement). This suggests that binding of plasminogen to VWF is mediated by a binding site that is cryptic in globular VWF. We next aimed to identify a plasminogen-binding site in VWF. VWF contains numerous lysine residues throughout the molecule (http://www.uniprot.org/uniprot/P04275). The most lysine-rich area in the mature molecule resides in the VWF A1 domain: KKKK1383–1386. We therefore investigated whether this domain has the capacity to bind plasminogen independently. First, we confirmed that plasminogen directly binds to the immobilized recombinant VWF A1 domain in a lysine-dependent manner (Figure 2F). Moreover, the binding of plasminogen to immobilized VWF was competitively inhibited by A1 domain in solution (Figure 2G). These experiments indicate that the VWF A1 domain contains a lysine-dependent binding site for plasminogen, which remains cryptic when VWF is in a globular state.

Streptokinase-Activated Plasminogen Degrades Platelet–VWF Complexes

We next investigated whether therapeutic thrombolytic agents that trigger plasminogen activation can be applied to degrade platelet–VWF complexes and bypass the need for uPA or ADAMTS13. Therefore, we activated plasminogen with streptokinase, which is used in the treatment of myocardial infarction and pulmonary embolism. Streptokinase-activated plasminogen gradually degraded purified VWF in solution (Figure IIIC in the online-only Data Supplement), as reported previously for purified plasmin.7 Furthermore, streptokinase-activated plasminogen rapidly cleaved platelet-covered VWF strings from endothelial cells under flow (Figure 3A; Movie VI in the online-only Data Supplement; quantification in Figure 3C). This cleavage was also lysine dependent; εACA protected against streptokinase-mediated degradation of platelet–VWF complexes (Figure 3B; Movie VII in the online-only Data Supplement). In similar experiments, we found that ADAMTS13 could directly degrade platelet–VWF complexes on HUVECs under flow, but neutrophil elastase, granzyme M, and granzyme B could not (Figure IVA through IVD in the online-only Data Supplement; quantification in Figure IVE in the online-only Data Supplement). We next investigated whether plasmin-mediated destruction of platelet–VWF complexes was restricted to the surface of endothelial cells, which are known to express lysine-dependent receptors for plasminogen.19 To that end, we triggered platelet agglutination by ristocetin in the presence of streptokinase-activated plasminogen. Although platelet–VWF complexes formed normally in the presence of plasmin activity, they became unstable and fell apart immediately after full agglutination (Figure 3D). In contrast, the addition of plasminogen alone, or in the presence of uPA or tPA, did not induce degradation of these complexes (which indicates a critical role for uPAR). Moreover, platelet agglutinates in suspension were protected against degradation by streptokinase-activated plasminogen in the presence of εACA (Figure 3E), and the addition of εACA after the onset of plasmin digestion immediately halted further degradation (Figure 3E). In similar experiments, we found that ADAMTS13, neutrophil elastase, and granzyme M and granzyme B were unable to directly (ie, without preexposure of VWF to these enzymes) degrade ristocetin-induced platelet agglutinates (Figure IVF in the online-only Data Supplement). Also in platelet-rich plasma, ristocetin-induced platelet agglutinates were sensitive to breakdown by streptokinase-activated
plasminogen in a lysine-dependent manner (Figure 3F). These data indicate that thrombolytic plasminogen activators can bypass ADAMTS13 and uPA to induce the breakdown of platelet agglutinates.

Uncontrolled plasmin activity can affect multiple important targets in the hemostatic system that are involved in platelet aggregation and hemostasis (eg, fibrinogen or platelet glycoprotein Ibα). We therefore investigated whether the levels of streptokinase-activated plasminogen that degrade platelet–VWF complexes also generally affect platelet aggregate formation. We triggered platelet activation by type I collagen or α-thrombin. Both of these physiological agonists induced complete and irreversible platelet aggregation in the presence of streptokinase-activated plasminogen (Figure 4A and 4B). However, we found that the initial phase of platelet aggregation was delayed, which suggests an auxiliary role for VWF during the initiation of aggregate formation. Under flow, platelet aggregate formation on immobilized collagen at arterial shear remained unaffected in the presence of streptokinase-activated plasminogen (Figure 4C; quantification of surface coverage in Figure 4D), even when whole blood was preincubated with a high dose of streptokinase.
(100 U/mL) for 5 minutes before perfusion (Movies VIII [control] and IX ([streptokinase] in the online-only Data Supplement). Together, these data indicate that neither globular plasma VWF nor collagen-bound VWF forms an efficient target for plasmin-mediated breakdown, whereas platelet–VWF complexes are rapidly degraded.

