I. Idiopathic Thrombotic Thrombocytopenia Purpura

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Clinical Presentations of TTP

Thrombotic thrombocytopenia purpura (TTP) is a severe microvascular occlusive "thrombotic microangiopathy" characterized by systemic platelet aggregation, organ ischemia, profound thrombocytopenia (with increased marrow megakaryocytes), and fragmentation of erythrocytes.[1] The RBC fragmentation occurs, presumably, as blood flows through turbulent areas of the microcirculation partially occluded by platelet aggregates. Schistocytes, or "split" red cells, appear on the peripheral blood smear (> 1% of total RBCs) as an indication of "microangiopathic hemolytic anemia." Serum levels of lactate dehydrogenase (LDH) are extremely elevated as a consequence of hemolysis and the leakage of LDH from ischemic or necrotic tissue cells.

The systemic platelet clumping in TTP is often associated with platelets below 20,000/μL. Occlusive ischemia of the brain or the gastrointestinal tract is common, and renal dysfunction may occur. A "pentad" of signs and symptoms was long associated with TTP: thrombocytopenia; microangiopathic hemolytic anemia; neurologic abnormalities; renal failure; and fever. In current clinical practice, thrombocytopenia, schistocytosis, and an impressively elevated serum LDH value are sufficient to suggest the diagnosis.[1] Clotting studies are usually normal.

A clinically similar thrombotic microangiopathy can occur weeks to months after exposure to mitomycin C; inhibitors of the Ca^{2+}-activated phosphatase, calcineurin (cyclosporine or tacrolimus [FK 506]); quinine; combinations of chemotherapeutic agents; total-body irradiation; or allogeneic bone marrow, kidney, liver, heart, or lung transplant.[1] The microvascular thrombi may be either predominantly renal or systemic in these heterogenous entities. Other thrombotic microangiopathies include the hemolytic-uremic syndrome (HUS), which can be acquired by ingestion of enterohemorrhagic bacteria that produce Shiga toxin (e.g., *Escherichia coli* of serotype 0157:H7).[1] Familial types of HUS are caused by a regulatory protein defect in the alternative complement pathway (plasma factor H or membrane-cofactor protein [CD46]) or defective intracellular cobalamin reduction/cofactor function. The discussion to follow is limited to TTP associated with deficient plasma von Willebrand factor (VWF)–cleaving metalloprotease (ADAMTS13) (**Table 1**).

Familial TTP is rare, usually (but not always) appears initially in infancy or childhood, and may recur as "chronic relapsing TTP" episodes at about 3-week intervals.[1-3] Acquired idiopathic ("out-of-the-blue") TTP has become a commonly recognized disorder that occurs in adults and older children[1,2] and, following successful treatment, recurs at irregular intervals in 11%–36% of patients. A small fraction of patients treated for arterial thrombosis with the platelet adenosine diphosphate receptor-inhibiting thienopyridine drugs, ticlopidine (Ticlid) or clopidogrel (Plavix) develop TTP within a few weeks after the initiation or therapy.[1] TTP occurs occasionally late in pregnancy or immediately after delivery.

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Table 1. ADAMTS13 deficiency and thrombotic thrombocytopenia purpura (TTP).

ADAMTS13 Plasma Activity Absent*	Clinical Presentation
ADAMTS13 mutations	Familial TTP; chronic relapsing TTP
Disease presentation in infancy/childhood	
Disease presentation delayed	
Autoantibodies against ADAMTS13	Acquired idiopathic TTP
Transient	Single episode TTP
Recurrent	Recurrent (intermittent) TTP
Thienopyridine-associated	Ticlopidine/clopidogrel-TTP
ADAMTS13 transient production or survival (?) defect	Acquired idiopathic TTP (?)
Pregnancy+	Pregnancy-associated TTP

* < 5% of normal
+ Autoantibodies may also be present

The ADAMTS13-Deficient Types of TTP

In 1982, "unusually large" (UL) VWF multimers found in plasma samples taken repeatedly from 4 patients with chronic relapsing TTP were proposed as the systemic agglutinating agents.[2] The patients described were believed to have defective ULVWF multimeric "processing."[2] Convincing evidence for ULVWF multimers

as the cause of platelet clumping in TTP has accumulated in the subsequent 22 years.[1]

Brief comments on VWF biochemistry and physiology are included here for orientation. Monomers of VWF (280,000 daltons) are linked by disulfide bonds into multimers with varying molecular masses that range into the millions of daltons. Multimers of VWF are constructed within megakaryocytes and endothelial cells, and stored within platelet α-granules and endothelial cell Weibel-Palade bodies. Most plasma VWF multimers are derived from endothelial cells. Both endothelial cells and platelets produce VWF multimers larger than the multimers in normal plasma. These ULVWF multimers bind more efficiently than the largest plasma VWF multimers to the glycoprotein (GP) Ibα components of platelet GPIb-IX-V receptors.[4,5] The initial attachment of ULVWF multimers to GPIbα receptors, and subsequently to activated platelet GPIIb-IIIa complexes, induces platelet adhesion and aggregation in vitro in the presence of elevated levels of fluid shear stress.[4] After retrograde secretion by endothelial cells, ULVWF multimers become entangled in subendothelial collagen, thereby maximizing the VWF-mediated adhesion of blood platelets to any subendothelium exposed by vascular damage and endothelial cell desquamation. An efficient "processing activity"[2,6] in normal plasma prevents the highly adhesive ULVWF multimers that are also secreted antegrade into the vessel lumen from persisting in the bloodstream.

The ULVWF "processing activity" is now known to be a specific VWF-cleaving metalloprotease present in normal plasma.[3,7,8] The enzyme degrades ULVWF multimers by cleaving 842Tyr-843Met peptide bonds in susceptible A2 domains of VWF monomeric subunits. The VWF-cleaving metalloprotease is number 13 in a family of 19 distinct ADAMTS-type enzymes identified to date. The ADAMTS enzymes are numbered 1–20, but ADAMTS5 and ADAMTS11 have been found to be identical. ADAMTS13 is *a* *d*isintegrin *a*nd *m*etalloprotease with eight *t*hrombo*s*pondin-1-like domains composed (**Figure 1**) of an amino-terminal reprolysin-type metalloprotease domain followed by a disintegrin domain; a thrombospondin-1-like domain; a cysteine-rich domain containing an arginine-glycine-aspartate (RGD) sequence and an adjacent spacer portion; 7 additional thrombospondin-1-like domains; and 2 similar CUB domains at the carboxyl-terminal end of the molecule. CUB domains, found only in ADAMTS13 among the ADAMTS enzymes, contain peptide sequences present in *C*omplement subcomponents C1r/C1s; embryonic sea *U*rchin protein egf; and *B*one morphogenic protein-1. ADAMTS13 is a Zn^{2+}- and Ca^{2+}-requiring 190,000 dalton glycosylated protein that is encoded on chromosome 9q34 and produced predominantly in the liver. ADAMTS13 activity is inhibited in vitro by ethylenediaminetetraacetic acid (EDTA) and, therefore, functional assays of the enzyme are usually performed using plasma anticoagulated with citrate.[3,7-11] Plasma anticoagulated with heparin, D-phenylalanylprolylarginyl chloromethyl ketone (PPACK), hirudin and other direct thrombin inhibitors, or even serum, may also be satisfactory for testing.

ULVWF multimers are cleaved by ADAMTS13 as they are secreted in long "strings" from stimulated endothelial cells[12,13] (**Figure 2A**). The ULVWF multimeric strings may be anchored in the endothelial cell membrane to P-selectin molecules that are secreted concurrently with the ULVWF multimers from Weibel-Palade bodies.[14] Endothelial cells are stimulated to secrete ULVWF multimers by histamine, Shiga toxin, tumor necrosis factor-alpha (TNFα), interleukin (IL)-8 and IL-6 in complex with IL-6 receptor.[15] CUB domains, as well as one or more of the thrombospondin-1-like domains, may be involved in binding the ADAMTS13 to ULVWF multimers as they are secreted by the endothelial cells. Specifically, ADAMTS13 enzymes may attach under flowing conditions to accessible A3 domains in the monomeric subunits of ULVWF multimers,[13] and then cleave Tyr 842-843 Met peptide bonds in adjacent A2 domains (**Figure 2B**). Partial unfolding of emerging ULVWF multimers by fluid shear stress may increase the efficiency of ADAMTS13 attachment to, or cleavage of, ULVWF multimers.[12]

Failure to degrade ULVWF multimers has long been suspected to cause the familial and acquired idiopathic types of

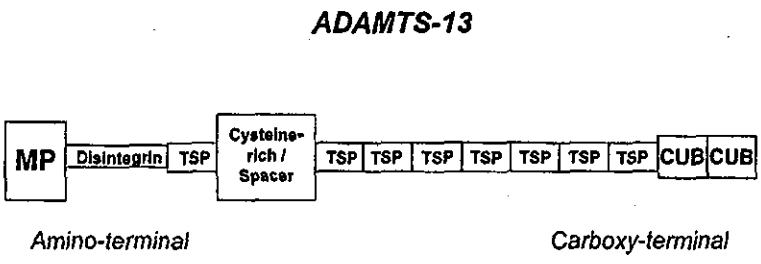
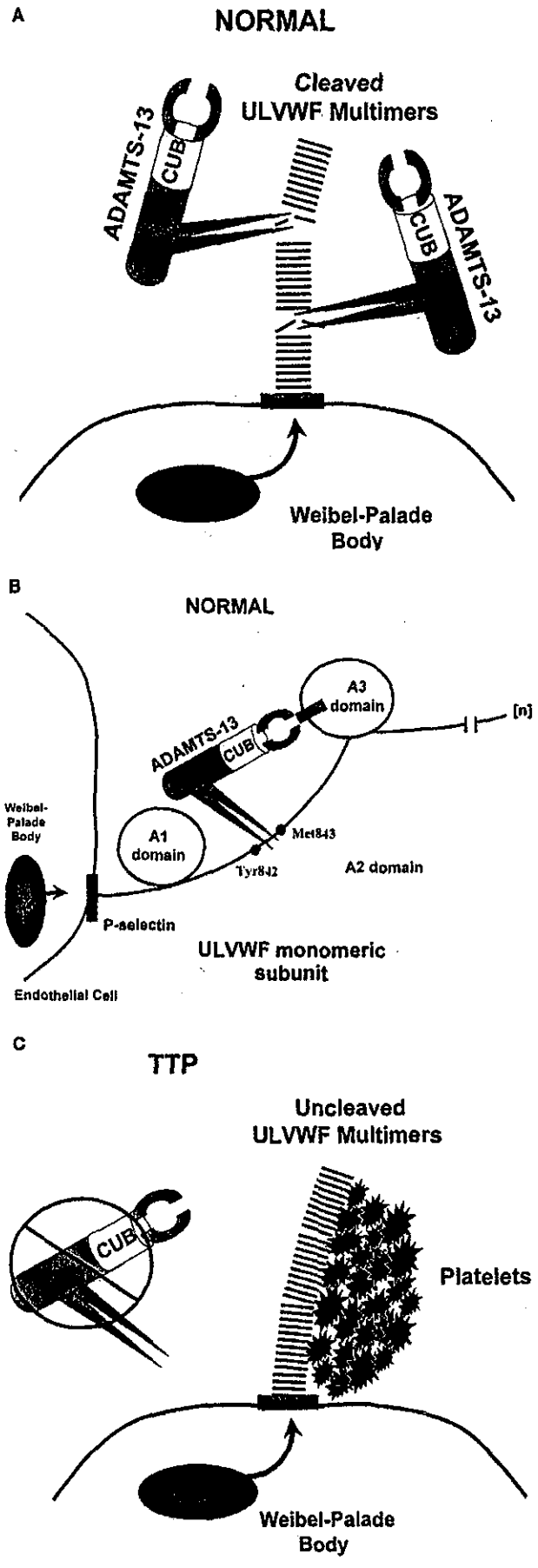


Figure 1. Domain structure of the plasma VWF-cleaving metalloprotease, ADAMTS13.

