

# Keeping von Willebrand Factor under Control: Alternatives for ADAMTS13

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## Abstract

Von Willebrand factor (VWF) is one of the most important proteins of the hemostatic system. Its multimeric state is essential for its natural function to guide platelets to sites of injury. ADAMTS13 is the key protease that regulates the multimeric state of VWF. Without ADAMTS13, VWF multimers can grow to pathologically large sizes. This is a risk factor for the life-threatening condition thrombotic thrombocytopenic purpura (TTP). Intriguingly, a complete ADAMTS13 deficiency does not cause continuous TTP, either in patients or genetically targeted mice. Instead, TTP occurs in episodes of disease, separated by extended periods of remission. This indicates that regulating factors beyond ADAMTS13 are likely involved in this pathologic cascade of events. This raises the question of what really happens when ADAMTS13 is (temporarily) unavailable. In this review, we explore the possible role of complementary mechanisms that are capable of modifying the thrombogenic potential of VWF.

## Keywords

- ▶ ADAMTS13
- ▶ plasmin
- ▶ VWF
- ▶ thrombotic thrombocytopenic purpura
- ▶ thrombotic microangiopathy

When the vessel wall ruptures during injury or cardiovascular events, platelets are the first to arrive on the scene. These platelets are guided to the damaged vessel wall by von Willebrand factor (VWF), which acts as molecular velcro. The multimeric state and conformation of VWF are key to its platelet-binding properties and enable platelets to bind to exposed subendothelium under the strong shear forces of flowing blood.<sup>1</sup> The platelet-binding properties of VWF need to be kept under constant control to prevent spontaneous intravascular platelet clustering, which can have dramatic pathologic consequences. When intravascular platelet clustering goes unchecked at sites of high shear forces, the microvasculature can become occluded by microthrombi.<sup>2</sup> Subsequently, organs rich in microvessels can become ischemic and damaged. The disease thrombotic thrombocytopenic purpura (TTP) is associated with waves of promiscuous platelet-clustering activity of VWF. These attacks of TTP are accompanied by hemolytic anemia and thrombocytopenia.

## ADAMTS13

The principal enzyme responsible for controlling the platelet-binding properties of VWF is ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13). Before its identification, it was reported that a VWF-cleaving enzyme is constitutively active in plasma.<sup>3</sup> This enzyme cleaves VWF in its A2 domain at a Tyr<sub>1605</sub>-Met<sub>1606</sub> bond (numbering includes the VWF propeptide, relating to positions 842–843 in mature VWF monomers). The VWF-cleaving capacity of this enzyme is dependent on metal-ions and promoted by conformational changes in VWF. In its globular form, VWF is resistant to proteolysis, while unfolding facilitates cleavage.<sup>4</sup> Later studies identified this enzyme as ADAMTS13.<sup>5,6</sup> The molecular mechanisms by which ADAMTS13 interacts with VWF to induce cleavage have been investigated in great detail and show us a unique, elegant, and tightly regulated process.<sup>7</sup> After VWF undergoes

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its conformational changes, ADAMTS13 binds to its substrate in multiple steps, positioning itself with high precision for cleavage of the Tyr<sub>1605</sub>-Met<sub>1606</sub> scissile bond.

## Thrombotic Thrombocytopenic Purpura

Lowered or absent ADAMTS13 activity can result in circulating VWF multimers that are unusually large. These VWF multimers are capable of spontaneous platelet recruitment in the circulation, especially under conditions of high shear. This can ultimately lead to the clinical syndrome of TTP. Reduced ADAMTS13 activity can be caused by a rare genetic deficiency (Upshaw-Shulman syndrome) but is more often the result of inhibitory autoantibodies (acquired TTP). TTP attacks are characterized by hemolytic anemia and thrombocytopenia, and are often accompanied by neurologic abnormalities, fever, and renal failure (although involvement of kidneys is less than in other forms of thrombotic microangiopathy<sup>2</sup>). The obstructive microthrombi are held responsible for these diverse pathologic features. If left untreated, TTP leads to organ failure with lethal consequences in the large majority of cases.