Plasminogen Activation During TTP Attacks

Given the effects of plasmin on platelet–VWF complexes, we hypothesized that plasminogen activation may also be triggered during attacks of microangiopathy in TTP. Therefore, we investigated the status of the plasminogen activation system in plasma samples from TTP patients, collected both during TTP attacks and during remission (baseline characteristics are provided in Table I in the online-only Data Supplement). First, we confirmed that ADAMTS13 activity was lowered during active TTP (median 0.0%, interquartile range [IQR] 0.0%–4.0%; n=26) compared with remission samples (median 6.5%, IQR 0.3%–32.0%; n=32; Figure 5A). A significant number of TTP patients in remission had little (8 patients <10%) or no (9

Figure 3. Streptokinase-activated plasminogen lysine-dependently degrades platelet–von Willebrand factor (VWF) complexes. Time-lapse morphology of stable platelet-covered VWF strings after addition of (A) streptokinase-activated plasminogen (Plg strep) and (B) streptokinase-activated plasminogen in presence of ε-aminocaproic acid (εACA Plg strep). Scale bars, 25 μm; images are representative of experiments that were repeated ≥3 times. C, String survival times during streptokinase-induced plasminogen activation with or without εACA (n=5 per condition; values are medians and interquartile ranges). D, Ristocetin-induced platelet agglutination in the presence of plasminogen (Plg) with or without streptokinase, tissue-type plasminogen activator (tPA), or urokinase-type plasminogen activator (uPA). E, Ristocetin-induced platelet agglutinates are protected by εACA during streptokinase-dependent plasmin activity (either added before or 9 minutes after onset of agglutination; indicated with black arrow). F, Streptokinase-dependent plasmin activity degrades ristocetin-induced platelet agglutinates in a lysine-dependent manner. D through F, Representative experiments that were performed ≥3 times.
patients) detectable ADAMTS13 activity, which underscores the fact that ADAMTS13 deficiency in itself is not sufficient to develop episodes of acute TTP.

After plasminogen activation in plasma, plasmin is allowed to function for a limited amount of time before it is inactivated by its natural inhibitor, α2-antiplasmin. This prevents off-target degradation of proteins. In a recent case report, a patient with a preexisting ADAMTS13 deficiency (10% activity) developed α2-antiplasmin deficiency during an episode of TTP, 21 potentially worsening ADAMTS13 deficiency. In the patient cohort in the present study, we found that α2-antiplasmin activity was somewhat lowered during acute TTP (median 98.4%, IQR 91.7%–106.6%; n=26) compared with patients in remission (median 104.0%, IQR 100.6%–113.3%; n=32) and healthy control subjects (median 104.7%, IQR 98.2%–107.5%; n=19; Figure 5B). However, no evidence for α2-antiplasmin deficiency was found among patients in the present study that could help to explain their lowered ADAMTS13 levels or TTP attacks.

Next, we investigated whether there was plasminogen activation in TTP patients. The determination of PAP complexes is commonly used to determine ongoing plasminogen activation, because this complex is cleared ≈5 times faster than the uncomplexed inhibitor22 or plasminogen zymogen.23 During acute TTP, PAP complex levels were elevated (Figure 5C; median 1.80 μg/mL, IQR 1.19–3.85 μg/mL; n=26) compared with patients in remission (median 1.19 μg/mL, IQR 0.99–1.51 μg/mL; n=32) and normal healthy donors (median 1.15 μg/mL, IQR 0.93–1.57 μg/mL; n=19). Because thrombocytopenia serves as a cardinal sign for microangiopathy, we categorized all patient samples by platelet counts and further investigated the association with PAP complex levels. We found that these complexes were predominantly elevated (median 3.89 μg/mL, IQR 1.65–5.99 μg/mL) in patients with platelet counts below 50×10^3 platelets/μL (n=11), which represents severe microangiopathy (Figure 5D). In the second category (50–100×10^3 platelets/μL; n=8), PAP complexes were only modestly increased (median 1.80 μg/mL, IQR 1.30–2.00 μg/mL) over the third and higher categories (>100×10^3/μL), which had normal PAP complex levels (median 1.08 μg/mL, IQR 0.98–1.47 μg/mL; n=28). This indicates that plasminogen activation mainly occurs in patients during severe thrombotic microangiopathy.

We next investigated the relationship between ADAMTS13 activity and plasminogen activation in further detail. In TTP patients with undetectable ADAMTS13 activity, PAP complexes were elevated, whereas they were normal in samples with low (1% to 20%) and moderate to normal activity (20% to 100%; Figure V A in the online-only Data Supplement). However, this negative association between ADAMTS13 activity and PAP complex levels only held true in acute TTP (Figure VB in the online-only Data Supplement) and was absent in patients in remission (Figure VC in the online-only Data Supplement). This suggests that the state of TTP disease activity (ie, microangiopathy), rather than the absence of ADAMTS13 activity, induces plasminogen activation in TTP patients. We further explored this rationale over time in repeated samples from 3 individual patients with acute TTP that were taken on admission (indicated by day 0) and on several days during plasmapheresis treatment (sampled...
before plasmapheresis). In the first patient, platelet counts were directly related to ADAMTS13 activity: With increasing ADAMTS13 activity, platelet counts were correspondingly higher (Figure 5E, patient A). Conversely, PAP complex levels were high in this patient when platelet counts were low and normalized with higher platelet counts (Figure 5E). In 2 other patients, platelet counts were corrected while ADAMTS13 activity remained undetectable (Figure 5E, patient B; Figure VD in the online-only Data Supplement, patient C). However, in agreement with results for the first patient, PAP complexes in these patients were elevated during severe thrombocytopenia but normalized with increasing platelet counts. These data indicate that plasmapheresis does not always restore ADAMTS13 activity and that thrombotic microangiopathy triggers plasminogen activation in human patients.