Abbreviations: MP, metalloprotease (proteolytic) domain; TSP, thrombospondin-1-like domain (a total of 8 are present); CUB, two similar domains containing peptide sequences found in the *c*omplement components, C1r/C1s, a sea *u*rchin protein, and a *b*one morphogenic protein.



TTP or predispose an individual to these disorders[1,2] (**Figure 2C**). Critical experiments verifying this concept were reported in 1997–1998. In 1997, Furlan et al[3] described 4 patients with chronic relapsing TTP who had a deficiency of VWF-cleaving protease activity in plasma. Because no inhibitor of the enzyme was detected, the deficiency was ascribed to an abnormality in the production, survival or function of the protease. The following year, elegant papers by Furlan et al[7] and Tsai and Lian[8] elucidated the pathogenesis of the more common acquired idiopathic type of TTP. The acquired idiopathic patients had little if any plasma VWF-cleaving protease activity during acute episodes; however, the activity returned to normal upon recovery. An IgG autoantibody produced transiently against the enzyme accounted for the lack of protease activity during the acquired idiopathic TTP episodes.[8]

Patients with familial, chronic relapsing TTP almost always have ULVWF multimers in their plasma[1,2] because they chronically have less than about 5% of normal plasma ADAMTS13—unless they have recently received plasma infusions. ULVWF multimers are also detectable in some patient plasma samples during acute episodes of acquired idiopathic TTP, but not after recovery,[1] due to transient inhibition of ADAMTS13 to less than about 5% of normal only during acute TTP episodes.[1,3,7,8,16] Severe deficiency of ADAMTS13 activity in TTP patient plasma often correlates with a failure to cleave ULVWF multimers as they emerge in

Figure 2. ADAMTS13 activity in normal and thrombotic thrombocytopenia purpura (TTP) plasma.

(A) In normal individuals, ADAMTS13 enzyme molecules from the plasma attach to, and then cleave, unusually large von Willebrand factor (ULVWF) multimers that are secreted in long "strings" from stimulated endothelial cells.

(B) The ULVWF multimeric strings may be anchored in the endothelial cell membrane to P-selectin molecules that are secreted concurrently with the ULVWF multimers from Weibel-Palade bodies. P-selectin molecules are retained in the endothelial cell membrane by a transmembrane portion. Each ADAMTS13 molecule may dock via one or both of its C-terminal CUB domains, possibly along with one or more thrombospondin-1-like domains, to exposed A3 domains in ULVWF monomeric subunits. The attached ADAMTS13 molecules then cleave Tyr 842-843 Met peptide bonds in the adjacent A2 domains of ULVWF monomeric subunits. The smaller VWF forms that circulate after cleavage do not induce the adhesion and aggregation of platelets during normal blood flow.

(C) Absent or severely reduced activity of ADAMTS13 in patients with TTP prevents the timely cleavage of ULVWF multimers secreted by endothelial cells. Uncleaved ULVWF multimers induce the adhesion and subsequent aggregation of platelets in flowing blood. Congenital deficiencies of ADAMTS13 activity caused by gene mutations or acquired defects of ADAMTS13 caused by autoantibodies result in TTP.

long strings from the surface of stimulated endothelial cells[12] (**Figure 2C**).

ULVWF multimeric strings are anchored to the endothelial cells[12,13] via P-selectin molecules, which have transmembrane domains and are secreted along with ULVWF multimers from Weibel-Palade bodies.[14] P-selectin molecules are predominantly retained in the endothelial cell membrane. Passing platelets adhere via their GPIbα receptors to the long ULVWF multimeric strings anchored to P-selectin.[12] (Platelets do not adhere to the smaller VWF forms that circulate after cleavage of ULVWF multimers, perhaps because the binding site for platelet GPIbα in VWF A1 domains is not exposed on smaller VWF forms.[5]) Many additional platelets subsequently aggregate under flowing conditions, probably via their activated IIb-IIIa complexes, onto the ULVWF multimeric strings to form large, potentially occlusive, platelet thrombi.

ULVWF multimeric strings are capable of detaching from endothelial cells in the absence of ADAMTS-13 activity, the presence of fluid shear stress, and the increasing torque generated by ULVWF-platelet adherence.[12,13] The detached ULVWF-platelet strings may "embolize" to microvessels downstream and contribute to organ ischemia. The formation of ULVWF-platelet thrombi and emboli may account for the detection of ULVWF multimers in the plasma of familial TTP patients chronically and of acquired idiopathic TTP patients during acute episodes.[1,2,6] Increased VWF antigen has been found by flow cytometry on platelets during episodes of familial or acquired TTP, and abundant VWF antigen (but not fibrinogen) has been observed by immunohistochemistry on platelet occlusive lesions in TTP.[1]

The absent or severely reduced plasma ADAMTS13 activity in familial TTP[1,3,7] is usually a consequence of homozygous (or double heterozygous) mutations in both of the ADAMTS13 alleles located at chromosome 9q34.[10] Mutations in familial TTP have been detected all along the gene, in regions encoding different domains.[10] In severe familial deficiency of ADAMTS13 activity, episodes of TTP usually commence in infancy or childhood (**Table 1**). In others, however, overt TTP episodes do not develop for years (e.g., during a first pregnancy), if ever. These latter observations suggest that (1) in vivo ADAMTS13 activity on ULVWF multimers emerging from stimulated endothelial cells may exceed the estimates of plasma enzyme activity using in vitro non-physiologic assays, and/or (2) that accentuated secretion of ULVWF multimers by endothelial cells induced by estrogen or pro-inflammatory cytokines[15] is required to provoke TTP episodes in some patients with severe plasma ADAMTS13 deficiency.

Many patients with acquired idiopathic TTP also usually have absent or severely reduced plasma ADAMTS13 activity only during an initial episode or later recurrence[1,7,8] (**Table 1**). IgG autoantibodies that inhibit plasma ADAMTS13 activity during acquired episodes can be detected using the nonphysiologic techniques currently available in 44%–94% of patients,[1,7,8] suggesting the presence of a transient, or intermittently recurrent, defect of immune regulation. Antibodies that inhibit plasma ADAMTS13 have also been demonstrated in a few patients with ticlopidine- or clopidogrel-associated TTP.[1] It is not known if there is a transient, severe defect of metalloprotease production or survival in patients with acquired idiopathic TTP who do not have detectable autoantibodies against ADAMTS13, or if the results are explained by the limited test sensitivity.

In one recent study[17] of ADAMTS-13 autoantibodies in 25 acquired TTP patients, the epitope targets *always included the cysteine-rich/spacer domain* (100% of autoantibodies), and were *exclusively* directed against the cysteine-rich/spacer domain in 3 of the 25 autoantibodies. The other 22 autoantibodies reacted with the cysteine-rich/spacer domain plus either the CUB domains (64%), the metalloprotease/disintegrin-like/1st thrombospondin-1-like domain combination (56%), or the 2nd–8th thrombospondin-1-like domain combination (28%). The propeptide region was also identified by 20% of autoantibodies,[17] indicating that propeptide removal is not required for secretion of active ADAMTS13. The recent demonstration of acquired idiopathic TTP caused by IgG autoantibodies against ADAMTS13 in identical twin sisters emphasizes that production of these autoantibodies is genetically determined.[18]

Plasma ADAMTS13 activity in healthy adults ranges from about 50% to 178%. Activity is often reduced below normal in liver disease, disseminated malignancies, chronic metabolic and inflammatory conditions, pregnancy, and newborns.[19] With the exception of those peri-partum women who develop overt TTP, the ADAMTS13 activity in these conditions is not reduced to the extremely low values (< 5% of normal) found in most patients with familial or acquired idiopathic TTP.

Neither bone marrow transplantation-associated thrombotic microangiopathy nor "classical" HUS is usually associated with absent or severely reduced plasma ADAMTS13 activity.[1,7] The explanation for VWF abnormalities in the plasma of a few chemotherapy/transplant-associated thrombotic microangiopathy patients is not known.

Therapy

In 1977, Byrnes and Khurana[20] reported that relapses of TTP could be prevented or reversed by the infusion

of only a few units of fresh-frozen plasma or its cryoprecipitate-poor fraction (cryosupernatant), without concurrent plasmapheresis. It was shown in 1985 that the processing of ULVWF multimers was restored in patients with familial, chronic relapsing TTP by transfusing fresh-frozen plasma or cryosupernatant.[6] Tsai and Lian[8] and Furlan et al[7] demonstrated in 1998 that these plasma products, as well as solvent/detergent-treated plasma,[1] contain active ADAMTS13.

The infusion about every 3 weeks of normal ADAMTS13 into familial TTP patients producing defective ADAMTS13 molecules[3,10] is sufficient to prevent TTP episodes. The plasma $t_{1/2}$ of infused ADAMTS13 activity is about 2 days, so perhaps ADAMTS13 molecules dock and cleave one secreted ULVWF multimeric string after another over a longer time period.[1,12,13]

The sequence of ADAMTS13 has been determined, and it has been partially purified[1,9] and produced in recombinant active form[21] for ultimate therapeutic use. Because plasma ADAMTS13 of only about 5% is often sufficient to prevent or truncate TTP episodes,[1,16] gene therapy may eventually extend remissions in familial, chronic relapsing TTP.

Adults and older children with acquired idiopathic TTP episodes associated with ADAMTS13 deficiency require daily plasma exchange. This combines plasmapheresis (which may remove circulating ULVWF multimer-platelet strings, agents that stimulate endothelial cells to secrete ULVWF multimers, and autoantibodies against ADAMTS13) and the infusion of fresh-frozen plasma or cryosupernatant (containing uninhibited ADAMTS13). Both solvent-detergent–treated plasma[1,8] and methylene blue/light-treated plasma (for inactivation of lipid envelope viruses) also contain active ADAMTS13; however, protein S activity is below normal in solvent-detergent plasma.

Plasma exchange allows about 80%–90% of acquired TTP patients to survive an episode, usually without persistent overt organ damage. Lower titers of ADAMTS13 autoantibodies are associated with better responses to plasma exchange procedures. Production of ADAMTS13 autoantibodies may be suppressed by high-dose glucocorticoids, 4–8 weekly doses of rituximab (monoclonal antibody against CD20 on B-lymphocytes) or removal of autoantibody-producing cells by splenectomy.

