

Deficiency of ADAMTS13 in Thrombotic Thrombocytopenic Purpura

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Abstract

Thrombotic thrombocytopenic purpura, characterized by the presence of systemic hyaline thrombi in the arterioles and capillaries, is a potentially fatal disease that responds to plasma infusion or exchange. Recent studies have demonstrated that a metalloprotease in the normal plasma cleaves endothelial von Willebrand factor to a series of multimers. A deficiency of the protease, due to autoimmune IgG inhibitors or genetic mutations, is detected in patients with thrombotic thrombocytopenic purpura. Positional cloning based on kindreds with a genetic deficiency of the protease and amino acid sequencing of the purified protein have identified the protease as a novel member of the ADAMTS (*a disintegrin and metalloprotease with thrombospondin type 1 repeat*) zinc metalloprotease family located on the long arm of chromosome 9. Mutations of the gene are detected in patients with the congenital form of thrombotic thrombocytopenic purpura. In the circulation, proteolysis of von Willebrand factor is critical in regulating vWF-platelet interaction. These advances in knowledge provide the basis for a rational approach to improving the diagnosis and treatment of thrombotic thrombocytopenic purpura.

Thrombotic thrombocytopenic purpura (TTP), first described by Moschowitz in 1924 [1], typically presents in previously healthy adolescents or adults with thrombocytopenia, hemolysis and fragmented red blood cells on the peripheral smears. The clinical manifestation of fever, neurological deficits, renal abnormalities, abdominal pain, elevated pancreatic enzymes, or cardiac arrhythmias is attributable to tissue ischemia or injury caused by thrombi in the arterioles and capillaries. Without treatment, the disease results in death, often within a few days, in greater than 90% of the cases [2]. When treated with plasma infusion or plasma exchange, 60~90% of the patients survive the acute episodes of TTP [3,4]. Although TTP is relatively uncommon, it shows a trend of increase in incidence [5]. The mysterious, abrupt development of platelet-rich thrombi in the arterioles and capillaries and the dramatic response to plasma infusion or plasma exchange are intriguing. Relapses occur in more than one third of the patients who achieve remission [6,7]. A subset of patients develops chronic TTP and requires long-term plasma exchanges. Splenectomy, vincristine, cyclophosphamide, azathioprine

and Staphylococcal protein A columns have been used empirically with success in some refractory cases.

The mechanism of thrombus formation in TTP has attracted great interests. Both endothelial injury and platelet aggregation induced by novel exogenous proteins have been implicated as the potential cause. However, the evidence supporting these hypotheses is preliminary and none provides an explanation for the occurrence of relapse and the efficacy of plasma therapy. Moake et al describe the presence of unusually large multimers of von Willebrand factor (vWF) in the plasma of patients with chronic relapsing TTP, suggesting that the homeostasis of vWF is abnormal in those patients [8]. Using electron microscopy and immunohistochemical techniques, Asada et al demonstrate that the thrombi in TTP consist mainly of vWF and platelets [9]. Flow cytometric analysis further reveals that the vWF on the surface of platelets is increased in the patients with TTP during the most thrombocytopenic phase [10]. These results suggest that the abnormal vWF-platelet binding occurs in TTP.

1. Proteolysis of von Willebrand Factor

VWF supports platelet adhesion and aggregation by binding to collagens and other components in the extracellular matrix and to glycoprotein receptors Ib/IX/V and alphaIIb beta3 on platelet surface. Levels of shear stress comparable to those encountered in the arterioles and capillaries promote vWF-mediated platelet deposition at sites of vessel injury. A deficiency in vWF activity results in a bleeding diathesis that may be fatal in severe cases.