Thrombolytic Therapy Attenuates Symptoms of Acute TTP in Adamts13−/− Mice

We previously found that exogenous plasminogen activators, such as streptokinase, can be used to degrade platelet–VWF complexes on endothelial cells and in suspension when ADAMTS13 is absent. We therefore continued to investigate whether thrombolytic agents have therapeutic value in vivo in a mouse model of acute TTP. Intravenous administration of a high dose of rhVWF into Adamts13−/− mice led to the formation of TTP-like symptoms, with thrombocytopenia as a cardinal sign. In continuation of our previous experiments, we chose to investigate the therapeutic potential of streptokinase, which specifically reacts with human plasminogen. To that end, we challenged mice by administration of rhVWF, while simultaneously administering

Figure 5. Plasminogen activation in patients with thrombotic microangiopathy. A, ADAMTS13 activity levels in patients with thrombotic thrombocytopenic purpura (TTP) in remission (n=32) and during episodes of active disease (n=26). α2-Antiplasmin activity (B) and plasmin–α2-antiplasmin (PAP) complex levels (C) in TTP patients in remission and during active disease and in normal healthy donors (NHD; n=19). D, PAP complexes in all TTP patients, categorized by platelet counts (solid symbols represent patients during active disease, open symbols represent patients in remission). Medians are indicated by bars in panels A through D. E, Changes in platelet counts, ADAMTS13 activity (open symbols; left y axis), and PAP complex levels (solid symbols; right y axis) in 2 individual acute TTP patients determined on several days during plasmapheresis treatment. The numbering over the symbols indicates the number of days after hospital admission.
human plasminogen. Twenty-four hours after this challenge, platelet counts in these mice were reduced to a median of 140.0×10³/μL (IQR 90.0–294.5×10³/μL; Figure 6A). In comparison, unchallenged mice had platelet counts of 490.5×10³/μL (IQR 455.8–528.3×10³/μL). In parallel, mean platelet volumes were elevated in rhVWF-challenged mice to 6.5 fL (IQR 5.75–6.85 fL) compared with unchallenged mice 4.1 fL (IQR 3.9–4.3 fL; Figure 6B). This is indicative of the presence of circulating platelet complexes as a long-term result of the challenge. When streptokinase was administered to these mice, either 5 or 15 minutes after the initial challenge, platelet counts normalized to 368.5×10³/μL (IQR 248.8–418.8×10³/μL) and 381×10³/μL (IQR 259.5–434.5×10³/μL), respectively, 24 hours later. In good agreement with these results, mean platelet volumes returned to 4.4 fL (IQR 4.1–5.0 fL) and 4.1 fL (IQR 4.1–4.9 fL), respectively, which indicates the absence of circulating platelet complexes. Finally, in control mice that were treated with thrombolytic treatment without being challenged with rhVWF, platelet counts remained normal at 518×10³/μL (IQR 454.5–557.5×10³/μL), and the mean platelet volume remained normal at 4.1 fL (IQR 4.0–4.3 fL). Histochemical analyses of sections from various tissues of the challenged mice revealed that the vasculature appeared to be largely normal 24 hours after rhVWF challenge. However, several thrombi were found dispersed in various tissues of challenged mice, but no thrombi were seen anywhere in mice that had received thrombolytic treatment (Figure 6C shows liver sections). Together, these experiments indicate that the induction of plasminogen activation in vivo attenuates thrombotic microangiopathy.