II. New ADAMTS13 Assays and Clinical Applications

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Thrombotic thrombocytopenic purpura is typically characterized by thrombocytopenia and microangiopathic hemolytic anemia, with variable degrees of renal failure, neurological dysfunction, and fever. In most cases the underlying mechanism of idiopathic TTP is explained by the accumulation of ULVWF multimers in the plasma, leading to the formation of platelet thrombi in microvessels. In turn, the accumulation of ULVWF multimers is caused by congenital or acquired deficiency of a metalloprotease, ADAMTS13. By 2001, this enzyme had been purified and cloned by several groups.[1] Since then, our understanding of TTP and ADAMTS13 has greatly increased.

von Willebrand factor and ADAMTS13

VWF is synthesized primarily by vascular endothelial cells. In the endoplasmic reticulum, VWF dimer formation occurs through disulfide bond formation near the C-termini. The pro-VWF dimers transit to the Golgi apparatus, where the pro-sequence is cleaved and multimers form by N-terminal disulfide bond formation. ULVWF multimers, disulfide-bonded at both N- and C-terminal domains, are formed in their mature form and stored in Weibel-Palade bodies or secreted into the plasma. ULVWF multimers are highly active in collagen binding and platelet aggregation. Under normal physiological conditions, ULVWF multimers depolymerize into smaller multimers ranging in size from 500 to 20,000 kDa. Depolymerization is catalyzed by a plasma metalloprotease, ADAMTS13, which specifically cleaves the peptide bond between Y1605 and M1606 in the A2 domain of VWF. Functional deficiency of ADAMTS13, caused by genetic mutation, inhibitory autoantibodies, or other etiologies, leads to the accumulation of ULVWF multimers in plasma. These multimers promote microvascular thrombi of platelets that result in platelet consumption and hemolysis. Once microvascular thrombi are formed in the brain or kidney, patients may suffer neurological dysfunction or renal failure.

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ADAMTS13 has a multidomain structure like other ADAMTS proteases and its characteristics have been described in Section I. The ADAMTS13 precursor consists of 1427 amino acids and contains a signal peptide, a short propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type I repeat (TSP1), a Cys-rich domain, a spacer domain, 7 additional TSP1 repeats, and 2 CUB domains.[1] The metalloprotease domain of ADAMTS13 is necessary but not sufficient for VWF cleavage, which appears to require other domains as well. A mutant with a common single nucleotide polymorphism leading to the P475S substitution in the Cys-rich domain, found in Japanese populations with an allelic frequency of about 5%, showed reduced VWF cleavage, indicating a significant role for the Cys-rich domain.[2] The analysis of sequentially truncated C-terminal mutants suggests that both the Cys-rich and spacer domains are indispensable for VWF-cleaving activity.[3,4] In addition, epitope analysis of ADAMTS13 autoantibodies in acquired TTP has shown that the Cys-rich and spacer domains usually are involved in antibody reactivity.[4,5] Taken together, the Cys-rich and spacer domains seem to be essential for the VWF-cleaving activity of ADAMTS13. Although removal of the propeptide sequence in ADAM family members is usually essential for their enzymatic activity, pro-ADAMTS13 exhibited normal proteolytic activity toward VWF, indicating that propeptide removal is not required for the activity of ADAMTS13.[6]

Assay Methods for ADAMTS13

As of 2002, five main assays for ADAMTS13 activity were in use to study ADAMTS13 activity in thrombotic microangiopathies and other pathophysiological conditions. However, more accurate and simpler methods were needed. This year, we developed a more convenient ADAMTS13 substrate based on the amino acid sequence of the A2 domain of VWF. In this section, I will describe conventional assay methods using multimeric VWF and then introduce our novel method, which uses a recombinant monomeric protein as the substrate. The assay methods for ADAMTS13 are summarized in **Table 2**.

The first assay method was developed by Furlan et al.[7] The substrate for this method was protease-free plasma VWF. The plasma samples are first diluted, then activated by barium chloride, mixed with substrate and dialyzed overnight against low ionic strength buffer, pH 8.0, containing 1.5 M urea. The reaction products were separated by sodium dodecyl sulfate (SDS)–1.4% agarose gel electrophoresis followed by immunoblotting. The resolution of the ladders of the degraded VWF multimers was excellent and reproducible, but the method required several days to complete.

Table 2. A comparison of ADAMTS13 assay methods.

Authors	Substrate	Denaturant	Incubation Time	Detection Method	Indication of VWF Proteolysis	Ref.
Furlan et al	purified VWF	1.5 mol/L urea	overnight	SDS-agarose gel electrophoresis, immunoblotting	decreased multimer size	7
Tsai	purified VWF	0.15 mol/L guanidine HCl	1 hour	SDS-polyacrylamide gel electrophoresis, immunoblotting	generation of dimer of 176 kDa fragment	8
Obert et al	recombinant VWF	1.5 mol/L urea	overnight	two-site immuno-radiometric assay	decreased VWF antigen	9
Gerritsen et al	VWF in EDTA-treated and dialyzed plasma	1.5 mol/L urea	2 hour	residual collagen binding	decreased collagen binding	10
Böhm et al	purified VWF	1.5 mol/L urea	overnight	residual ristocetin cofactor activity	decrease of ristocetin induced platelet aggregation	11
Kokame et al	recombinant VWF fragment, VWF73	none	20–60 min	SDS-polyacrylamide gel electrophoresis, immunoblotting	generation of proteolytic fragment	13
Whitelock et al	VWF A2 domain	none	2 hour	enzyme linked immunosorbent assay	decrease of intact A2 domain	14
Zhou and Tsai	recombinant VWF fragment, VWF73	none	3 hour	enzyme immunoassay	decrease of VWF73	16

Abbreviations: VWF, von Willebrand factor

The second method was reported by Tsai.[8] Plasma samples were incubated with guanidine HCl-treated protease-free VWF for one hour. Next, the products were separated by SDS–polyacrylamide gel electrophoresis, followed by immunoblotting for dimers of C-terminal 176-kDa fragments of VWF. An advantage of this method is that dimer formation directly reflects the cleavage of the scissile bonds.

Obert et al reported an elegant and high-throughput method,[9] in which the plasma samples were incubated with recombinant VWF overnight and the degraded VWF fragments were detected by two-site immunoradiometric assay in microtiter plates. This test can be performed in the setting of a hospital laboratory and the cumbersome immunoblot technique is not required. In 102 healthy volunteers, the range (± 2 SD) of ADAMTS13 activity was 50%–178%, where the mean was 114%.

Gerritsen et al reported a functional assay based on the preferential binding of high molecular weight forms of VWF to collagen.[10] Plasma that was treated with EDTA and dialyzed against the buffer was used as substrate. Since EDTA is a chelator of divalent cations, EDTA treatment of plasma abolishes the VWF-cleaving activity. Depolymerized forms of VWF showed impaired binding to microtiter plates coated with collagen type III, and collagen-bound VWF was quantified using a specific anti-VWF antibody. In 177 healthy volunteers, ADAMTS13 activity was widely distributed, ranging from 40% to 170%.

Böhm et al used the ristocetin-induced platelet aggregation activity to quantitate the residual VWF multimers for their ADAMTS13 assay.[11] This assay is based on the positive correlation between VWF multimer size and ristocetin cofactor activity of platelet aggregation. The method requires no special laboratory equipment or expertise. In 80 healthy volunteers, the ADAMTS13 activity ranged from 52% to 134%.

To evaluate the various assays for ADAMTS13 activity, a pilot multicenter comparison of the four different assays—the quantitative immunoblotting of VWF (Furlan's method), the two-site immunoradiometric assay (Obert's method), the residual collagen binding assay (Gerritsen's method), and the residual ristocetin cofactor assay (Böhm's method)—has been performed.[12] The test consisted of 30 plasma samples from patients with hereditary and acquired TTP and other conditions associated with ADAMTS13 levels ranging from < 3% to > 100%. All assays identified plasma with severe ADAMTS13 deficiency (< 5%), notwithstanding some exceptions observed using the collagen-binding assay, suggesting that all were useful to screen for suspected TTP. For samples with normal to moderately reduced activity, results were less concordant.

Specific Minimum Recombinant Substrate for ADAMTS13 Activity

Coagulation proteases such as thrombin or factor Xa that are classified as "serine proteases" have a restricted substrate specificity recognizing short amino acid sequences on the N-terminal side of the scissile bonds. Based on these sequences, peptidyl substrates specific for many of these proteases were developed and utilized for activity assays. ADAMTS13 is a metalloprotease rather than a serine protease, and it cleaves a peptide bond between Y1605 and M1606 in the A2 domain of VWF. The development of peptidyl substrates for ADAMTS13 has been attempted but not achieved, suggesting that cleavage depends not only on specific residues in the close vicinity of the scissile bond, but also on more remote sequences in VWF.

We utilized a recombinant protein approach to develop an ADAMTS13 substrate, using the amino acid sequence of the A2 domain of VWF that contains the scissile bond.[13] To identify the minimal region of VWF required for cleavage by ADAMTS13, we prepared 5 recombinant proteins, each containing a region of the A2 domain of VWF (**Figure 3A**). Two criteria were set: (1) the region must contain the cleavage site by ADAMTS13, and (2) it must not contain Cys residues that often interfere with the proper folding of recombinant proteins. The longest region that meets the criteria was D1459 to R1668 of VWF. For purification and detection, the protein was flanked with N-terminal glutathione-*S*-transferase (GST) and C-terminal 6xHis (H) tags. This was designated GST-D1459R1668-H (substrate A). We also prepared 4 shorter recombinant proteins (substrates B-E), as shown in **Figure 3A**.