Plasma exchange and immunosuppressive therapy are valuable in TTP caused by an acquired ADAMTS13 deficiency. However, exciting developments may lead to more therapeutic options in the near future. First, recombinant forms of ADAMTS13 are currently under development,<sup>8,9</sup> some of which are resistant against neutralizing autoantibodies.<sup>10</sup> The common aim of these therapies is to restore ADAMTS13 activity in patients, which, in turn, should facilitate cleavage of VWF to destroy VWF-rich microthrombi. Furthermore, there is evidence that VWF multimer size can be pharmacologically targeted with *N*-acetylcysteine (NAC). This Food and Drug Administration (FDA)-approved drug is (among others) useful for treatment of acetaminophen toxicity and chronic obstructive lung disease. Interestingly, NAC has been shown to be able to reduce disulfide bonds in VWF, reducing size and activity of VWF multimers. Case reports on the treatment of TTP with NAC describe varying outcomes.<sup>11,12</sup> A clinical trial is currently ongoing to test its efficacy under controlled conditions (ClinicalTrials.gov Identifier: NCT01808521). Finally, baboon experiments previously demonstrated that inhibition of the platelet GPIIb-VWF interaction with a nanobody (camelid single-chain antibody) is an effective and safe strategy for managing acquired TTP.<sup>13,14</sup> A clinical trial has been performed (TITAN Trial; ClinicalTrials.gov Identifier: NCT01151423) to investigate the therapeutic potential of VWF-targeting nanobody caplacizumab (ALX-0681) in conjunction with plasma exchange for treatment of TTP.<sup>15</sup> The first published results from this trial are very promising: nanobody treatment reduces the time to platelet count normalization and reduces the need for plasma exchange.<sup>16</sup>

## The Mysterious Erratic Nature of TTP

### Lessons from Patients

During attacks of acquired TTP, ADAMTS13 activity is generally lower than it is during remission in the same patients.<sup>17</sup> Variations in autoantibody levels may help explain this variation in ADAMTS13 activity: when autoantibody titers

against ADAMTS13 are high, its activity decreases.<sup>18</sup> Based on the attack/remission clinical phenotype seen in TTP, it is attractive to hypothesize that ADAMTS13 activity levels need to remain above a threshold for the maintenance of a healthy perfusion of the microvasculature and prevention of TTP.<sup>19</sup> However, several lines of evidence argue against this concept: (1) patients with acquired TTP can have undetectable ADAMTS13 activity while being in remission and (2) patients with congenital ADAMTS13 do not experience continuously ongoing TTP but develop attacks of TTP in a pattern that closely resembles that of acquired TTP patients. Indeed, despite their complete congenital ADAMTS13 deficiency, such patients can remain completely asymptomatic for years.<sup>20-22</sup>

### Lessons from Mouse Models

The findings from congenital ADAMTS13-deficient patients have been corroborated in congenitally deficient ADAMTS13-deficient mice: these mice also do not spontaneously develop symptoms of TTP nor experience continuous thrombotic microangiopathy.<sup>23</sup> They only develop features that approximate TTP after exposure to pathogenic toxins,<sup>23</sup> or after injection with large amounts of recombinant multimeric VWF.<sup>9</sup> Intriguingly, congenital ADAMTS13 deficiency alone is not sufficient to increase the susceptibility of these mice for these triggers. TTP-like symptoms mainly develop in ADAMTS13-deficient mice with a very specific genetic background (i.e., CASA/Rk), but not in ADAMTS13 deficient mice with other genetic backgrounds. This strongly indicates that additional genetic risk factors modulate the capacity of these mice to develop TTP-like symptoms. Although this strain produces more VWF than the other strains, this is not held responsible for the heightened sensitivity of these mice to develop TTP-like symptoms.<sup>23</sup> The identification of additional genetic risk factors in the CASA/Rk strain may have significant impact on our understanding of TTP.

These combined observations from human TTP patients, as well as mice, strongly suggest that ADAMTS13 deficiency is an important risk factor for the development of TTP. However, it is also clear that additional factors modulate the disease phenotype.