**Discussion**

Attacks of TTP are hallmark by occlusion of the microvasculature by platelet–VWF complexes and are life-threatening unless treated. ADAMTS13 deficiency forms an important risk factor for TTP attacks. Intriguingly, both patients and knockout mice with continuous deficiency do not present with continuous symptoms of TTP, which suggests that additional susceptibility factors beyond ADAMTS13 deficiency modify the development of TTP attacks. In the present studies, we explored the hypothesis that a second enzyme can substitute for ADAMTS13 in its absence. Several candidate enzymes have been identified in vitro, such as thrombin, neutrophil elastase, granzyme B, and plasmin. Here, we chose to investigate the potential involvement of plasmin in TTP. Our in vitro experiments showed that platelet–VWF complexes can be degraded efficiently through uPA-dependent plasminogen activation on endothelial cells (Figure 1). This can take place through direct lysine-dependent binding of plasminogen to VWF (Figure 2). Furthermore, exogenously added thrombotic agents degrade platelet–VWF complexes with equal efficacy and can be securely controlled by the administration of lysine analogues (Figure 3). In contrast, plasminogen activation has little effect on primary hemostasis in vitro (Figure 4). These findings indicate that platelet–VWF complexes, rather than platelet aggregates in general, are susceptible to degradation by plasmin. The gradual cleavage of globular VWF by plasmin has been reported previously. We here propose that the efficiency of VWF cleavage by plasmin is a function of its conformation: Plasmin has limited affinity for the binding to and cleavage of globular VWF, but unfolding of VWF strongly enhances this process. As a result, uPA-triggered plasminogen

![Figure 6](http://circ.ahajournals.org/)
activation destroyed platelet–VWF complexes on endothelial cells and in suspension in a matter of seconds, which could be recapitulated by streptokinase-activated plasminogen. On the basis of these in vitro findings, we propose a working model of the activation of plasminogen in response to obstructive platelet–VWF complexes (Figure 7). In the complete absence of ADAMTS13, platelets, as well as ultralarge VWF multimers, are present in the circulation. Obstructive microthrombi do not form spontaneously until an elusive triggering event occurs. At that moment, vascular endothelial cells are the first to register differences in oxygenation and respond to hypoxia through expression of extracellular uPAR. In a final step, plasminogen is activated on the activated endothelial cell surface, with the aim to remove the obstructions.

In the past, there has been enigmatic evidence for plasminogen activation in acute TTP patients. In our studies, we found elevated PAP complexes, representing plasminogen activation, in a group of TTP patients with acute microangiopathy (Figure 5). However, in TTP patients in remission (ie, without microangiopathy), no plasmin was generated. This corresponds well with our proposed model (Figure 7): Microvascular obstructions cause hypoxia, which results in plasminogen activation on endothelial cells. It is seemingly paradoxical that plasminogen activation takes place, especially in those patients who have acute TTP. Apparently, in these patients, plasmin activity is not able to fully prevent clinical problems. However, it is most likely that an avalanche-like sequence of intravascular events precedes the moment of clinical presentation in the hospital: In the first phase, continuously forming platelet agglutinates begin obstructing the microvasculature. In later stages, these obstructions grow in size and accumulate more platelets until clinical symptoms appear. In the situation in which plasmin is able to clear obstructed vessels at an early stage, a TTP patient would not present to the hospital and would not be included in our patient group. In other words, patients who experienced an attack of microangiopathy that was successfully countered by endogenous plasminogen activation were by definition not included in the present study. However, we propose that even in the present group of acute TTP patients, the observed plasminogen activation is not without purpose; it may help to slow disease progression. Indeed, it is difficult to estimate what would happen if a TTP patient did not have the capacity to recruit plasmin at sites of microvascular occlusion. Mice that are deficient in ADAMTS13 do not spontaneously develop TTP-like symptoms unless challenged with Shiga toxin or supraphysiological amounts of rhVWF. It remains unknown what factors beyond ADAMTS13 deficiency modify the susceptibility of mice to develop TTP, but certain strains are more susceptible than others. Targeting factors of the plasminogen activation system in mouse models for TTP may help to provide new insights.

In the present group of 32 acute TTP patients, we found no evidence that ADAMTS13 deficiency was related to α2-antiplasmin deficiency, in contrast to a recently published case report. How to reconcile these apparently contradicting findings? In the case report, the cause for the (transient) α2-antiplasmin deficiency remained uncertain. However, α2-antiplasmin deficiency can be caused by genetic abnormalities, or α2-antiplasmin can become depleted through consumption. This is possible because plasminogen is more abundantly present in the circulation (2.4 μmol/L) than its inhibitor, α2-antiplasmin (1 μmol/L). Subsequently, the continuous formation of plasmin would result in the continuous formation of PAP complexes until the inhibitor runs out. We hypothesize that this was the case in the α2-antiplasmin–deficient TTP patient. We propose the following sequence of events: (1) The patient had a preexisting ADAMTS13 deficiency. (2) After a trigger, the patient developed an episode of microangiopathy (when the remaining ADAMTS13 was insufficiently protective). (3) Plasminogen activation was triggered to degrade VWF in microvascular obstructions until all α2-antiplasmin was consumed. Finally, the remaining uncontrolled plasmin slowly degraded the remaining ADAMTS13 (as collateral damage).