Five recombinant proteins were expressed in *E coli* and purified by affinity chromatography. The purified proteins were incubated with plasma for 1 hour at 37°C and the reaction mixture was electrophoresed on an SDS-polyacrylamide gel. The cleaved products were then detected by Western blot using anti-GST antibodies. Four out of 5 recombinant proteins were successfully cleaved with plasma ADAMTS13; however, substrate E (the shortest) was not (**Figure 3B**). Therefore, the minimum substrate for ADAMTS13 was substrate D, which contains the region from D1596 to R1668 (73 amino acid residues). We designated substrates D and E as GST-VWF73-H and GST-VWF64-H, respectively. The amino acid sequence of substrate D is shown in **Figure 3C**. As described, substrate E was resistant to cleavage. Therefore, the region of nine amino acid residues (EAPDLVLQR) underlined in **Figure 3C** was likely essential for cleavage by ADAMTS13. Mass spec-

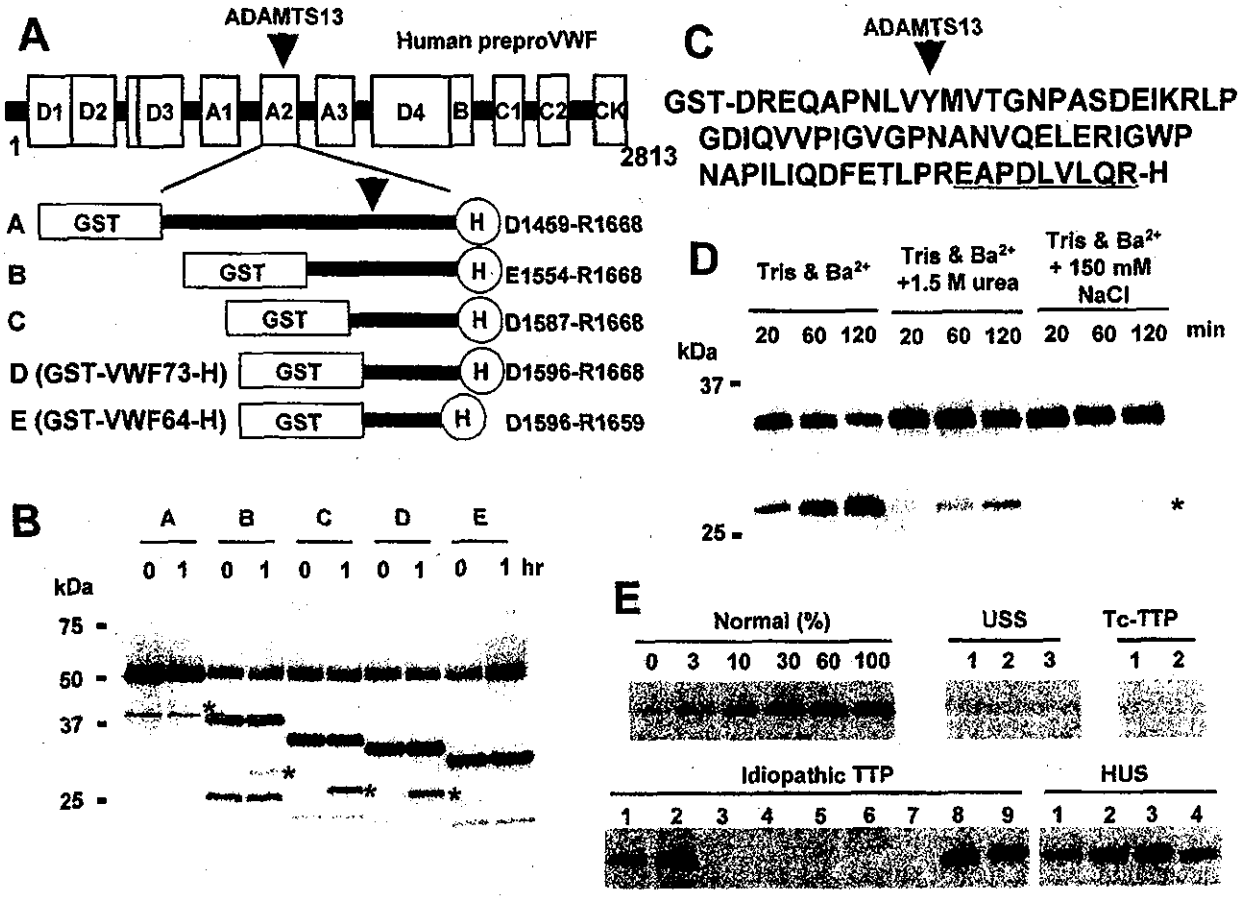

Figure 3. A novel assay for ADAMTS13.

(A) Structures of substrates A–E. The domain structure of human preproVWF is depicted. Five recombinant proteins flanked with GST- and H-tags were expressed for the ADAMTS13 assay. Substrates D and E were designated as GST-VWF73-H and GST-VWF64-H, respectively.

(B) Cleavage of recombinant proteins. The recombinant proteins were incubated with normal plasma at 37°C for 1 hour. The reaction products were run by SDS-polyacrylamide gel electrophoresis and the products were detected by anti-GST antibodies. The product bands are indicated by asterisks. The nonspecific bands of approximately 50 kDa are plasma albumin.

(C) Amino acid sequence of VWF73. Nine amino acid residues indispensable for cleavage are underlined.

(D) Effect of urea and ion concentration on cleavage. GST-VWF73-H was incubated with plasma for the indicated time in reaction buffer (5 mM Tris-HCl, 10 mM BaCl2, pH 8.0) or in the same buffer supplemented with 1.5 M urea or 150 mM NaCl. The products are marked by an asterisk.

(E) Cleavage of GST-VWF73-H by patients' plasma. GST-VWF73-H was incubated with serially diluted normal plasma (0%–100%) or with plasma from patients with Upshaw-Schulman syndrome (USS), ticlopidine-associated thrombotic thrombocytopenic purpura (Tc-TTP), idiopathic TTP, or hemolytic uremic syndrome (HUS), and the products were detected by anti-GST antibodies.

A part of these figures were originally published in *Blood*. Kokame K, Matsumoto M, Fujimura Y, Miyata T. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. Blood. 2004:103:607-612.

trometry analysis of both substrates, VWF73-H and VWF64-H, showed that their estimated molecular weights were exact, indicating no modification. To understand the substrate specificity of ADAMTS13, we determined the solution structure of ^{1}H and ^{15}N double-labeled substrates VWF73-H and VWF64-H by nuclear magnetic resonance (NMR). The results indicated an extended structure for both peptides, suggesting an induced-fit substrate recognition mechanism (unpublished observations).

It was previously reported that ADAMTS13 cleaves VWF in vitro in the presence of urea and low ionic strength conditions. In hypotonic buffer containing 5 mM Tris-HCl and 10 mM $BaCl_2$, GST-VWF73-H was cleaved by plasma in a time-dependent manner (**Figure 3D**). The supplementation of 1.5 M urea or 150

mM NaCl reduced the cleavage, indicating low ADAMTS13 activity under these conditions. GST-VWF73-H was cleaved in a dose-dependent manner with high sensitivity. This assay was able to detect as little as 3% activity (**Figure 3E**).[13] GST-VWF73-H was not cleaved by either plasma from patients with congenital deficiency of ADAMTS13 activity due to *ADAMTS13* mutations (designated as Upshaw-Schulman syndrome [USS]) or plasma from patients with acquired TTP associated with ticlopidine (Tc-TTP). ADAMTS13 activity was observed in 4 patients with idiopathic TTP, but no activity was found in 5 other patients. All patients with hemolytic uremic syndrome (HUS) exhibited activity. Most patients with acquired TTP have autoantibodies that inhibit ADAMTS13 activity in their plasma. GST-VWF73-H was also useful for the detection of autoantibody when normal plasma was incubated with plasma from the patients with acquired TTP.[13]

Another attempt at developing recombinant substrates has been reported in which the *E coli*–expressed A2 domain was used and proved to be a substrate for ADAMTS13 (**Table 2**).[14]

There are several advantages to our method. First, ours is a direct assay for measuring ADAMTS13 product generation, which should provide a more accurate analysis than indirect methods that measure substrate depletion. Second, the bacteria-expressed protein avoids the need to make protease-free VWF. The recombinant protein is simple to prepare: we can produce 1 mg of pure GST-VWF73-H, sufficient for more than 2000 assays, from 100 mL of *E coli* culture in 2 days. Third, the bacterial expression system is easily modified. For example, we have expressed a mouse version of GST-VWF73-H to measure the mouse ADAMTS13 activity.[15] Fourth, having the substrate tagged with two different molecules makes it easy to modify the detection of product. For example, Zhou and Tsai used GST-VWF73-H to develop an ELISA system to measure ADAMTS13 activity in a 96-well format (**Table 2**).[16] Finally, VWF73 will be used as the lead compound to develop more convenient and rational substrates for ADAMTS13 activity assays.

One potential disadvantage of this method is that GST-VWF73-H is not a natural substrate. A recent report showed that ADAMTS13 may recognize the A3 domain of VWF.[17] Therefore, if the defects of the enzyme in patients with TTP affect the ADAMTS13 binding site for the A3 domain, cleavage of GST-VWF73-H will not reflect these defects.

Clinical Applications

TTP is a life-threatening disease. If untreated, mortality may exceed 90%, but plasma exchange therapy significantly reduces mortality. Therefore, measurement of ADAMTS13 activity could be useful for early diagnosis, which is essential for successful treatment of an acute episode. For example, in July 2003, a Japanese pharmaceutical company began ADAMTS13 monitoring, using GST-VWF73-H, for the early diagnosis of patients with suspected TTP associated with ticlopidine. This approach may be effective in preventing the morbidity caused by drug-associated TTP. In addition, periodical ADAMTS13 measurements may be important for monitoring the therapeutic efficacy of plasma exchange or plasma infusion.

Low levels of ADAMTS13 activity have been observed occasionally in several conditions, including heparin-induced thrombocytopenia, severe sepsis, and HUS.[18] But ADAMTS13 activity in these conditions is consistently detectable, and severe ADAMTS13 deficiency appears to be specific for TTP.[19,20] Consequently, ADAMTS13 assays may help in the differential diagnosis of thrombocytopenia, especially when accompanied by microangiopathic changes. In particular, the discrimination of TTP from HUS can be an urgent issue for treatment. Therefore, an assay for ADAMTS13 activity with a narrow normal range is needed. We are currently developing more rapid and quantitative methods for determining ADAMTS13 activity. One of our goals is to develop a bed-side assay for ADAMTS13 activity, for the diagnosis of congenital or autoimmune TTP.

III. Clinical Course and Long-Term Outcomes of Thrombotic Thombocytopenic Purpura

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Diagnosis of Thrombotic Thrombocytopenic Purpura

The evaluation of patients with suspected thrombotic thrombocytopenic purpura is difficult because the diagnostic criteria—(1) thrombocytopenia, (2) micro-

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angiopathic hemolytic anemia, and (3) no other clinically apparent etiology—are not specific.[1] The value of ADAMTS13 measurements for establishing the diagnosis of TTP and determining the indication for plasma exchange treatment remains uncertain. Although a severe deficiency of ADAMTS13 activity (< 5%) may be an abnormality that is specific for TTP,[2] the presenting symptoms of patients with severe ADAMTS13 deficiency are often not severe and not distinguishable from many other common illnesses. Patients can have the characteristic presenting features and clinical course of TTP without severe ADAMTS13 deficiency or even with normal ADAMTS13 activity (> 50%),[3] and also patients can have severe ADAMTS deficiency for many years with no illness.[4]

Patients with TTP and severe ADAMTS13 deficiency are heterogeneous, with remarkably variable presenting features (**Table 3**).[3,5] Nine of the 22 patients with severe ADAMTS13 deficiency (< 5%) in the Oklahoma TTP-HUS Registry (**Table 3**) had no neurologic abnormalities, not even mild symptoms such as headache and confusion. Nonspecific symptoms, such as weakness, abdominal pain, nausea, vomiting, and diarrhea, were common presenting complaints. Some patients had had symptoms for 3 weeks before their diagnosis; the median duration of symptoms prior to diagnosis was 6 days. The presenting symptoms of these patients were similar to patients diagnosed with idiopathic TTP-HUS but who did not have severe ADAMTS13 deficiency.[3]

Patients with severe illnesses that can mimic the presenting clinical features of TTP, such as sepsis and preeclampsia, may have low but detectable levels of ADAMTS13.[3,5,6] The clinical importance of less severe ADAMTS13 deficiencies is unknown. Furthermore, because of the long half-life of ADAMTS13 in plasma, estimated to be 2.6 days,[7] transfusion of red cells and platelets before the diagnosis of TTP is considered can increase plasma ADAMTS13 activity.