### A Second Hit for TTP

As ADAMTS13 deficiency alone cannot explain the onset of all TTP attacks, it is probable that a triggering event is needed. It is attractive to hypothesize that an infection constitutes this trigger. In the case of Shiga toxin-mediated hemolytic uremic syndrome (ST-HUS), another form of thrombotic microangiopathy, the responsible pathogens have been identified. These are *Shigella dysenteriae* type 1 and certain strains of *Escherichia coli*, which produce shigatoxin.<sup>24</sup> Exposure to these pathogens via food intake can trigger severe thrombotic microangiopathy, independent of ADAMTS13 activity. On a macroscopic level, ST-HUS is clinically more commonly associated with acute kidney injury than TTP.<sup>2</sup> On a microscopic level, analyses of the composition of obstructive microthrombi reveal the involvement of fibrin in ST-HUS, whereas

microthrombi in TTP are (enigmatically) fibrin-poor.<sup>25</sup> These differences between ST-HUS and TTP suggest that, if there is a pathogen responsible for triggering TTP, it may be different from the pathogens that cause ST-HUS.

There is scientific evidence that links infection to TTP: recent studies showed that acute TTP attacks are associated with elevated levels of circulating DNA-histone complexes, as well as other components that can be traced back to release by neutrophils.<sup>26</sup> These neutrophil-derived components are able to trap bacterial pathogens and assist in their destruction<sup>27</sup> and were originally discovered in severe bacterial septicemia.<sup>28</sup> Their identification in TTP leads to the attractive hypothesis that these DNA-histone complexes, rather than the underlying infection, constitute the second hit that elicits an attack. However, are the DNA-histone complexes that are seen in TTP really *caused* by infection or are they a *consequence* of thrombotic microangiopathy? This same study shows neutrophil-released components in several other forms of thrombotic microangiopathy (unrelated to ADAMTS13 deficiency).<sup>26</sup> Furthermore, neutrophil release products have been reported in a variety of prothrombotic states that are not directly linked to infection,<sup>29–33</sup> and are released during *ex vivo* storage of red blood cells.<sup>34</sup> TTP attacks are often associated with hemolytic anemia, resulting from shear-related cellular damage to red blood cells at sites of vascular occlusions. It is attractive to speculate that in TTP neutrophils undergo a fate that is similar to that of erythrocytes, resulting in the release of their contents in a shear-related, but infection-unrelated, manner. This process, in turn, could worsen TTP symptoms by providing a “second hit” that reinforces microthrombosis.<sup>35</sup>

### Assistance for ADAMTS13 to Keep VWF Activity “Under Control”

It is evident that ADAMTS13 is a central physiologic regulator of VWF multimer size in blood. However, is it also certain that ADAMTS13 is the *only* protein that regulates the thrombogenicity of VWF? The proverb “Amicus certus in re incerta cernitur” by the roman poet Quintus Ennius (239 BC–c. 169 BC: <http://en.wikipedia.org/wiki/Ennius>) can be popularly translated as “A friend in need is a friend indeed.” The observation that congenital ADAMTS13-deficient persons and mice can be symptom free for extended periods of time suggests that ADAMTS13 may get help to keep VWF activity under control.

### The Complement System

In complement-mediated thrombotic microangiopathy, excessive activation of the complement system leads to thrombus formation. Mutations in factors H and I, which together control the alternative pathway of the complement system, are associated with this disease.<sup>36,37</sup> The complement system influences VWF release and function on multiple levels. First, unregulated formation of membrane-attack complexes causes endothelial cell damage and platelet activation. This can trigger VWF release, which may contribute to microthrombosis. Second, endothelial-cell

tethered platelet-VWF complexes can recruit and activate components of the alternative complement pathway.<sup>38</sup> Under normal conditions, this is kept under tight control by factors I and H (which can directly bind to VWF),<sup>38,39</sup> Third, factor H may directly influence the cleavage of VWF by ADAMTS13. A recent study showed that factor H enhances the cleavage of soluble VWF by ADAMTS13,<sup>39</sup> while another study showed that VWF cleavage by ADAMTS13 was inhibited by factor H on the surface of endothelial cells.<sup>40</sup>