In a final set of experiments, we have shown that thrombolytic therapy is therapeutic in a mouse model of acute TTP (Figure 6). Administration of thrombolytic therapy resolved thrombocytopenia, and platelet complexes were no longer detectable in the circulation or found in the microvasculature. Although this therapeutic opportunity needs to be explored further, it is an attractive idea that conventional and commonly

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**Figure 7.** A model of plasminogen activation during microangiopathy. In the complete absence of ADAMTS13, platelets and ultralarge von Willebrand factor (VWF) multimers are present in the circulation. A triggering event induces the formation of platelet–VWF complexes until microvascular occlusions start to develop. Nearby endothelial cells (EC) are sensitive to hypoxia and present urokinase-type plasminogen activator receptors (uPAR) on their surface. Finally, plasminogen is activated on the activated EC surface with the aim of clearing the obstructed vessel. UPA indicates urokinase-type plasminogen activator.
available therapeutic agents may be valuable in the treatment of TTP attacks. Intuitively, it appears dangerous to administer thrombolytic drugs (with bleeding as a well-known side effect) to thrombocytopenic patients. However, the present experiments suggest that thrombolysis may actually be helpful in restoring primary hemostasis by restoring platelet counts (Figure 6A), while removing harmful obstructions from the vasculature at the same time. Additionally, the dosing of thrombolysis required to degrade a platelet–VWF complex is expectedly lower than the dose required to degrade a fibrin-rich thrombus: In the present experiments, we applied a single dose of streptokinase that approximately represented 20% of the loading dose (in concentration) commonly given to resolve pulmonary embolism and stroke in human patients. Finally, should the desired effects of thrombolysis in TTP patients be achieved, we expect that it will be possible to limit further plasmin activity through administration of lysine analogs, such as eACA (Figure 3E). The majority of TTP patients have ADAMTS13 deficiency as a result of inhibitory antibodies against this metalloprotease. Currently, high volumes of plasma exchange are required to overcome these antibodies. It is attractive to consider the use of thrombolytic therapy to effectively bypass anti-ADAMTS13 antibodies.

In conclusion, we propose that endogenous plasminogen activation as is seen during TTP-related microangiopathy is meant for local clearance of microvascular obstructions. This last line of proteolytic defense can operate independent of other enzymes. On the basis of our findings, we propose that the plasminogen activation system forms a modulating factor in the events that cause microangiopathy, which may help to explain the episodic nature of TTP. Future studies will need to identify the reasons the plasminogen activation system falls short of completely preventing microangiopathy in patients who present with episodes of acute TTP. However, stimulation of plasminogen activation with readily available conventional thrombolytic agents may have therapeutic value by stimulating the breakdown of vascular obstructions in thrombotic microangiopathy.

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Disclosures
None.

References
Patients with thrombotic thrombocytopenic purpura (TTP) experience life-threatening episodes of microangiopathy that damage kidneys, heart, and brain. Although triggers for these attacks remain elusive, deficiency in ADAMTS13 is an important risk factor. This metalloprotease cleaves von Willebrand factor (VWF) multimers to prevent formation of obstructive platelet–VWF complexes. However, complete ADAMTS13 deficiency does not cause continuous disease, which suggests that additional pathophysiological processes modulate the clinical phenotype of TTP attacks. Plasmin, the key enzyme of the fibrinolytic system, can be activated on vascular endothelium during cellular stress. We set out to investigate whether plasmin can serve as a backup enzyme for ADAMTS13 in the degradation of obstructive pathological platelet–VWF complexes. We identified that plasmin efficiently degrades platelet–VWF complexes. This can take place on the endothelium and may occur at sites of vascular obstructions to mediate clearance. We found confirmatory evidence for such a mechanism in TTP patients, in whom plasmin formation occurs during attacks in a manner that correlates with the extent of microangiopathy. Therapeutic stimulation of plasminogen activation with the thrombolytic agent streptokinase has therapeutic value in a mouse model of TTP by degrading circulating platelet–VWF complexes while restoring primary hemostasis through attenuation of thrombocytopenia. Our findings indicate that plasminogen activation during TTP is a previously unidentified modulator in the clinical presentation of microangiopathy. These findings may aid in our understanding of physiological plasminogen activation and the effectiveness of thrombolytic therapy. Furthermore, our in vivo studies offer primary evidence for the clinical efficacy of thrombolytic agents in the management of TTP.
Plasmin Cleavage of von Willebrand Factor as an Emergency Bypass for ADAMTS13 Deficiency in Thrombotic Microangiopathy
Claudia Tersteeg, Steven de Maat, Simon F. De Meyer, Michel W.J. Smeets, Arjan D. Barendrecht, Mark Roest, Gerard Pasterkamp, Rob Fijnheer, Karen Vanhoorelbeke, Philip. G. de Groot and Coen Maas

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Supplemental Material
Supplemental methods

Blood collection

Blood was collected from healthy volunteers (without anticoagulant- or antiplatelet treatment for at least 10 days) under approval by the University Medical Center Utrecht Ethics Committee. Blood was anticoagulated with trisodium citrate (final concentration of 0.32% w/v). Platelet-rich plasma (PRP) and washed platelets (WP) were prepared by centrifugation as described before\(^1\). Platelet counts were determined using CellDyn1800 (Abbott, Hoofddorp, the Netherlands) and set to 200x10\(^9\)/L.