VWF is secreted from endothelial cells as a very large ($>20 \times 10^6$ daltons) disulfide-bonded polymer of vWF dimers [11]. In the endoplasmic reticulum, VWF peptides are linked near the carboxyl termini to form a dimeric form of pro-vWF [12]. As the dimers are translocated to the Golgi complex and the storage granules, the prosequence is cleaved and disulfide bonds are formed near the amino termini, linking the dimers to polymers. In the circulation, a protease cleaves the endothelial vWF, generating a series of multimers. In some endothelial cell cultures, oligomers of pro-vWF are secreted constitutively [12]. These oligomers of pro-vWF are different from the multimers observed in normal plasma.

In the circulation, vWF is cleaved at the peptide bond of Tyr842-Met843. This cleavage generates a 140kd-140kd and a 176kd-176kd dimers [13,14]. Since vWF is not cleaved by the protease *in vitro*, the identity of the protease responsible for this cleavage and its role in hemostasis remained unknown until recently. The discovery that high levels of shear stress promote proteolysis of vWF leads to the detection of the protease activity in normal plasma [14,15]. Biochemical studies demonstrate that the protease, approximately 200 kd in size, is similar to but distinct from the family of matrix metalloprotease [14-17].

In studies using atomic force microscopy, Siedlecki et al report that the conformation of vWF is responsive to shear stress: it exists in a globular conformation under static conditions, and unfolds to an elongated, filamentous form upon exposure to levels of shear stress comparable to those encountered in the arterioles and capillaries [18]. Previously, studies using rotary-shadow electron microscopy also observed variation in the conformation of vWF [19]; presumably some vWF molecules underwent unfolding during the sample preparation that included a spraying step. These findings provide a physical basis for understanding why shear stress promotes proteolytic cleavage of von Willebrand factor: in its static conformation, the globular conformation of vWF makes its cleavage sites inaccessible to the protease; unfolding under high shear stress conditions exposes the cleavage sites, making the vWF molecule susceptible to the protease.

In contrast to normal vWF, which is susceptible to proteolysis only after exposure to high levels of shear stress, type 2A group 2 von Willebrand factor mutants are proteolyzed by the protease under static conditions

[17]. Mutations of the vWF polypeptide, most of which are located in the A2 domain, are believed to alter the conformation of the vWF polymer, making the type 2A vWF mutants susceptible to proteolysis under static conditions. Based on these findings, it is likely that proteolysis of type 2A vWF occurs continuously in the circulation, resulting in decreased large multimers. The large multimers are further decreased *in vitro* unless the protease activity is suppressed with EDTA [20,21]. These findings suggest that modulating the proteolysis of vWF may correct the bleeding diathesis in patients with type 2A von Willebrand disease.

What is the biologic function of vWF proteolysis? According to the current hypothesis, the large size makes the vWF molecule flexible, enabling it to unfold under conditions of high shear stress and expose multiple binding sites for its ligands. On the other hand, the unfolded forms, if allowed to accumulate, may increase the risk of vWF-platelet binding, platelet-platelet aggregation, and microvascular thrombosis. Multiple platelets are brought to proximity when they are bound to the same vWF molecule. This closeness may facilitate platelet-platelet interaction and aggregation. In this scheme, proteolysis of the unfolded forms of vWF by the plasma metalloprotease represents an anti-thrombotic mechanism that prevents vWF-platelet binding in the circulation. This model explains why a lack of this proteolytic process leads to the formation of vWF-platelet thrombi in patients with TTP [22,23]. Physical entrapment may account for the restriction of the thrombi to arterioles and capillaries. Alternatively but not exclusively, the high shear stress in this part of the circulation may promote vWF unfolding and facilitate vWF-platelet binding.

According to this scenario, unfolding by shear stress is essential for vWF to function under conditions of high shear stress. In support of this model, large vWF multimers, after a brief exposure to shear stress, are more active in supporting platelet aggregation. With small multimers, the capacity for supporting platelet aggregation is not increased after exposure to shear stress. Similarly, shear stress also increases the binding of large vWF multimers, but not the small forms, to immobilized collagen. These findings suggest that under static conditions, vWF exists in a conformation in which most binding sites are inaccessible. Shear stress increases platelet aggregation or collagen binding by exposing the binding sites on vWF. The responsiveness of the adhesive capacity to shear stress provides an explanation for two unique features of vWF activity: shear stress increases vWF-supported platelet aggregation by causing a conformational unfolding in vWF; and large multimers are more active than small forms in supporting platelet aggregation because they are more responsive to shear stress.