Finally, detailed biochemical studies identified a direct influence of factor H on VWF that is unrelated to its role in the complement system: factor H directly modifies multimeric VWF in a nonenzymatic manner through reduction in large soluble multimers.<sup>41</sup> Together, these studies offer an explanation for the genetic association between factor H mutations and (complement-mediated) thrombotic microangiopathy: factor H has multiple protective roles, which help control the thrombogenicity of VWF. It is important to note that this form of thrombotic microangiopathy is largely unrelated to ADAMTS13 deficiency.<sup>2</sup>

### Enzymes

A variety of enzymes can cleave VWF *in vitro*. These enzymes include thrombin and plasmin,<sup>42</sup> as well as cathepsin G, neutrophil elastase, proteinase 3, matrix metalloproteinase 9,<sup>43</sup> and granzyme M and B.<sup>44</sup> The molecular mechanisms by which these enzymes cleave VWF are not uniform. Though some enzymes target the Tyr<sub>1605</sub>-Met<sub>1606</sub> bond in VWF A2 domain (which is represented in the fluorogenic FRET-VWF73 peptide<sup>45</sup>) that is also targeted by ADAMTS13, other enzymes have been shown to target different sites. Most of the reported enzymes can be secreted in active form by circulating cells or become activated in blood plasma during clot formation or breakdown. It is attractive to hypothesize that these enzymes contribute to the proteolysis of VWF *in vivo* and have protective roles during attacks of TTP.

### Neutrophil Enzymes

Neutrophils release enzymes during their activation. These enzymes can cleave VWF close to or at the same position in the A2 domain as ADAMTS13, even in the presence of physiological inhibitors.<sup>42,43</sup> Neutrophil-release products are seen during attacks in various forms of thrombotic microangiopathy<sup>26</sup> and correlate well with disease activity.<sup>46</sup> It is attractive to hypothesize that neutrophil enzymes are released for the degradation of VWF in obstructive microthrombi. However, experimental evidence points out that neutrophil enzymes are not (continuously) involved in the degradation of VWF: neutrophil depletion in *Adamts13*<sup>-/-</sup> mice does not affect VWF multimer composition.<sup>47</sup> Taken together, the overall contribution of neutrophil activation to acute TTP episodes appears ambivalent: on the one hand, the release of NETs may provide a “second hit” that worsens microthrombosis, on the other hand, neutrophil enzymes may help ADAMTS13 degrade VWF during microangiopathy.

### Granzymes

Granules of cytotoxic lymphocytes contain a collection of secretable enzymes named granzymes. Through a precise mechanism that is dependent on formation of immunologic synapses and perforin, granzymes are transferred to compromised target cells to cleave caspases and initiate apoptosis. Granzymes are also produced by other cell types, such as mast cells, that do not have this sophisticated cell-targeting capacity. In this case, granzymes are secreted into the extracellular compartment after degranulation. One of these is granzyme B, which can cleave extracellular matrix proteins, as well as fibrinogen and VWF.<sup>48</sup> It cleaves VWF effectively when it is unfolded and destroys its platelet-binding capacity. In contrast, granzyme M cleaves VWF in its globular conformation. This does not affect its platelet-binding capacity but instead abrogates the capacity of VWF to carry coagulation factor VIII.<sup>49</sup> The cleavage sites for both granzymes in VWF differ from each other, explaining the selectivity of their actions on VWF. Granzymes have been implicated in a variety of inflammatory conditions,<sup>50</sup> where they may modulate the activities of VWF.<sup>44</sup>

### Thrombin

The coagulation cascade generates thrombin at sites of vessel injury. This versatile enzyme is a key regulator of both primary and secondary hemostasis. In a side-by-side comparison of multiple VWF-cleaving enzymes, thrombin has a modest capacity to cleave VWF in comparison to neutrophil elastase.<sup>42</sup> Nonetheless, these same studies revealed that purified thrombin removes adherent platelets from a collagen surface. This experiment suggests that thrombin modulates platelet adhesion to collagen by cleaving VWF, effectively overruling the well-known platelet-activating properties of thrombin.<sup>42</sup> This leads to the question: could thrombin influence the role of VWF in TTP?