Human umbilical vein endothelial cell (HUVEC) isolation and cell culture

HUVEC were freshly isolated from umbilical cords by trypsinisation as described previously\(^2\). Isolated cells were cultured for 2 passages in HUVEC medium (EndoPrime Base Medium, enriched with EndoPrime Kit Supplements (2% Fetal Bovine Serum (FBS)), PAA, Pasching, Austria) in culture flasks coated with 5 µg/mL fibronectin (purified according to Klebe et al\(^3\)).

Immunofluorescence microscopy

Confluent HUVEC were pre-activated with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Zwijndrecht, the Netherlands) for 60 minutes. Cells were fixed with 4% paraformaldehyde and blocked with 10% normal goat serum in phosphate buffered saline (21 mM Na\(_2\)HPO\(_4\), 2.8 mM NaH\(_2\)PO\(_4\), 140 mM NaCl, pH=7.4; PBS). uPAR was visualized with mouse anti-human uPAR (R&D systems, Abingdon, United Kingdom) and goat anti-mouse-AF488 (Invitrogen, Bleiswijk, the Netherlands). VWF was stained using rabbit anti-human VWF (Dako, Heverlee, Belgium) and goat anti-rabbit-AF488. Where indicated, HUVEC were incubated for 60 minutes at 37°C with 216 µg/mL plasminogen (purified in-house by lysine-sepharose affinity chromatography) and 10 ng/ml uPA (Komabiotech, Seoul, Korea) or either
individually. Cells were embedded with DAPI prolonged gold (Invitrogen) and permeabilised with 0.5% Triton-X100 for 5 minutes prior to embedding where indicated.

**Enzyme-linked immunosorbent assay (ELISA) for VWF release**

HUVEC were stimulated with PMA as before. Next, the medium was replaced with fresh medium (removing soluble non-cell bound VWF), containing 2% FCS, 216 µg/mL plasminogen, 10 ng/mL uPA, 200 mM ε-aminocaproic acid (εACA), 10 ng/mL tPA (Actilyse, Boehringer Ingelheim, Alkmaar, the Netherlands), combined or individually. Cell remnants were removed by centrifuging the supernatant for 10 minutes at 1000xg. MaxiSorp 96-wells plates (Nunc Thermo Scientific, Waltham, USA) were coated with rabbit anti-human VWF (Dako) in 40 mM Na₂CO₃, 35 mM NaHCO₃, 3 mM NaN₃, pH 9.6. Wells were washed with PBST (PBS, 0.05% Tween20) and blocked at 37°C with PBS, 1% bovine serum albumin (BSA). Samples and standards (purified VWF in culture medium) were incubated followed by rabbit anti-human VWF/HRP (Dako) as a detection antibody. Finally, 100 μL/well TMB substrate (3,3',5,5'-Tetramethyl-benzidine; Tebu-Bio Heerhugowaard, the Netherlands) was added and the reaction was stopped with 50 μL/well 1M H₂SO₄ and absorbance was measured at 450 nm.

**Platelet aggregation on collagen**

For platelet aggregation experiments on collagen surfaces under flow, coverslips were attached to polydimethylsiloxane (PDMS) perfusion chambers. The channels were precoated with 0.1 mg/mL type I collagen and blocked with 1% human serum albumin. Whole blood that was preincubated with 100 U/mL streptokinase for 5 minutes at 37°C, was perfused at a shear rate of 1600 sec⁻¹ for 5 minutes. Recordings were made by differential interference contrast (DIC) microscopy, using a 40x/1.25 oil or 100x/1.25 oil EC-plan Neofluar objective (Carl Zeiss). Images were analyzed with AxioVision software (Release 4.6, Carl Zeiss) and ImageJ software (Release 1.41, National Institutes of Health, Bethesda, USA). For
quantification, string survival times were determined by following 5 strings per experimental condition for 5 minutes after introduction of experimental variables.

**Plasminogen binding ELISA**

Nunc PolySorp plates (Thermo Scientific, Breda, the Netherlands) were coated with 5 μg/mL VWF or 1 μg/mL recombinant VWF A1 domain (U-protein Express, Utrecht, the Netherlands) and blocked with 2% BSA in PBS for 1 hour. Plasminogen binding was analyzed in PBS containing 50 μM PPACK and 50 μM GGACK (Haematologic Technologies Inc, Vermont, USA) to block proteolysis and 200 mM εACA where indicated. Competition ELISAs were performed by determining the binding of 100 μg/mL plasminogen (suboptimal binding) in the presence of a concentration range of VWF A1 domain, globular VWF or εACA. Plates were washed with PBS 0.05% Tween and incubated with goat anti-plasminogen (Affinity Biologicals, Ancaster, Canada), followed by RAGPO (Dako) to detect plasminogen binding. Plates were stained with TMB (Tebu-Bio) and H₂SO₄. Absorbance was read at 450 nm.