Initial studies reported that a severe deficiency of ADAMTS13 distinguished syndromes described as TTP from the related syndromes described as HUS.[8] Although this distinction is valid in many patients, overlap of these syndromes can occur. Acute renal failure, the characteristic clinical feature that is often used to designate syndromes as HUS, may rarely occur in adults with acquired severe ADAMTS13 deficiency.[3] Children with congenital TTP caused by mutations of the *ADAMTS13* gene may have severe renal failure.[9,10] Also children with typical HUS caused by *E coli* 0157:H7 infection may rarely have severe ADAMTS13 deficiency.[11]

Clinical Course of Thrombotic Thrombocytopenic Purpura

Although the value of ADAMTS13 measurements for establishing the diagnosis of TTP is uncertain, demonstration of severe ADAMTS13 deficiency and documentation of the inhibitor titer may help to anticipate the clinical course of patients with acquired TTP. Four

Table 3. Presenting features of 22 consecutive patients with severe deficiency (< 5%) of ADAMTS13 activity.*

Age	39 years (range, 19–71)	
Gender	18 (82%) female	
Race	10 (45%) African-American	
Obesity	12 (55%) BMI ≥ 30 kg/m^2	
Presenting symptoms:		
neurologic abnormalities	13	(59%)
nausea, vomiting, diarrhea	6	(27%)
abdominal pain	6	(27%)
weakness	4	(18%)
chest pain	3	(14%)
hematuria	2	(9%)
menorrhagia	1	(5%)
purpura	1	(5%)
flank pain	1	(5%)
Duration of symptoms	6 days (1–21)	
Neurologic abnormalities:		
severe	10	(45%)
mild	3	(14%)
none	9	(41%)
Renal function:		
acute renal failure	1	(5%)
renal insufficiency	8	(36%)
normal	13	(59%)
Platelet count	9000/μL (range, 4000–27,000/μL)	
Hematocrit	21% (range, 13%–30%)	
LDH	1431 U/L (range, 436–3909 U/L)	

* Data are presented on 22 consecutive patients from the Oklahoma TTP-HUS Registry, November 13, 1995-December 31, 2003, who had a severe deficiency of ADAMTS13 activity at the time of their initial presentation. This experience represents an extension of our previously published data.[3] The number of major presenting symptoms exceeds 22 because some patients had multiple major symptoms. The duration of symptoms describes the patient's history of the initial onset of symptoms until diagnosis of TTP-HUS. Severe neurologic abnormalities included coma, stroke, seizure, or fluctuating focal signs; mild neurologic abnormalities included headache, blurred vision, ataxia, or mental status changes with transient confusion. Acute renal failure and renal insufficiency have been previously defined.[3] Lactate dehydrogenase (LDH) values were adjusted to an upper limit of normal value of 200 U/L. Age, duration of symptoms, and laboratory data are median values. Laboratory data are the most abnormal values at the time of diagnosis of TTP-HUS ± 7 days, to account for transient effects of transfusion and potential worsening after diagnosis.

*Table 4. Risks for death and relapse in thrombotic thrombocytopenic purpura (TTP) related to ADAMTS13 deficiency.**

Case Series (no. of patients)	ADAMTS13 Deficient			ADAMTS13 Not Deficient		
	Total No. Patients	Death (no.)	Relapse (no.)	Total No. Patients	Death (no.)	Relapse (no.)
Veyradier et al[15] (63)	44	–	10	19	–	0
Mori et al[12] (18)	12	2	–	6	4	–
Raife et al[14] (107)	50	4	–	57	10	–
Zheng et al[13]						
idiopathic (20)	16	3	6	4	0	1
non-idiopathic (17)	0	–	–	17	10	1
Vesely et al[3]						
idiopathic (67)	20	4	7/16	47	7	3/40
non-idiopathic (118)	2	0	1/2	116	48	2/68

* Data from 5 case series describing the occurrence of death and relapse among patients distinguished by severe deficiency or lack of severe deficiency of ADAMTS13 activity.

Data from Veyradier et al[15] presented here are on 63 of 111 patients who were described as having TTP rather than hemolytic uremic syndrome (HUS). Deaths were not reported. Patients were distinguished as "sporadic" or "intermittent." For this table, sporadic was assumed to be a single episode while intermittent was assumed to indicate the occurrence of relapses.

Data from Mori et al[12] presented here are on 18 of 27 patients who were described as having TTP rather than HUS. Relapses were not reported.

Data from Raife et al[14] presented here are on 107 consecutive patients described as having thrombotic microangiopathy; syndromes resembling TTP or HUS were not distinguished. Relapses were not reported.

All 37 patients in the study of Zheng et al[13] were described as having TTP. Patients who were described as idiopathic had no apparent pre-existing illness. Patients described as non-idiopathic had had hematopoietic stem cell transplantation, were pregnant or postpartum, had systemic lupus erythematosis, or had taken FK506, mitomycin C, or clopidogrel.

Data from Vesely et al[3] presented here are on 185 patients who had ADAMTS13 measurements at the time of their initial presentation, representing 90% of the 206 patients enrolled in the Oklahoma TTP-HUS Registry, November 13, 1995-December 31, 2003. Data on 142 of these patients, up to June 30, 2002, have been previously published.[3] Patients defined as idiopathic had no apparent etiology or associated clinical conditions. Patients described as non-idiopathic had had hematopoietic stem cell transplantation, were pregnant or postpartum, had taken a drug associated with TTP-HUS, had a bloody diarrhea prodrome, or had an additional/alternative diagnosis. Deaths are reported if they occurred within 30 days of stopping plasma exchange treatment. Relapses are reported in patients who survived for more than 30 days following their plasma exchange treatment.

case series[3,12-14] have reported lower mortality among patients diagnosed with TTP who had severe ADAMTS13 deficiency (**Table 4**). In the two studies[3,13] that distinguished patients who had idiopathic TTP from patients who had potential etiologies and associated conditions that could have contributed to their illness, the mortality rates were less in the patients with idiopathic TTP. These reports suggested that the high rates of mortality among patients who did not have severe ADAMTS13 deficiency may have been caused by other serious conditions, such as systemic infections. Among the four case series reporting mortality in patients with severe ADAMTS13 deficiency,[3,12-14] overall mortality was 13% (**Table 4**). Among the 3 case series reporting relapses in patients with severe ADAMTS13 deficiency, the frequencies of relapse were 10 of 44 (23%),[15] 6 of 16 (38%),[13] and 7 of 16 patients (44%)[3] (**Table 4**).

Zheng et al[13] have reported a further distinction among patients with severe ADAMTS13 deficiency, between patients who had no demonstrable inhibitor at presentation and those patients who had a high titer of inhibitory activity. In patients with no demonstrable inhibitor, plasma exchange was effective in 8 of 9 patients; none of the 8 patients who recovered have subsequently relapsed. In contrast, 4 patients with severe ADAMTS13 deficiency and a high titer inhibitor had more prolonged courses with more frequent serious complications; all suffered relapses and 2 have died, in spite of additional intensive immunosuppressive treatment.

The demonstration of severe ADAMTS13 deficiency with a high titer inhibitor may be an appropriate indication for intensive immunosuppressive treatment. Current clinical practice is inconsistent regarding the use of glucocorticoids and other immunosuppressive agents.[1] Part of this inconsistency is related to the uncertain initial diagnosis in many patients, and the presumed lack of efficacy of immunosuppressive agents for patients with non-idiopathic TTP. However, even among patients with acquired idiopathic TTP and severe ADAMTS13 deficiency, some respond promptly and completely to short courses of plasma exchange, without additional glucocorticoid treatment.[3,13] Patients who respond quickly and completely with only plasma exchange treatment may have only minimal inhibitor

activity or an inhibitor that is not detectable by current methods. Patients with high-titer inhibitors may require not only glucocorticoids but also more intensive immunosuppressive treatment with agents such as rituximab or cyclophosphamide.

However, the correlation between the clinical course and ADAMTS13 levels is not consistent. Several patients have been described who had prolonged stable hematologic remissions in spite of persistent undetectable ADAMTS13 activity.[13,16] These observations, similar to observations of patients with congenitally absent ADAMTS13 activity who may live many years or even a lifetime without evidence of TTP,[4] demonstrate that severe ADAMTS13 deficiency alone is not sufficient to cause the clinical syndrome of TTP. The suggestions of clinical correlation are also tempered by observations that some assays for ADAMTS13 activity may not be consistent across different laboratories, although a multicenter study documented excellent agreement among most assay techniques.[17] Therefore management decisions should remain based upon the clinical course. Patients who respond promptly and completely to plasma exchange may require no additional treatment. Patients whose platelet count does not increase within several days, or in whom recurrent thrombocytopenia recurs when plasma exchange treatments are diminished or discontinued, will likely benefit from glucocorticoid treatment. Patients who have a more severe course, with more severe neurologic abnormalities, and who either do not respond or exacerbate in spite of continuing plasma exchange and glucocorticoid treatment may benefit from more intensive immunosuppressive treatment.

Risk for Relapse of Thrombotic Thrombocytopenic Purpura

The major concern of patients who achieve remission from TTP is the risk for relapse. Current data suggest that the risk for relapse is almost totally restricted to patients who have severe ADAMTS13 deficiency, and severe ADAMTS13 deficiency is usually, but not always, restricted to patients who have idiopathic TTP. Data from the Oklahoma TTP-HUS Registry are presented in **Table 5**. Relapse has not occurred among survivors who had TTP following stem cell transplantation or that was associated with dose-dependent drug toxicity, or who had a prodrome of bloody diarrhea. Two patients with an immune-mediated drug association have relapsed, but these recurrent episodes were caused by repeated ingestion of quinine. Three women whose initial episode of TTP was associated with pregnancy have relapsed. In 1 woman who had recurrent mid-trimester fetal losses, the diagnoses of both the initial and recurrent episode of TTP were uncertain. One woman had 2 episodes of TTP during the first trimester of each of her first 2 pregnancies, and then a third episode when she was not pregnant; ADAMTS13 ac-

Table 5. Relapses in 301 consecutive patients from the Oklahoma TTP-HUS Registry with a clinical diagnosis of thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP-HUS).*

Clinical Category	Patients (Total No.)	Survivors (No.)	Relapse (No.)	Comments Regarding Patients with Relapses
Stem cell transplantation	23	6	0	
Pregnant/postpartum	25	23	3 (13%)	1 patient, recurrent fetal loss, ? TTP. 1 patient, 2 relapses, 1 not with pregnancy. 1 patient, 4 relapses, none with pregnancy, initial ADAMTS13 < 5%.
Drug-association:				
immune-mediated	19	15	2 (13%)	Recurrent quinine use
dose-dependent	17	14	0	
Bloody diarrhea prodrome	19	13	0	
Additional/alternative diagnosis	79	34	2 (6%)	1 patient, scleroderma, 3 relapses, ADAMTS 13%–40%. 1 patient, HIV infection, 4 relapses, initial ADAMTS13 60%, 5th episode < 3%.
Idiopathic	119	96	19 (20%)	11 patients, 1 relapse; 6 patients, 2 relapses; 2 patients, 4 & 5 relapses ADAMTS13 assay on 12 patients; < 5% at initial or subsequent episode in 11 (92%)

* Data are presented on all 301 consecutive patients in the Oklahoma TTP-HUS Registry, 1989-2003. The total number of patients in each clinical category is presented; definitions of the clinical categories have been previously published.[3] Survival is defined as more than 30 days after the last plasma exchange treatment. Relapse is defined as a diagnosis of TTP-HUS in a patient who has survived more than 30 days following their last plasma exchange.

tivity was not measured. The third woman, whose initial episode of TTP was diagnosed at the time of delivery of her first child, had severe ADAMTS13 deficiency with a low titer inhibitor (**Figure 4**, Patient 7); she had 4 relapses during the following year when she was not pregnant; cholecystectomy and splenectomy were performed following her fourth relapse; she has been asymptomatic since that time and has had 2 subsequent uncomplicated pregnancies.