A dynamic process involving the balance among endothelial secretion, shear stress-dependent proteolytic cleavage and consumption in vWF-platelet binding determines the vWF multimeric size distribution in the circulation. This complex process explains why vWF

multimers undergo a biphasic change during the course of TTP. When a patient presents with acute TTP, the plasma metalloprotease activity is undetectable, the level of platelet-bound vWF is increased, and the large multimers are decreased. Presumably, in the absence of the protease activity, large vWF multimers unfold and bind to platelets, resulting in the increase of the platelet-bound vWF and the decrease of large multimers. Plasma exchange, by raising the protease activity, diminishes the vWF-platelet binding. As a result, the platelet count increases. Ultra large vWF multimers are detected at this stage because the protease level remains very low and vWF cleavage remains inadequate. Eventually, the protease activity rises to the normal range and the multimer pattern also normalizes. In patients with chronic relapsing TTP, ultra large multimers are detected because these patients are investigated before reaching the stage of severe thrombocytopenia. The subsequent course may evolve in either direction: during exacerbation of TTP, vWF-platelet binding is increased; after plasma therapy, a higher protease activity leads to increased cleavage of vWF. Thus, both processes, by different mechanisms, cause a decrease of the large multimers [24,25].

2. Deficient vWF Cleavage in Thrombotic Thrombocytopenic Purpura

Patients with idiopathic TTP, but not randomly selected hospitalized patients, or patients who have thrombocytopenia, hemolysis, or thrombosis from other causes, are found to be deficient in the protease activity that cleaves vWF [22,23]. IgG isolated from patients with acquired TTP inhibits the protease activity [22,23]. Thus, autoimmune reaction to the protease is responsible for the protease deficiency observed in acquired TTP. In analogy to the development of cold agglutinins in some individuals following infections of mycoplasma or Epstein-Barr virus, the inhibitors of the vWF-cleaving protease may be induced by exposure to infectious etiologies or medications such as ticlopidine [26] and are self-limited when the inciting agents are withdrawn. In some cases, the immune response persists, resulting in chronic disease. Such cases may benefit from measures that decrease antibody production. In patients with intermittent relapses, serial determinations of the protease activity and inhibitor titer are needed to determine if the immune reaction persists at low levels between relapses.

The presence of inhibitors of the vWF cleaving metalloprotease in TTP explains why plasma infusion or exchange is efficacious in the treatment of TTP: plasma infusion provides the missing protease; plasma exchange is more effective because the patients receive large volume of plasma without the risk of fluid overload. Removal of the inhibitors during the exchange may also contribute to the efficacy.

Similar to the experience in patients with inhibitors of factor VIII, in which replacement therapy is effective only in patients with inhibitor titers less than 5~10 U/ml, the inhibitor titers are very low in most patients

of TTP. In one study, only 2 of the 41 cases investigated had a titer greater than 5 U/ml and none had a titer >10 U/ml [27]. The low titers explain why plasma therapy is effective in most cases. The reason for the low inhibitor titers has not been determined. Presumably, the patients become ill from thrombotic complications soon after the protease level is suppressed to very low levels and therefore do not have sufficient time to accumulate high titers of inhibitors. Alternatively, the immunoglobulin may be directed against other antigens but exhibit cross-reactivity to the protease. Occasionally, the titer of inhibitors increases during the course, leading to treatment failure and a fatal outcome [28].