**Platelet agglutination and aggregation**

Agglutination assays with WP: 10 μg/mL purified VWF was mixed with 10 U/mL streptokinase, 216 μg/mL plasminogen, 200 mM εACA, 10 ng/mL tPA or combinations thereof. The mixed samples were immediately added to pre-warmed WP (200x10³/μL) containing 200 μM RGDW (NKI, Amsterdam, the Netherlands), 100 μg/mL iloprost (Bayer, Leverkusen, Germany) and 0.6 mg/mL ristocetin (Biopool Us Inc, Jamestown, NY). Similar agglutinations were performed with 5 μg/mL ADAMTS13 (in the presence of 5 mM CaCl₂ and 20 μM ZnCl₂), 20 nM elastase, 300 nM granzyme B or -M. Agglutination assays in PRP were performed by adding 10 U/mL streptokinase with or without 200 mM εACA to pre-warmed PRP (200 x10³ platelets /μL) containing 200 μM RGDW, 100 μg/mL iloprost and 1.6 mg/mL ristocetin. Agglutination was recorded for 30 minutes at 37°C with an aggregometer.
(Chrono-log model 700, Stago, Leiden, the Netherlands). Platelet aggregation experiments were performed with WP, to which 1 mg/mL purified fibrinogen and 10 μg/mL VWF were added. Aggregation was induced by the addition of 1 IU/mL α-Thrombin or 5 μg/mL type I collagen (Horm-collagen; Nycomed, Linz, Austria) in the presence or absence of 10 U/mL streptokinase and 216 μg/mL plasminogen and 200 mM εACA where indicated.

**HUVEC-dependent plasminogen activation assay**

HUVEC were stimulated for 60 minutes with 100 nM PMA and then incubated with a concentration range of uPA, and 216 μg/mL plasminogen for 15, 30 or 60 minutes at 37°C. The supernatant was centrifuged for 10 min at 1000xg to spin down any cell remnants. 0.2 mM fluorogenic plasmin substrate (I-1390; Bachem, Bubendorf, Switzerland) was added to the samples and the fluorescence (at an excitation wavelength of 390nm and emission wavelength of 460nm) was read after 1 hour at 37°C with a microplate reader (Versamax, Molecular Devices, Sunnyvale, United States).

**Western blotting**

10 μg/mL purified VWF was incubated with 10 U/mL streptokinase (Streptase, CSL Behring, Ontario, Canada) and 216 μg/mL plasminogen for 0, 15, 30, 45, 60 or 90 seconds, and 2.5, 5 or 10 minutes at 37°C. The samples were mixed 1:1 with sample buffer containing DTT and boiled for 10 minutes at 95°C. SDS-PAGE was performed using a 6% running and 4% stacking polyacrylamide gel and proteins were transferred to a polyvinylidenefluoride membrane (Millipore, Amsterdam, the Netherlands). Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NB, USA) was used for blocking and as diluent for primary antibody (anti-VWF, Dako) and secondary antibody incubations (goat anti-rabbit AF680, A21057, Invitrogen). Protein bands were visualized with the Odyssey Imaging system (LI-COR Biosciences). Quantification was performed using ImageJ software (Release 1.41).
Supplemental Figures and Tables

**Supplemental Figure 1.** Time-lapse morphology of stable platelet-covered VWF strings after addition of **A)** Plg only or **B)** uPA only. Scale bars represent 25 µm. Images are representative for experiments that were repeated at least three times.
**Supplemental Figure 2.** Activated endothelial cells express uPAR and support plasminogen activation by uPA. 

A) Immunofluorescent staining of uPAR (red) on HUVEC (nuclei in blue) without or with PMA. Scale bars represent 20 μm. Images are representative for experiments that were repeated at least three times. 

B) PMA-activated HUVEC were incubated for 15, 30 or 60 minutes in the absence or presence of 216 μg/mL plasminogen and a concentration series of uPA (n=3; median (range)). Fluorescence of converted plasmin substrate was determined in supernatants at 390 (excitation) - 460 (emission) nm after 1 hour at 37°C.
Supplemental Figure 3. Soluble globular VWF does not compete for plasminogen binding. 