Elevated thrombin-antithrombin (TAT) complexes, prothrombin fragment F1 + 2, and D-dimer levels are seen in ST-HUS. These markers indicate prothrombin activation that may be attributed to tissue factor expression.<sup>51,52</sup> The direct thrombin inhibitor hirudin prevents the lethal effects of Shiga toxin in a canine model for ST-HUS,<sup>53</sup> suggesting that the overall role of thrombin in HUS is pathologic. However, the role of thrombin in TTP is less clearly defined. Similar to HUS, case reports of acute TTP patients describe elevated plasma markers for thrombin activity.<sup>52,54–56</sup> Paradoxically, this does not appear to lead to fibrin-rich thrombi: microthrombi observed in TTP pathology are generally classified as platelet and VWF-rich, but poor in fibrin.<sup>25</sup> This remarkable absence of fibrin from these thrombi suggests that thrombin activity in TTP is limited (if present at all). However, these findings certainly do not rule out the possibility that thrombin directly modulates the thrombogenicity of VWF during TTP attacks.

### Plasmin

Plasmin is essential for fibrinolysis (i.e., the breakdown of fibrin polymers in blood clots). The liver produces plasminogen as precursor, which can be activated into plasmin by tissue-type plasminogen activator (tPA). This enzyme mainly

becomes effective as an activator of plasminogen in the presence of fibrin.<sup>57</sup> Both tPA and plasminogen itself bind to fibrin polymers in a lysine-dependent manner, facilitating the enzymatic crosstalk required for successful clot breakdown.

Through an alternative mechanism, plasminogen activation can be triggered on the surface of cells by urokinase-type plasminogen activator (uPA). This occurs in a receptor-mediated mechanism and does not require the presence of fibrin. The uPA receptor (uPAR) is present on endothelial cells, certain types of leukocytes (e.g., neutrophils), and platelets.<sup>58</sup> Furthermore, a large number of cellular receptors for plasminogen have been identified, which are thought to direct the multitude of diverse functions that plasmin is thought to execute.<sup>59</sup> Plasminogen activator inhibitor-1 (PAI-1) is an important regulator of plasminogen activation. This serine protease inhibitor can rapidly bind and inactivate both uPA and tPA, preventing plasmin formation. In similar manner, plasmin is inhibited by  $\alpha_2$ -antiplasmin. When plasmin is bound to fibrin or to a receptor at the cell surface, it is temporarily shielded from inactivation, allowing plasmin to act longer on the cell surface than it would in solution.

Besides its role in fibrinolysis, plasmin can also cleave VWF,<sup>42,60,61</sup> as well as ADAMTS13,<sup>62,63</sup> and is able to disaggregate platelet-VWF complexes in the absence of ADAMTS13 *in vitro*.<sup>42</sup>

### Plasmin in TTP: Saint or Criminal?

We recently explored the possibility that plasmin plays an important protective role in the pathogenesis of TTP by degradation of VWF in microthrombi.<sup>64</sup> We confirmed that plasmin can cleave VWF and is capable of breaking down platelet-VWF complexes *in vitro*. These findings support the proposed protective function of plasmin during attacks of thrombotic microangiopathy. However, our findings in patients with TTP are more complex to interpret. In our studies, we found that levels of plasmin- $\alpha_2$ -antiplasmin (PAP) complexes (representing recent plasminogen activation) are mainly elevated in plasma samples of patients during acute attacks of TTP. In detail, levels of PAP complexes correlate with the extent of thrombocytopenia, which serves as a marker for disease activity as low platelet counts are seen as a consequence of consumptive microthrombosis. This observation appears counterintuitive: if plasmin mediates degradation of microthrombi, why is plasminogen activation seen during active disease, exactly when microthrombi are not sufficiently cleared?