3. Genetic Deficiency of the vWF Cleaving Metalloprotease

A genetic deficiency of the protease causes thrombocytopenia and microangiopathic hemolysis soon after birth. Schulman described the first case of what is now believed to be a congenital form of TTP in a case whose thrombocytopenia and microangiopathic hemolysis responded to plasma infusion [29]. Upshaw noted in a similar case that plasma infusion corrected the shortened survival time of the red blood cells and postulated that the patient lacked a plasma factor that was essential for preventing destruction of the red blood cells and platelets in the normal circulation [30]. The presence of unusually large vWF multimers in the plasma further suggests that Schulman-Upshaw syndrome is a congenital form of TTP [31].

Genetic studies have been conducted in ten cases with features of Schulman-Upshaw syndrome [32]. These cases are characterized by the onset of disease soon after birth, either in the patients or their siblings. Anemia and severe jaundice requiring transfusion or whole blood exchange are common immediately after birth. Ultra large vWF multimers are present in each case. Analysis of these patients and their family members from 7 kindreds reveals that the patients have a protease level less than 10% of control; the parents are partially deficient (49-68%); and the other genetically linked members in the family are equally divided between the normal group (79-127%) and partially deficient group. The family members not genetically linked to the patients have normal protease levels, indicating that the deficiency of the protease is not caused by environmental factors.

Genome-wide linkage analysis performed on 4 kindreds localizes the defect to chromosome 9q34. Sequence analysis of genomic DNA in this region detects mutations in ADAMTS13, a novel gene of the ADAMTS zinc metalloprotease family [32]. Northern blotting reveals that liver is the main organ expressing ADAMTS13. In RT-PCR, the full-length mRNA and/or its alternatively spliced isoforms are expressed in the brain, placenta, ovaries, and other tissues. The functions of the isoforms are not known.

ADAMTS (*a disintegrin and metalloprotease with thrombospondin type 1 repeat*) is a recently recognized

family of zinc metalloprotease with a characteristic domain structure: a signal sequence, a propeptide sequence, a metalloprotease domain with zinc-binding motif (Hxx-GHxxGxxHD), a thrombospondin type 1 repeat, a cysteine-rich sequence, and a spacer sequence, which is followed by one or more thrombospondin repeats [33]. Among members of the ADAMTS family, ADAMTS13 is unique in that it has a very short propeptide sequence and a CUB domain, which may be critical for substrate recognition [34]. An RGD sequence, also found in ADAMTS2, is located after the first TSP1 repeat. Deficiency of ADAMTS1 is associated with developmental abnormalities and uretero-pelvic fibrosis. Mutations of ADAMTS2, which cleaves procollagen I, causes Ehler-Danlos type VIIC. ADAMTS3 cleaves procollagen II. ADAMTS4 and ADAMTS5/11 cleaves aggrecan, a proteoglycan in the cartilage, and are believed to be involved in the destruction of cartilage in patients with arthritis. The functions of other ADAMTS members remain unknown.

Twelve mutations have been detected in 14 of the 15 disease alleles: one splice, two frame-shift and 9 missense mutations. The missense mutations are found in the metalloprotease domain (3 mutations), the thrombospondin repeats no. 1, 3, 5, and 6 (4 mutations), the cysteine-rich region (1 mutation) and the CUB domain (1 mutation) [32]. These domains are likely to be essential for the integrity of proteolytic activity. No recurrent mutations have been detected, except in one pedigree, in which all three cases are homozygous for a single mutation. The parents of these three cases come from the same town and may have a common ancestry.

These genetic data confirm that a deficiency of ADAMTS13 causes TTP. In most genetic cases, the deficiency of ADAMTS13 causes a persistent disease with onset during the neonatal period, requiring periodic plasma infusion every 2-3 weeks to prevent severe

thrombocytopenia and other serious complications. Other cases require plasma infusion during periods of exacerbation, which is often precipitated by stressful conditions such as fever, infection, diarrhea, surgery, and pregnancy. The variability in clinical severity indicates that disease manifestation is modified by other genetic or environmental factors. In some cases, the distinction between congenital and acquired forms of TTP may not be clinically apparent and requires kinetic studies of the ADAMTS13 level following plasma infusion, systemic investigation of the family members or meticulous assays for the presence of inhibitors (Table 1). In congenital cases, the distribution of ADAMTS13 is essentially intravascular and its activity declines with an approximate half-life of 2 days. In the few cases of acquired TTP that have been investigated, no mutations in ADAMTS13 gene have been detected.