Two patients who had additional disorders that may have contributed to the development of TTP have had multiple relapses. One woman with scleroderma had 3 relapses; her ADAMTS13 activity was not measured initially but was 40% at the time of her first relapse. One man with characteristic features of TTP was incidentally diagnosed with HIV infection at the time of his initial episode when his ADAMTS13 activity was 60%. He subsequently has had 4 relapses and his ADAMTS13 activity has decreased with each episode; ADAMTS13 activity was undetectable at the time of his fourth relapse.

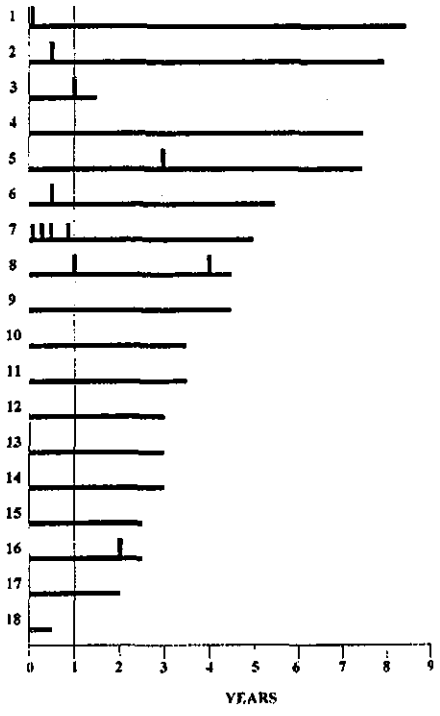

Figure 4. Occurrences of relapses among 18 surviving patients in the Oklahoma TTP-HUS Registry who had severe ADAMTS13 deficiency documented at the time of their initial diagnosis.

Patients are presented consecutively, from patient 1 diagnosed in December 1995 to patient 18 diagnosed in December 2003. During this time 4 patients with severe ADAMTS13 deficiency died during their first episode, before achieving remission and are not presented in this figure. Follow-up is complete to the present time on all patients. Patient 3 died of acute myocardial infarction during her first relapse. Patient 7 had a splenectomy after her fourth relapse. Horizontal bars indicate the duration of follow-up; vertical bars indicate the occurrence of a relapse. Median follow-up duration is 3.3 years; one year of follow-up is indicated by the vertical line.

Among patients with idiopathic TTP, 19 have relapsed (**Table 5**). Twelve of these patients have had ADAMTS13 activity measurements; 11 patients had undetectable activity either at the time of their initial diagnosis or at the time of a subsequent relapse. The exception is a woman with 3 episodes of TTP who had ADAMTS13 measured only at the time of her third episode; ADAMTS13 activity was 100%. Three of these 12 patients had ADAMTS13 activities of 5%–25% at the time of their initial episode and then undetectable ADAMTS13 activity at the time of a relapse. Their initial higher levels of ADAMTS13 activity may have been related to multiple red cell and platelet transfusions given prior to consideration of the initial diagnosis of TTP-HUS.

Figure 4 illustrates the course following recovery from the initial episode of TTP in 18 patients who initially had severe ADAMTS13 deficiency. Four additional patients had severe ADAMTS13 activity but died during their initial episode, 3 from unresponsive disease and 1 from acute hemorrhage related to central venous catheter insertion. Among the 18 survivors, 8 (44%) have had 1 or more relapses; the occurrence of relapse did not appear to be related to the strength of inhibitor activity. Among the patients who have relapsed, 1 (patient 5) had no demonstrable inhibitor, 3 (patients 6, 7, and 8) had trace or mild inhibitory activity, 3 (patients 1, 3, and 16) had moderate inhibitory activity, and 1 patient (patient 2) had strong inhibitory activity.[3] At the time of his relapse, patient 2 was diagnosed with systemic lupus erythematosus and this has been his continuing major problem. Of the 6 patients who had strong inhibitory activity (patients 2, 4, 9, 11, 12, 15), only 1 has relapsed. Although the follow-up of these patients is limited, with a median duration of 3.3 years, these observations suggest that in most patients who will relapse, the initial relapse will occur within the first year (6 of 8 patients). These observations also suggest that in patients who relapse, most will have only 1 relapse (6 of 8 patients). Therefore these observations demonstrate the difficulty of assessing the benefit of any treatment to prevent future relapses. For example, reports of success of splenectomy for preventing future relapses may only be observing the natural history of TTP, with diminishing risk for relapses over time from the initial diagnosis.[18,19]

The observations that essentially all patients who have recurrent episodes of TTP have severe ADAMTS13 deficiency, and that nearly all of these patients have

demonstrable inhibitory activity, suggests that it is appropriate to begin immunosuppressive therapy together with plasma exchange for management of patients with a recurrent episode.

Although almost all recurrent episodes of TTP are immediately recognizable, recent observations suggest the potential for diagnostic difficulty. Three women have been reported who had stroke symptoms following recovery from TTP without accompanying thrombocytopenia; recurrent TTP was considered and symptoms improved with plasma exchange treatment. Subsequently the diagnosis of TTP was supported by finding absent ADAMTS13 activity.[20,21]

Risk for Recurrent Thrombotic Thrombocytopenic Purpura with Subsequent Pregnancies

Since TTP primarily affects women, and since pregnancy appears to be associated with TTP,[22] the risks of a future pregnancy are a common concern among women who have recovered from TTP. Case reports suggest that the risk for recurrent TTP with a subsequent pregnancy is high; among 49 women who have been reported to have 70 subsequent pregnancies, 36 (73%) had recurrent TTP with a pregnancy.[23] However, these reports may be biased by descriptions of unusual patients with complex outcomes.[23] Our experience with 19 women who have had 30 pregnancies following recovery from TTP is more encouraging. Only 5 (26%) women have been diagnosed with recurrent TTP during a subsequent pregnancy, each only during 1 subsequent pregnancy.[23] In 3 of these 5 women, the diagnosis of recurrent TTP was uncertain because of coexisting complications: severe preeclampsia, intrauterine fetal death, and extreme hypertension in a woman with chronic renal failure. This experience emphasizes the difficulty of diagnosing TTP in the presence of other pregnancy-related complications, especially when anticipation is high because of a previous diagnosis of TTP. However, this experience, with complete documentation of all uncomplicated pregnancies, also suggests that recurrent TTP with a subsequent pregnancy is uncommon.

Other Long-Term Outcomes of Thrombotic Thrombocytopenic Purpura

Although the risk for relapse is the major concern of patients who have recovered from TTP, it is not the only concern. Many patients describe persistent cognitive abnormalities for many years following recovery that can be documented by tests of new learning and recent memory.[24] The etiology of these abnormalities is unclear. Perhaps successful treatment with plasma exchange and immunosuppressive therapy will reveal additional long-term sequelae that require further study and perhaps additional supportive care.

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VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13

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ADAMTS-13 was recently identified as a new hemostatic factor, von Willebrand factor (VWF)–cleaving protease. Either congenital or acquired defects of the enzymatic activity lead to thrombotic thrombocytopenic purpura (TTP). ADAMTS-13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF. Here, we determined the minimal region recognized as a specific substrate by ADAMTS-13. A series of partial deletions in the A2 domain flanked with N- and C-terminal tags were expressed in *Escherichia coli* and affinity-purified. These purified proteins were incubated with human plasma, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blot. Judging from mobility shifts, all constructs except one were cleaved at the expected site. Data suggested that a minimal region as a functional substrate consisted of 73 amino acid residues from D1596 to R1668 of VWF, designated VWF73, and that further deletion of the E1660-R1668 region led to the loss of cleavage by ADAMTS-13. VWF73 was not cleaved by plasma from patients with congenital or acquired TTP, but cleaved by plasma from patients with hemolytic uremic syndrome, suggesting that VWF73 is a specific substrate for ADAMTS-13. Thus, VWF73 will be a useful seed to develop a new rapid assay to determine ADAMTS-13 activity. (Blood. 2004;103:607-612)

Introduction

Thrombotic thrombocytopenic purpura (TTP), a serious disease with high mortality, is typically characterized by 5 features: thrombocytopenia, microangiopathic hemolytic anemia, renal failure, fever, and neurologic dysfunction.[1] In patients with TTP, formation of platelet thrombi within the microvasculature is observed. TTP can be categorized into congenital and acquired types. In congenital cases with neonatal onset, the patients quickly respond to infusion of fresh frozen plasma, but the episodes of thrombocytopenia and hemolytic anemia are repeated. TTP with neonatal onset and frequent relapses is often diagnosed as Upshaw-Schulman syndrome (USS).[2] The majority of clinically observed TTP is acquired, often affecting adolescents and adults.

The 2 key molecules involved in the pathogenesis of TTP are the plasma proteins, von Willebrand factor (VWF)[3-6] and ADAMTS-13.[7-11] The platelet-adhesive blood-coagulation protein, VWF, is synthesized primarily in vascular endothelial cells and released into plasma as large multimeric forms, which are highly active in interactions with platelets and collagen.[12,13] In patients with both congenital and acquired TTP, unusually large VWF multimers circulate in plasma, resulting in the promotion of microvascular thrombosis, platelet consumption, and hemolysis. In normal plasma, VWF multimers are rapidly cleaved into smaller forms ranging in size from 500 to 20 000 kDa. This physiologically important cleavage is achieved by a newly identified plasma protease, ADAMTS-13. Functional deficiency of ADAMTS-13 caused by genetic mutation,[14-18] inhibitory autoantibodies,[19,20] or other etiologies leads to the accumulation of unusually large VWF multimers in plasma.