Acute TTP attacks are associated with increased tPA levels, as well as increased PAI-1 levels.<sup>65</sup> This could be the result of endothelial cell activation. Surprisingly, fibrin degradation product (FDP) and D-dimer levels remain normal, which indicates that fibrin degradation is low or absent during attacks. It is attractive to hypothesize that plasmin is formed by tPA on platelet-VWF complexes.<sup>66</sup> However, we found that these complexes do not stimulate tPA-mediated plasminogen activation,<sup>64</sup> leaving them intact in the combined presence of tPA and plasminogen. In contrast, we found that endothelial



cells can swiftly generate sufficient amounts of plasmin (via the urokinase system) to break down nearby platelet-VWF complexes under shear flow. Hence, we believe that this latter mode of plasminogen activation takes place during TTP attacks. This leads to the question: how is the urokinase system activated during TTP attacks? Plasminogen activation on the endothelium can be triggered by hypoxia<sup>67,68</sup> and takes place on the endothelium of ischemic tissue during thrombotic microangiopathy: postmortem kidney sections demonstrate that uPAR expression is highly increased in the kidney vasculature of TTP patients, compared with control subjects.<sup>69</sup> We therefore propose plasminogen activation in TTP is triggered only *after* microthrombotic vascular obstructions trigger endothelial cell activation (► Fig. 1). This plasmin subsequently degrades VWF aiming to eliminate these obstructions, but it may also cause collateral damage by degrading ADAMTS13 when its activity is improperly controlled.<sup>62</sup> This helps to explain why plasminogen activation will coincide with TTP attacks, but why is this enzyme is not always fully capable of averting disease?

Clinical evidence from patients provides an important lead: acute TTP episodes are accompanied by *decreased* uPA levels, indicating that this plasminogen activator is being consumed.<sup>65</sup> We hypothesize that in a situation where obstructive microthrombi are successfully cleared by plasmin, clinical symptoms will not become evident (i.e., the patient will stay in an apparent remission state). However, when the endogenous capacity for plasmin formation becomes exhausted (e.g., after prolonged formation of microthrombi), obstructions are no longer sufficiently cleared with pathologic results. This concept may extend beyond TTP: there is evidence to suggest that an imbalanced plasminogen activation system also has dangerous consequences for other forms of thrombotic microangiopathy. First, plasminogen activation is reported to take place in patients with ST-HUS.<sup>51</sup> Recent studies identified that genetic variations in the plasminogen gene that reduce its expression form an important independent genetic risk

factor for this disease,<sup>70</sup> indicating a protective function of plasmin against HUS. In conclusion, we (as well as others<sup>71</sup>) believe that plasmin can act as an enzymatic “last resort” for the removal of vascular obstructions by cleaving fibrin, VWF, or both.

### A Possible Therapeutic Opportunity: Thrombolysis for TTP Treatment

#### Bypassing Autoantibodies in TTP: A Clinical Challenge

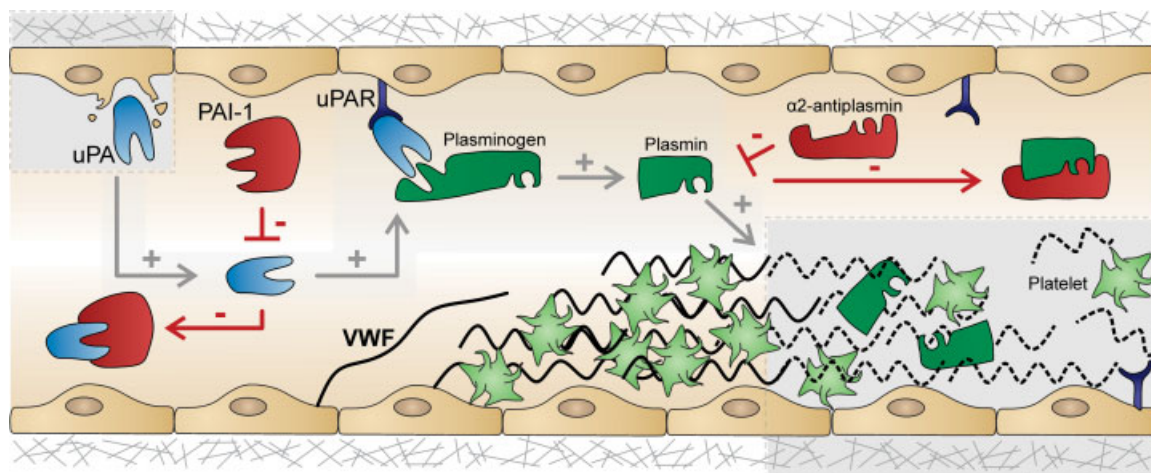
Autoantibodies against ADAMTS13 form the underlying cause for ADAMTS13 deficiency in most patients with TTP. These antibodies also complicate plasma transfusion therapy by neutralizing exogenous ADAMTS13. Based on our recent findings, we propose that endogenous plasmin acts as a natural “emergency replacement” for ADAMTS13. If this is the case, thrombolytic agents may have therapeutic value for patients with TTP. These drugs would help break down obstructive microthrombi but would not be neutralized by autoantibodies.