Three groups of investigators have purified the protease from normal plasma and mapped the partial amino acid sequence to the same ADAMTS13 gene [35-38]. Together, these results establish that ADAMTS13 is the plasma protease that cleaves vWF.

4. ADAMTS13 Activity in Other Microangiopathic Disorders

Hemolytic uremic syndrome (HUS), originally described as a separate entity because it characteristically develops in young children after a bout of hemorrhagic diarrhea and has a clinical course dominated by acute renal failure, is frequently applied broadly to disorders that have the common features of microangiopathic hemolysis, thrombocytopenia and renal failure. As a result, the distinction between TTP and HUS becomes arbitrary. To avoid the inclusion of patients with HUS or other microangiopathic disorders, our series of TTP cases exclude those patients that have plausible causes, diarrhea prodrome, prominent renal failure (maximal creatinine >353 mmol/L or requirement of dialysis), or an age less than 10 years. In this series, which now include more than 120 cases, all the patients have plasma ADAMTS13 activity less than 10% of normal control. Among 16 cases of typical hemolytic uremic syndrome following *E. coli* O157:H7 infection, the ADAMTS13 activity was found to be normal [39]. Analysis of serial samples obtained in a subset of these patients revealed that vWF multimer size decreased with the onset of HUS. The decrease of large multimers was associated with an evidence of increased proteolysis of vWF, presumably due to abnormal shear stress in the microcirculation. Histochemical studies of the thrombi in renal glomeruli detected the presence of fibrin but not vWF. These results confirm that TTP and *E. coli*-associated HUS are distinct in pathogenesis, although they overlap in the clinical manifestations. Other studies detect low ADAMTS13 activity in some cases of HUS [22,40,41]. Those studies might have included patients with TTP. As will be discussed later, variation of the assays may also contribute to the low ADAMTS13 levels reported by some studies.

Table 1.

Distinction between Congenital and Acquired TTP.

Type	Congenital acquired	
Age of onset	Early infancy*	Adolescent-adult
Adolescent-adult	Early childhood	
Family history	Yes, no	No, yes
Course	Chronic	Acute
Intermittent	Intermittent	
	Chronic	
ADAMTS13 < 10%	Yes	No
Inhibitors	No Yes**	
ADAMTS13 mutations	Yes	No***
ADAMTS13 response	Yes	No
to plasma (10-15 ml/kg)		

* The predominant presentation is in bold.

** Detection may require the isolation of the IgG molecules.

*** Need confirmation in large number of cases.

Microangiopathic hemolysis and thrombocytopenia occasionally develop in patients with metastatic cancers, bone marrow transplants, HIV infection, autoimmune collagen-vascular diseases, certain medications, disseminated intravascular coagulopathy, or pregnancy. The syndrome has been variably referred to as TTP, HUS, or TTP/HUS, without knowledge of the underlying mechanisms. The discovery of ADAMTS13 provides a laboratory assay for determining whether microvascular thrombosis involves ADAMTS13 deficiency in these disorders. The status of ADAMTS13 and vWF proteolysis has been investigated in a small number of cases. Decreased ADAMTS13 activity due to inhibitors of the protease is detected in patients with ticlopidine use [26] but not in the patients with HELLP syndrome [23], bone marrow transplants (42;43) or neoplastic disorders [44]. The glomerular thrombi in the thrombotic microangiopathy following bone marrow transplantation contain fibrin and vWF, indicating that it is different from TTP or *E.coli* O157:H7-associated HUS [43]. The negative results do not exclude the possibility that TTP with ADAMTS13 inhibitors may occasionally occur in patients with these disorders.