Human ADAMTS-13 was purified from plasma[21-23] and its cDNA was cloned.[23-25] At the same time, the *ADAMTS13* gene was also identified as a gene responsible for congenital TTP by linkage analysis.[14] ADAMTS-13 mRNA is predominantly expressed in liver.[14,23,24,26] The protease consists of 1427 amino acid residues, containing an N-terminal signal peptide, a propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type-1 motif (TSP1), a cysteine-rich domain, a spacer domain, 7 more TSP1 repeats, and 2 CUB domains. The only known physiologic substrate for ADAMTS-13 is VWF multimers. The spacer domain of ADAMTS-13 is necessary for normal VWF-cleaving activity, and the more C-terminal domains are dispensable for the catalytic activity in vitro.[27,28]

ADAMTS-13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF.[29-32] Although several methods have been developed to measure plasma ADAMTS-13 activity, they are not widely used at the clinical level due to various difficulties. The symptoms of TTP are similar to hemolytic uremic syndrome (HUS), a syndrome that is also characterized by thrombocytopenia, microangiopathic hemolytic anemia, and renal failure. HUS occurs mostly in young children after *Escherichia coli* O157 infection, but in some cases, it is difficult to discriminate between TTP and HUS. Therefore, the diagnosis of ambiguous TTP/HUS is made occasionally. For adequate therapy, the establishment of a consistent

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diagnosis system for TTP is eagerly anticipated by physicians and patients. The clinical assay of ADAMTS-13 activity is the most effective instrument for the diagnosis of TTP.[33,34]

To develop a more rapid and convenient method than previously described, an artificial specific substrate that can be easily processed by ADAMTS-13 will be useful. Here, we report that the minimal substrate for ADAMTS-13 is composed of 73 amino acid residues, and we designate this substrate as VWF73.

Materials and methods

Materials

Human plasma was obtained by centrifugation from whole blood that had been anticoagulated with 1:10 volume of 3.8% sodium citrate. Plasma from 3 patients with USS (congenital TTP), 6 patients with acquired TTP, 3 patients with HUS, and healthy individuals were used to measure the ADAMTS-13 activity.

Construction of bacterial expression vectors

Plasmid DNA to express partial regions of human VWF tagged with N-terminal glutathione *S*-transferase (GST) and C-terminal 6xHis (H) were constructed as follows. First, the D1459-R1668 region of VWF was amplified by reverse transcription–polymerase chain reaction (RT-PCR) using total RNA prepared from cultured human umbilical vein endothelial cells. We used 2 primers for amplification: 5′-cgggatccGACCTTGCCCCTGAAGCCCCTC-3′ and 5′-cggaattcTCAGTGATGGTGATGGTGATGCCTCTGCAGCACCAGGTCAGGA-3′. Lowercase letters indicate added restriction enzyme sites, and the underlined sequence is the inserted C-terminal H-tag. The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the corresponding site of pGEX-6P-1 (Amersham Biosciences, Buckinghamshire, England), a *Schistosoma japonicum* GST fusion expression vector. The other plasmids for E1554-R1668, D1587-R1668, D1596-R1668, and D1596-R1659 regions of VWF were also prepared in the same way by combinational use of primers as follows: 5′-cgggatccGAGGCACAGTCCAAAGGGGACA-3′, 5′-cgggatccGACCACAGCTTCTTGGTCAGCC-3′, 5′-cgggatccGACCGGGAGCAGGCGCCCAACC-3′, and 5′-cggaattcTCAGTGATGGTGATGGTGATGTCGGGGGAGCGTCTCAAAGTCC-3′.

Expression and purification of recombinant proteins

To obtain the different recombinant proteins, expression vectors encoding GST-D1459R1668-H, GST-E1554R1668-H, GST-D1587R1668-H, GST-D1596R1668-H, and GST-D1596R1659-H were introduced into *E coli*, BL21 (Stratagene, La Jolla, CA). After isopropyl-β-D-thiogalactoside (IPTG) induction in liquid culture, bacterial cells were collected and lysed with CelLytic B (Sigma, St Louis, MO), followed by centrifugation to separate soluble and insoluble fractions. GST-D1587R1668-H, GST-D1596R1668-H, and GST-D1596R1659-H were collected in soluble fractions, whereas GST-D1459R1668-H and GST-E1554R1668-H were in insoluble fractions. First, all these proteins were purified by Ni-NTA Spin Kit (Qiagen, Hilden, Germany) in a denaturing condition containing 8 M urea and 20 mM 2-mercaptoethanol according to the instruction. The eluates (pH 4.3) were diluted to a 40-times volume of phosphate-buffered saline and left overnight at 4°C for refolding. Then, the proteins were purified by MicroSpin GST Purification Module (Amersham Biosciences) according to the instruction. Eluted proteins (10 mM glutathione) were dialyzed against 20 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 8.0) and quantified by DC Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Cleavage of recombinant proteins by plasma

Purified recombinant proteins (300 ng) were incubated with 1 μL plasma in 40 μL reaction buffer (5 mM Tris-HCl, 10 mM $BaCl_2$, and 1 mM amidinophenylmethanesulfonyl fluoride hydrochloride, pH 8.0) at 37°C for the indicated time. The reaction was stopped by adding 10 μL sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA (ethylenediaminetetraacetic acid), 0.1% bromophenol blue, and 30% glycerol; pH 6.8). Alternatively, to detect inhibitors of ADAMTS-13 in plasma from patients, normal plasma was preincubated with an equal volume of heat-inactivated patient plasma for one hour at room temperature, and then incubated with recombinant substrate proteins at 37°C for 1 hour.

Western blot analysis

The samples were subjected to SDS-PAGE (10%-20% gradient gel) and transferred to a polyvinylidene fluoride membrane (Bio-Rad). Following blocking with 3% skim milk, the membrane was incubated with 1 μg/mL anti-GST (Molecular Probes, Eugene, OR) and then with 0.1 μg/mL peroxidase-labeled anti–rabbit immunoglobulin G (IgG; Kirkegaard & Perry Laboratories, Gaithersburg, MD). Chemiluminescence was developed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Warrington, United Kingdom) and detected on an image analyzer LAS-1000plus (Fujifilm, Tokyo, Japan).

Results

Preparation of substrate proteins

To identify the minimal region of VWF recognized as a substrate by ADAMTS-13, we prepared 5 recombinant proteins containing a partial region of human VWF. First, 2 criteria were set: (1) The region should contain the cleavage site by ADAMTS-13, Y1605 and M1606, in the A2 domain of VWF. (2) It should not contain any cysteine residues that often interfere with the proper folding of artificially engineered proteins. The longest region that satisfied the criteria ranged from D1459 to R1668 of VWF. These 210 amino acid residues were flanked with N-terminal GST and C-terminal H tags for convenient purification and detection, and designated GST-D1459R1668-H or substrate I (Figure 1). The other 4 substrates, GST-E1554R1668-H (substrate II), GST-D1587R1668-H (substrate III), GST-D1596R1668-H (substrate IV), and GST-D1596R1659-H (substrate V), were shorter derivatives of this VWF region.

When expressed in *E coli*, a band corresponding to the expected size of each substrate was visualized (substrate I, 50.8 kDa; II, 40.4 kDa; III, 36.7 kDa; IV, 35.7 kDa; V, 34.7 kDa) after IPTG induction

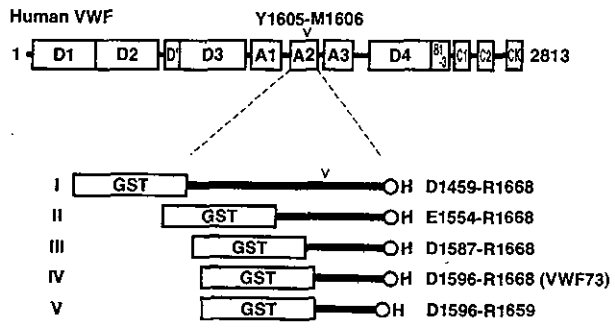

Figure 1. Structures of VWF and fusion proteins for ADAMTS-13 substrate. The domain structure of human preproVWF is shown above the structures of recombinant fusion proteins designed in the present study. Amino acid residues of preproVWF are numbered from the initiating Met codon. The locations of 5 kinds of structural domains (A, B, C, D, and CK) are indicated. The mature VWF secreted from cells consists of 2050 residues (S764-K2813) from the D′ domain to the C-terminal CK domain. ADAMTS-13 cleaves the Y1605-M1606 peptidyl bond in the A2 domain (D1498-L1664). We made 5 different recombinant proteins flanked with GST- and H-tags: GST-D1459R1668-H (I), GST-E1554R1668-H (II), GST-D1587R1668-H (III), GST-D1596R1668-H (IV), and GST-D1596R1659-H (V).

(Figure 2A). Substrates I and II were collected from the insoluble fractions (inclusion bodies), whereas substrates III, IV, and V were mainly recovered in soluble fractions (Figure 2B). All of the recombinant proteins were purified by 2 steps, nickel-ion chelating column chromatography and glutathione-affinity column chromatography, using C-terminal H and N-terminal GST tags, respectively (Figure 2C).

Cleavage of substrate proteins by normal plasma

If ADAMTS-13 cleaves the expected site of substrates I, II, III, IV, and V, the sizes of N-terminal portion including the GST-tag will be 43.1, 32.7, 29.0, 28.0, and 28.0 kDa, respectively. To explore the proteolytic effects of human plasma, these substrate proteins were incubated with normal plasma and analyzed by Western blot using an anti-GST antibody (Figure 3). When substrate I was incubated with normal human plasma for one hour, a very faint band (arrowhead in lane 2) appeared with the apparent size of approximately 43 kDa; this band was not detected before incubation (lane 1). This implied that substrate I was cleaved by some protease in plasma. In the presence of 50 mM EDTA, the substrate was not cleaved (data not shown), suggesting that this cleavage was catalyzed by a metalloprotease, possibly ADAMTS-13. For substrates II, III, and IV, the N-terminal fragments with expected sizes were also detected only after incubation with plasma (arrowheads in lanes 4, 6, and 8). Substrates III and IV were cleaved more effectively than I and II. This might be caused by different refolding efficiency during purification, because substrates I and II were recovered from inclusion bodies. Adding urea to the reaction, which is expected to expose the proper cleavage site of these substrates, did not enhance the cleavage (data not shown). Interestingly, substrate V was not cleaved by plasma, suggesting that it was not recognized as a substrate by ADAMTS-13. Thus, the shortest cleavable substrate in these, IV (GST-D1596R1668-H), was characterized further. Hereinafter, the peptide with 73 amino acid residues corresponding to the region from D1596 to R1668 of VWF is referred to as VWF73.