#### Proof-of-Concept Experiments

To explore the hypothesis that thrombolytic agents may be therapeutic in TTP, we administered a single dose of the direct-acting thrombolytic agent streptokinase to ADAMTS13-deficient mice that had been subjected to a model for TTP (via injection of a high dose of recombinant VWF).<sup>64</sup> We found that his treatment attenuated symptoms of TTP and corrected the thrombocytopenia.

#### Safety Aspects

Bleeding is the most well-known side effect of thrombolytic treatment. The risk of thrombolysis-related bleeding is strongly associated with anticoagulant therapy (that many of these patients use) and increases steeply with age.<sup>72</sup> In these patients, thrombocytopenia is a contraindication for the use of thrombolytic agents. Intuitively, it can be assumed that there will be a severe bleeding risk associated with the



**Fig. 1.** Model: Plasminogen activation on endothelial cells mediates degradation of platelet-VWF complexes. PAI-1, plasminogen activator inhibitor-1; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VWF, von Willebrand factor.

treatment of TTP patients with thrombolytic agents, especially as these patients are thrombocytopenic. This safety aspect is of critical importance, and should be completely and carefully investigated in a preclinical setting.

However, it is also very important to note that the main body of clinical experience on the relationship between thrombolysis and bleeding is based on the treatment of types of thrombosis that do clinically not resemble TTP at all. Actually, there is little to no clinical experience with thrombolytic agents in TTP. We would like to challenge the dogma that thrombolytic treatment of TTP will carry a high risk of bleeding; we believe that it will be possible to safely use thrombolytic agents for treatment of TTP.

- **Dosing:** It is logical to assume that the treatment regimen (dosage, length, and intensity) of thrombolytic therapy that will be required for TTP is the same as that is needed for other types of thrombosis. However, this may not be the case: we found that a single dose of the direct-acting plasminogen activator streptokinase was sufficient to attenuate symptoms of TTP in an ADAMTS13-deficient mouse model (at ~20% of the loading dose by plasma concentration that is given for pulmonary embolism [PE]).<sup>64</sup> Prolonged continuous infusion of thrombolysis was not required (this is indicated for treatment of PE or deep vein thrombosis [DVT]). We found no macroscopic evidence for bleeding or a perturbed secondary hemostasis after this treatment. Dosing studies in various preclinical models will be helpful to estimate how much plasminogen activation is actually needed for the management of TTP. This is important as the incidence of bleeding events is directly related to the intensity of thrombolytic therapy.<sup>72</sup> Only after the required dosage is established can any potential off-target effects of thrombolysis on primary and secondary hemostasis really be evaluated.
- **Molecular mechanisms:** microthrombi in TTP are VWF-rich but fibrin-poor. Thrombi in other forms of thrombosis generally contain fibrin, which is protected from degradation by plasmin through a variety of molecular mechanisms. As a result, thrombus breakdown by plasmin is relatively slow and their destruction requires a prolonged exposure to therapeutic plasminogen activators. This treatment can lead to a partial consumption of fibrinogen by plasmin. For example, treatment of ischemic stroke patients with tPA leads to a 26% decrease in fibrinogen levels. This decrease is associated with an increased risk on thrombolysis-related bleeding, especially in patients with low fibrinogen levels prior to treatment.<sup>73</sup> In comparison to “classic” breakdown of fibrin, plasmin-mediated destruction of platelet-VWF complexes occurs relatively rapid.<sup>68</sup> Furthermore, VWF is not protected from cleavage by dedicated molecular mechanisms (as far as we know). These differences may strongly influence the amount of plasmin that is required for TTP treatment, as well as the risk on off-target effects.
- **Patient characteristics:** Age is a strong risk factor for the development of cardiovascular events. Moreover, age is a