5. Implications in Diagnosis and Treatment

In patients who are presumed to have TTP, a normal ADAMTS13 activity has led to the discovery of other causes of thrombotic microangiopathy that require different treatments. Serial analysis of ADAMTS13 activity and its inhibitors in patients with TTP has provided new insights into why relapse occurs. In some patients, inhibitors persist at low levels, with flaring of the autoimmune response occurring at the time of relapse. The autoimmune nature of acquired TTP suggests that immunosuppressive therapy is a potentially effective treatment. Rituximab, a chimeric monoclonal antibody against CD20, has been used successfully in patients who fail conventional immunosuppressive therapies [45].

6. Assays of the ADAMTS13 Activity

After the initial studies demonstrating an association

between ADAMTS13 deficiency and TTP were published, several studies have reported conflicting results (Table 2) [40,41,46-48]. A review of the literature shows that the assays for determination of ADAMTS13 activity are different. Since vWF in its static form is not cleaved by ADAMTS13, assay of the proteolytic activity requires a step of substrate unfolding. Shear stress is the most physiologic approach to unfolding vWF. For practical reasons, the assays of ADAMTS13 activity use either urea or guanidine hydrochloride. In assays that use the method originally designed by Furlan et al [22], mixtures of vWF and test samples are dialyzed against 1.5 M urea, in which the cleavage of vWF proceeds slowly and requires an overnight incubation. It also requires the addition of barium chloride, whose potential contribution to assay variability has not been explored. Cleavage of vWF is measured by a decrease in the multimer size [22], a decrease in the vWF species that bind both monoclonal antibodies directed against the regions of vWF on each side of the cleavage site [49], or a decrease in collagen-binding activity [50]. Binding of vWF to collagen is notably unreliable because it is affected by plasma factors. In the assay used in our laboratory, the vWF substrate is treated with 1.5 M guanidine hydrochloride before being added to a test sample at a dilution of 1:10 [23]. The low final concentration of guanidine hydrochloride in the reaction mixtures exhibits does not affect the protease activity. Since zinc cation is part of the metalloprotease, the assay does not require additional metallic cations. Cleavage of vWF occurs immediately, reaching a maximum within 60 minutes. Proteolysis is measured by the increase of the 176kd-176kd dimer. A comparison of the values in normal individuals reveals that some assays produce wide normal ranges, detecting no or very low protease levels in some normal subjects and in patients without TTP. Only one assay has correctly identified the carriers of ADAMTS13 mutations, a critical step for the success of positional cloning [23]. A low ADAMTS13 level that is not associated with the presence of IgG inhibitors or ultra large vWF multimers should raise suspicion on the validity of the assay result.

7. Summary and Future Directions

Studies on how von Willebrand factor multimers are generated have led to new concepts on the regulation of vWF-platelet interaction and the identification and cloning of a novel metalloprotease ADAMTS13. Genetic mutations of ADAMTS13 result in congenital TTP, while autoimmune inhibitors of ADAMTS13 cause the acquired form of the disease. The discoveries have provided new insights into the role of vWF in the pathogenesis of TTP. The assay of ADAMTS13 activity is extremely useful in the management of patients with a suspected diagnosis of TTP. Unfortunately, reliable assays are not widely available. Plasma exchange, used in many patients with or without TTP, is associated with potentially serious complications. The future development of ADAMTS13 concentrates may eliminate

Table 2.
Potential Causes of Discrepant ADAMTS13 Results.

Findings	Causes
Deficient ADAMTS13 in normal subjects	Assay not accurate
patients without microangiopathy	Assay not accurate
patients with other microangiopathies	Incorrect diagnosis Assay not accurate
Normal ADAMTS13 in patients with TTP (uncommon)	Incorrect diagnosis

the need of plasma exchange in many patients.

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