A) Binding of plasminogen (Plg) to immobilized VWF in the presence of increasing concentrations of soluble globular VWF (n=6; median (IQR)). B) Binding of Plg to immobilized VWF in the presence of increasing concentrations of εACA (n=6; median (IQR)). C) Streptokinase-activated plasminogen degrades purified VWF in solution. 10 μg/mL VWF was incubated with streptokinase-activated plasminogen in a time series until 10 minutes incubation at 37°C and analyzed by western blot under reducing conditions (representative for three individual experiments).
Supplemental Figure 4. The direct effects of ADAMTS13, neutrophil elastase, granzyme M and granzyme B on platelet-VWF complexes. A) Time-lapse morphology of stable platelet-covered VWF strings after addition of A) ADAMTS13, B) elastase, C) granzyme M and D) granzyme B. Scale bars represent 25 μm. Images are representative for experiments that were repeated at least three times. E) String survival times (n=5 per condition; median (IQR)) F) Ristocetin-induced platelet agglutination in the presence of ADAMTS13, elastase, granzyme M and -B. Shown figures are representative for experiments that were performed at least three times.
Supplemental Figure 5. Plasminogen activation and ADAMTS13 activity in thrombotic thrombocytopenic purpura. PAP-complexes in A) all TTP patients, B) acute TTP and C) TTP patients in remission. D) Changes in platelet counts, ADAMTS13 activity (open symbols; left y-axis) and PAP-complex levels (closed symbols; right y-axis) in an individual acute TTP patient (patient C) determined on several days during plasmapheresis treatment. The numbering over the symbols indicates the number of days after hospital admission.
**Supplemental Table 1.** Baseline characteristics of TTP patients and healthy control donors.

<table>
<thead>
<tr>
<th></th>
<th>Acute TTP</th>
<th>Remission TTP</th>
<th>Normal healthy donors</th>
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<tr>
<td><strong>n =</strong></td>
<td>26</td>
<td>32</td>
<td>19</td>
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<td>Gender (% female)</td>
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<tr>
<td>Other autoimmune disease</td>
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**Movie legends**

**Supplemental Movie 1.** Platelet-VWF complexes on endothelial cells are degraded by uPA-dependent plasmin activity. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s⁻¹. At the indicated timepoint, plasminogen and uPA are simultaneously added without preincubation (216 μg/mL and 10 ng/mL, respectively). The movie is representative for experiments that were performed at least three times.

**Supplemental Movie 2.** Platelet-VWF complexes on endothelial cells are protected from uPA-dependent plasmin degradation by a monoclonal antibody against uPAR. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s⁻¹ in the constant presence of monoclonal anti-uPAR (2 μg/mL). At the indicated timepoint, plasminogen and uPA are simultaneously added without preincubation (216 μg/mL and 10 ng/mL, respectively). The movie is representative for experiments that were performed at least three times.

**Supplemental Movie 3.** Control: Platelet-VWF complexes on endothelial cells in the presence of uPA only. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s⁻¹. At the indicated timepoint, uPA is added (10 ng/mL). The movie is representative for experiments that were performed at least three times.

**Supplemental Movie 4.** Control: Platelet-VWF complexes on endothelial cells in the presence of Plg. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s⁻¹. At the indicated timepoint, Plg is added (216 μg/mL). The movie is representative for experiments that were performed at least three times.
Supplemental Movie 5. Platelet-VWF complexes on endothelial cells are protected from uPA-dependent plasmin degradation by the soluble lysine analog εACA. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s$^{-1}$ in the constant presence of 200 mM εACA. At the indicated timepoint, plasminogen and uPA are simultaneously added without preincubation (216 μg/mL and 10 ng/mL, respectively). The movie is representative for experiments that were performed at least three times.

Supplemental Movie 6. Platelet-VWF complexes on endothelial cells are degraded by streptokinase-dependent plasmin activity. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s$^{-1}$. At the indicated timepoint, plasminogen and streptokinase are simultaneously added without preincubation (216 μg/mL and 10 U/mL, respectively). The movie is representative for experiments that were performed at least three times.

Supplemental Movie 7. Platelet-VWF complexes on endothelial cells are protected from streptokinase-dependent plasmin degradation by the soluble lysine analog εACA. Platelet-VWF complexes form on PMA-stimulated HUVEC at a shear rate of 300 s$^{-1}$ in the constant presence of 200 mM εACA. At the indicated timepoint, plasminogen and streptokinase are simultaneously added without preincubation (216 μg/mL and 10 U/mL, respectively). The movie is representative for experiments that were performed at least three times.

Supplemental Movie 8. Platelet aggregate formation on type I collagen at arterial shear. Whole blood was perfused over immobilized type I collagen on glass coverslips at a shear rate of 1600 s$^{-1}$. The movie is representative for experiments that were performed at least three times.
**Supplemental Movie 9.** Platelet aggregate formation on type I collagen at arterial shear in the presence of streptokinase. Whole blood was preincubated with 100 U/mL streptokinase for 5 minutes at 37°C prior to perfusion over immobilized type I collagen on glass coverslips at a shear rate of 1600 s⁻¹. The movie is representative for experiments that were performed at least three times.

**Supplemental references**