strong risk factor for thrombolysis-related bleeding in these patients. In contrast, TTP patients may develop attacks at much earlier times in life (with potential implications for bleeding risk). Furthermore, it is expected that TTP patients will not receive anticoagulant therapy (a strong risk factor for thrombolysis-related bleeding). Finally, thrombolysis-related bleeding generally occurs in larger vessels that were previously occluded (e.g., intracerebral hemorrhage follows after stroke). In contrast, patients with TTP experience thrombosis of the microvasculature, where blood flow, blood pressure, and vessel wall composition are strikingly different. Also, these determinants may influence the risk on thrombolysis-related bleeding.

- **Consumptive thrombocytopenia—prohemostatic effects of antihemostatic agents:** During thrombocytopenia in general, any treatment that further compromises hemostasis (either primary or secondary) can logically be expected to elicit bleeding problems. However, thrombocytopenia in TTP is the result of an ongoing consumptive reaction, in which platelets accumulate in microvessels and become unavailable for hemostasis. Any treatment that destroys microthrombi would “break this circle,” thereby attenuating thrombocytopenia and reducing bleeding risk. For example, it was recently reported that full in vivo neutralization of VWF with nanobody ALX-0681 did not induce bleeding in a primate model for acquired TTP during thrombocytopenia.<sup>13</sup> This can be attributed to restoration of platelet counts (prohemostatic), despite full blockade of VWF by ALX-0681 (a potent antihemostatic agent). A clinical study with ALX-0681 in patients with TTP has recently confirmed these findings.<sup>16</sup> In a similar manner, platelet counts normalized when we treated ADAMTS13-deficient mice in a mouse model for TTP with thrombolysis. This suggests that plasmin may exert a prohemostatic role in the consumptive thrombocytopenia that hallmarks TTP, and that this would eventually reduce the bleeding risk.

### The Optimal Thrombolytic Agent

We expect that not all types of thrombolytic agents will be equally effective for the treatment of TTP. Direct-acting plasminogen activators may be useful because plasmin has a direct binding affinity for unfolded VWF.<sup>64</sup> However, the immunogenic potential of these agents limits their attractiveness for repeated use in TTP patients (as they are of bacterial origin). The major clot-busting drug tPA can directly bind to fibrin, but it is unable to target platelet-VWF complexes for degradation in vitro.<sup>64</sup> In contrast, uPA-triggered plasminogen activation leads to rapid cleavage of VWF, but only in the presence of cell-surface expressed uPAR. Because uPA levels are reportedly reduced during TTP attacks,<sup>65</sup> we believe that reinforcement of the urokinase system provides a viable target for further investigation as a therapy. Clinical experience with uPA is very limited but appears positive: a case-report describes that uPA was repeatedly used successfully for the treatment of TTP.<sup>74</sup> Ultimately, it may be attractive to develop a new type

of thrombolytic agent (e.g., by development of an antibody fusion protein<sup>75</sup>) that directly targets VWF-rich microthrombi.

**Conclusion**

ADAMTS13 is a key regulator of VWF and its deficiency is strongly linked to TTP. However, the absence or presence of ADAMTS13 alone does not fully explain the clinical picture of this condition. This literature overview shows that auxiliary mechanisms are in place to keep VWF under control. We hypothesize that plasmin is an important endogenous regulator of the thrombogenicity of VWF and plays a role in the etiology of TTP. Plasminogen activation takes place on the endothelium near sites of vascular obstruction. We propose that stimulation or reinforcement of plasminogen activation (in conjunction with other treatments) has therapeutic value for the treatment of TTP.

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