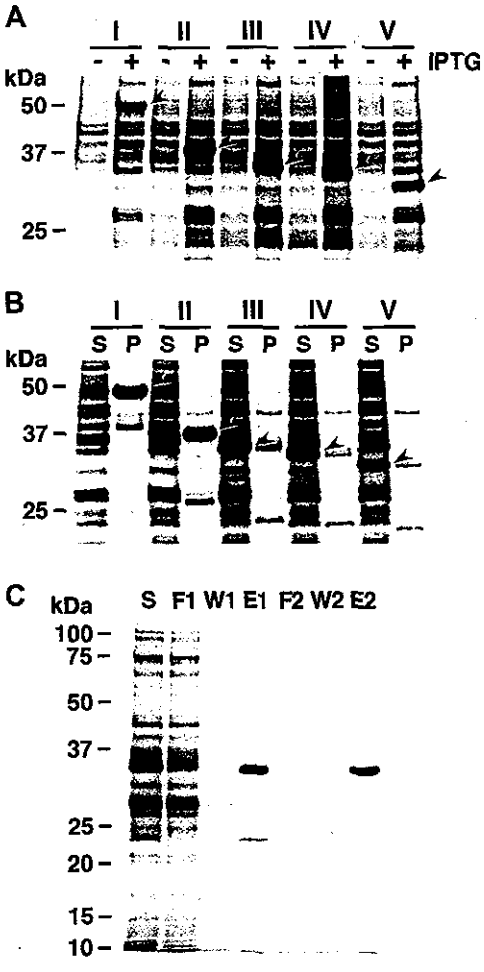

Figure 2. Preparation of bacterial recombinant proteins. (A) Expression. Arrowheads indicate 5 recombinant proteins expressed in *E coli* after IPTG induction: GST-D1459R1668-H (I), GST-E1554R1668-H (II), GST-D1587R1668-H (III), GST-D1596R1668-H (IV), and GST-D1596R1659-H (V). Gels after SDS-PAGE were stained with GelCode Blue (Pierce, Rockford, IL). The sizes of the protein standards are indicated at the left. (B) Fractionation. Recombinant proteins I and II were collected in pellet fractions (P) after centrifugation, whereas III, IV, and V were in soluble fractions (S). (C) Purification. All the recombinant proteins were purified by 2 sequential column-chromatography procedures, nickel-ion chelating chromatography and glutathione-affinity chromatography. The representative pattern of GST-D1596R1668-H is shown. S indicates soluble fraction of bacterial lysate; F1, flow-through of nickel-ion column; W1, wash; E1, eluate; F2, flow-through of glutathione column; W2, wash; and E2, eluate.

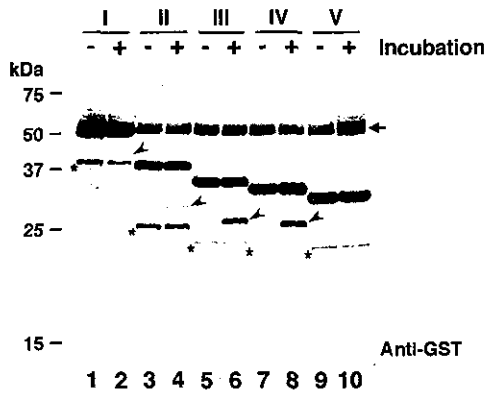

Figure 3. Cleavage of recombinant proteins by normal plasma. The recombinant substrates (I-V) were incubated with normal plasma at 37°C for 0 hours (lanes 1, 3, 5, 7, and 9) or 1 hour (lanes 2, 4, 6, 8, and 10). Both substrates and products were detected by Western blot using anti-GST. The product bands including N-terminal GST-tag are indicated by arrowheads. Substrates III and IV were cleaved more efficiently than I and II, and substrate V was not cleaved. The arrowed bands observed in all lanes are nonspecific signals derived from plasma albumin. The bands with asterisks, probably contaminating degradation products, are reproducible background signals.

Characterization of cleavage

It was previously reported that ADAMTS-13 cleaves VWF in vitro preferentially in the presence of urea and in low ionic strength.[4] We examined the effect of urea and NaCl on the cleavage efficiency of GST-VWF73-H. In hypotonic buffer including 5 mM Tris-HCl and 10 mM $BaCl_2$, GST-VWF73-H was efficiently cleaved by normal plasma in a time-dependent manner (Figure 4, lanes 1-3). In the presence of either 1.5 M urea or 150 mM NaCl, however, the production of the N-terminal fragment was quite low (lanes 4-9). The inhibitory effect of physiologic ionic strength was consistent with a previous report.[4] No requirement of urea for efficient cleavage suggests that the structure surrounding the Y1605-M1606 peptidyl bond is different between GST-VWF73-H and intact VWF multimers.

Cleavage of GST-VWF73-H by patient plasma

To confirm that the cleavage of GST-VWF73-H is catalyzed by ADAMTS-13, the substrate was incubated with plasma prepared from patients with congenital TTP, USS (Figure 5). Previously, we confirmed that these 3 patients have no VWF-cleaving activity,[2]

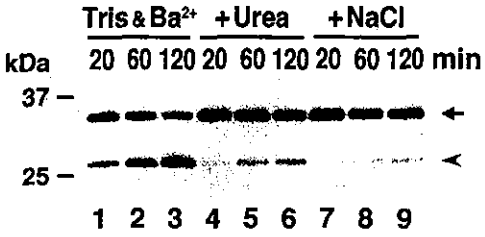

Figure 4. Effect of urea and ion strength on cleavage. GST-VWF73-H was incubated with normal plasma for the indicated time in reaction buffer (5 mM Tris-HCl, 10 mM $BaCl_2$, pH 8.0) (lanes 1-3) or in the same buffer supplemented with either 1.5 M urea (lanes 4-6) or 150 mM NaCl (lanes 7-9). The substrate and product bands are shown by an arrow and an arrowhead, respectively.

and that USS patients 2 and 3 are a homozygote of the ADAMTS-13 Q449X mutation and a compound heterozygote of R268P/C508Y, respectively.[15] In the present assay, none of their plasma cleaved GST-VWF73-H, strongly suggesting that the cleavage of GST-VWF73-H by normal plasma is catalyzed by ADAMTS-13. The plasma derived from patients with ticlopidine-associated TTP also showed no cleavage of the substrate (Figure 5), consistent with our previous reports.[35,36] Among 9 patients with idiopathic TTP examined, 5 patients had no ADAMTS-13 activity, but the remaining 4 patients had some apparent activity. This result was also consistent with previous data.[37] Conversely, the plasma from 4 patients with HUS produced a fragment of the substrate. Thus, the recombinant substrate, GST-VWF73-H, was confirmed to be a specific substrate for ADAMTS-13.

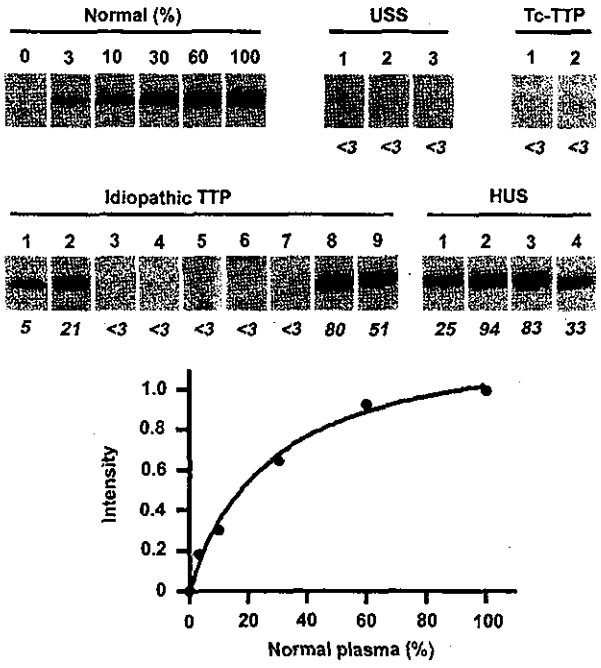

Figure 5. Cleavage of GST-VWF73-H by patients' plasma. GST-VWF73-H was incubated with serially diluted normal plasma (0%-100%) or with plasma from patients with USS, ticlopidine-associated TTP (Tc-TTP), idiopathic TTP, and HUS. The band intensities of reaction products were measured by chemiluminescence on Western blot, and the relative activities of patients' plasma (%, shown by italic numbers) to normal plasma were calculated from nonlinear regression by serially diluted normal plasma. USS patients 1 to 3 correspond to ST-III-4, SY-III-1, and KI-III-2 by previous report.[2] Tc-TTP patients 1 and 2 were reported by Sugio et al[35] and Orimo et al,[36] respectively. The idiopathic TTP patients 1 to 9 correspond to the case numbers 1-3, 5, 6, 11-13, and 17, and HUS patients 1 to 4 correspond to the case numbers 2, 4, 6, and 9 in the previous report.[37]

Inhibitors of ADAMTS-13 in plasma from patients

Most patients with acquired TTP have autoantibodies that inhibit ADAMTS-13 activity in their plasma.[19,20] No inhibitors are detected in plasma from patients with USS.[19] After incubation of normal plasma with plasma from the patients with USS or acquired TTP, the cleavage of GST-VWF73-H was examined (Figure 6). Preincubation with plasma from 3 patients with acquired TTP inhibited the cleavage of GST-VWF73-H, whereas preincubation with plasma from 3 USS patients had no effect. This indicates that the assay system using recombinant substrate VWF73 can be also useful to measure inhibitors of ADAMTS-13.

Discussion

Several assay methods have been reported to measure plasma ADAMTS-13 activity. The original method was developed by Furlan et al[31] and Tsai,[32] independently. They purified human VWF and incubated it with plasma in the presence of urea or guanidine-HCl as well as divalent cations such as Ba^{2+} and Ca^{2+}. Subsequently, Furlan et al separated the degraded material by SDS-agarose gel electrophoresis followed by Western blot using anti-VWF antibodies to detect a decrease in VWF-multimer ladders. Alternatively, Tsai separated the reaction materials by SDS-PAGE and detected the degraded products by Western blot. The former is visually attractive and sensitive, but time and skill are required. From an enzymologic viewpoint, the latter approach is superior in that it visualizes the product of the reaction, and not the disappearance of the substrate.

Gerritsen et al developed a different method based on the preferential binding of high–molecular-weight forms of VWF to collagen.[38] The proteolytic degradation of VWF leads to low-molecular-weight forms of VWF, which show impaired binding to microtiter plates coated with collagen. The collagen-bound VWF is quantified using antibodies against VWF. Obert et al reported an immunoradiometric assay using 2 site-directed monoclonal antibodies to VWF.[39] In this assay, the residual full-length VWF after proteolytic incubation was estimated by a sandwich enzyme-linked immunosorbent assay. Böhm et al recently reported a method based on the positive correlation between VWF multimeric size and Ristocetin cofactor activity.[40] After digestion of VWF with plasma, the residual cofactor activity of the samples was assessed to calculate the ADAMTS-13 activity of the samples. Although these assay methods may be more suitable for clinical applications because they require less time to complete, they provide only an indirect detection of the cleavage reaction compared with the original methods developed by Furlan et al[31] and Tsai.[32]

In the present study, we provide a new substrate for ADAMTS-13, VWF73, by which convenient clinical assays can be developed.

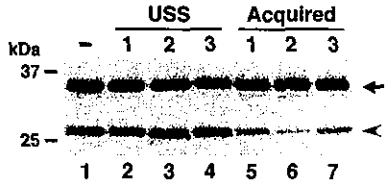

Figure 6. Inhibitory activity of plasma from patients. Normal plasma was preincubated with equal volumes of heat-inactivated plasma from patients with USS (lanes 2-4), Tc-associated TTP (lanes 5-6), and idiopathic TTP (lane 7). Then, cleavage of GST-VWF73-H was compared with normal plasma without preincubation (lane 1). Plasma from patients with acquired TTP but not with USS inhibited substrate cleavage.