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A shear-based assay for assessing plasma ADAMTS13 activity and inhibitor in patients with thrombotic thrombocytopenic purpura

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Abstract

Background—Severe deficiency of plasma ADAMTS13 activity is a frequent finding in patients with hereditary and acquired thrombotic thrombocytopenic purpura (TTP). To date, plasma ADAMTS13 activity is determined by cleavage of either pre-denatured von Willebrand factor (VWF) or small peptides derived from the VWF-A2 domain. The physiological relevance of the assay results is uncertain.

Methods—We sought to develop a novel shear-based assay to assess plasma ADAMTS13 activity and inhibitor. We also compared this assay with a fluorogenic peptide assay.

Results—We found that an incubation of purified plasma VWF with 0.5–1.0 µl of citrated plasma under constant vortexing at 2,500 rpm for 60 minutes in the presence of 5 mM CaCl₂, 1.7 µM ZnCl₂ and low concentration of NaCl resulted in the maximal cleavage of VWF. The cleavage product could be separated by a 2.5% agarose gel and detected by Western blotting. The assay revealed that plasma and recombinant ADAMTS13 are highly sensitive to inhibition by zinc and chloride ions. Under the optimal conditions, the shear-based assay appeared to be more sensitive than the guanidine-denaturation assay for determining plasma ADAMTS13 activity.

Conclusions—Our fluid shear-based assay may be useful for investigating basic biological function and regulation of ADAMTS13 metalloprotease. It may also be applicable for assessing plasma ADAMTS13 activity and inhibitors in TTP patients.

Introduction

ADAMTS13 cleaves von Willebrand factor (VWF) at the Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ bond^{1,2}, thereby reducing the sizes of circulating VWF multimers. The proteolytic cleavage of VWF by ADAMTS13 under physiologic conditions may occur on the surface of endothelial cells^{3,4} and in blood^{2,5}. Upon stimulation, newly released unusually large (UL) VWF polymers anchored on endothelial cells are rapidly cleaved by ADAMTS13 in the presence^{3,6} and in the absence of fluid shear stress^{4,7}. However, the released VWF from endothelial cell membrane remains ultra large in size, revealed by agarose gel electrophoresis and Western blotting⁴, suggesting that further proteolytic cleavage of UL-VWF in circulation (or in solution) may be necessary to further reduce the sizes of UL-VWF multimers that are normally seen plasma. The key to regulate proteolytic cleavage of soluble VWF by ADAMTS13 is fluid shear stress^{5,8}. High shear stress causes conformational changes of

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inter and intra-domains of VWF and A2 domain, which allows ADAMTS13 to bind and access the sessile bond for cleavage⁸⁻¹⁰. Coagulation factor VIII¹¹ and platelets¹² either alone or in combination¹³ have been shown to accelerate this process under fluid shear stress, but not under static/denaturing conditions. These results suggest that the conformational changes induced by fluid shear stress and by denaturants may be fundamentally different. ADAMTS13 is primarily synthesized in the liver¹⁴ and also detectable in endothelial cells¹⁵ and megakaryocytes or platelets¹⁶. Plasma concentrations of ADAMTS13 in healthy individuals range from 0.5 to 1.0 µg/ml¹⁷. Severe deficiency of ADAMTS13 activity has been linked to the pathogenesis of congenital¹⁸⁻²¹ and acquired idiopathic TTP²²⁻²⁴. ADAMTS13 deficiency may be caused by mutations of the *ADAMTS13* gene^{20,21} or by autoantibodies against ADAMTS13 protein^{23,24}. Several studies have shown that severely deficient plasma ADAMTS13 activity with or without positive identification of autoantibodies against ADAMTS13 may be specific findings for idiopathic TTP²³. However, this is not the case in patients with bone marrow transplant associated TTP^{25,26} and other related thrombotic microangiopathies such as hemolytic uremic syndrome (HUS)¹⁹. These patients usually show normal to moderate reduction of plasma ADAMTS13. Therefore, knowing the status of plasma ADAMTS13 activity and autoantibodies in patients with clinical suspicion of TTP helps confirm the diagnosis, provide rationale for installation of adjunctive immunosuppressive therapies, and predict the disease relapses and outcomes^{24,27}. Over the years, many assays have been developed to assess plasma ADAMTS13 activity, which are classified into 3 categories: 1) assessing proteolytic cleavage of multimeric VWF under denaturing conditions^{19,23}; 2) assessing the cleavage of truncated peptidyl substrates such as FRETs-VWF73^{28,29}; 3) assessing the cleavage of cell bound UL-VWF under flow conditions^{3,6}. These assays have provided us with invaluable knowledge of ADAMTS13 status in patients with TTP and many other arterial thrombotic disorders. However, there are drawbacks with each one of these assays. For instance, the denaturants added into the reaction may inactivate ADAMTS13 enzyme or potential cofactors that accelerate VWF proteolysis by ADAMTS13¹¹. It may also result in dissociation of antibody-antigen complexes. The peptidyl substrates, however, lack the ancillary binding domains that are critical for interaction with C-terminal domains of ADAMTS13^{30,31}. Moreover, the assays based on endothelial cell culture are technically challenging and difficult to be performed reproducibly^{32,33}. Here, we describe a novel vortex-based assay for assessing plasma ADAMTS13 activity and inhibitors in patients with TTP. Under fluid shear stress, plasma and recombinant ADAMTS13 proteases are sensitive to zinc ion and sodium chloride, which makes this assay particularly suitable for investigating the function and regulation of ADAMTS13 activity. With further optimization, the vortex-based assay may be useful for assessing ADAMTS13 activity and inhibitor in patients with TTP.

Materials and methods

Materials

Recombinant VWF73 mutant (D1596C/R1668C) was labeled with 5'-maleimide fluorescein (Thermo Fisher, Rockford, IL) in column and purified to homogeneity as described previously^{34,35}. The final product, designated as rF-VWF73, was stored in 0.5% trifluoroacetic acid (TFA) (Sigma, St. Louis, MO) at -80 °C. Human VWF was purified from cryoprecipitate as described in detail in our recent publication¹³. Ultra-large VWF (UL-VWF) released from human umbilical vein endothelial cells (HUVEC) upon histamine stimulation or constitutively was prepared as described previously⁴. Recombinant human ADAMTS13 protease was expressed in stable HEK293 cells and purified using three-step column chromatographies according to our published procedures^{31,36}. Protein concentrations were determined by using the following molecular weights and extinction

coefficient (E_{280} , 1 mg/ml): ADAMTS13, 195 kDa, 0.68; VWF, 250 kDa per monomer, 1.0)^{31,36}.

Plasma

Pooled normal human plasma (NHP) was purchased from George King Biomedical, Overland Park, Kansas). Patient plasma was collected from TTP patients receiving plasma exchange therapy. Venous blood (5 ml) was collected into a tube containing 3.8% sodium citrate as anticoagulant²⁴ either prior to the first plasma exchange or after several plasma exchange therapies as indicated in Table 1. The citrated plasma was obtained after centrifugation at 3,000 rpm for 15 min. Plasma aliquots were stored at -80°C until assay. The Institutional Review Board of the Children's Hospital of Philadelphia approved the study. Informed consent was obtained from patients participating in the study.

Plasma ADAMTS13 activity and inhibitors by rF-VWF73

Plasma (3 μ l) was diluted with 97 μ l of assay buffer (5 mM Bis-Tris, 25 mM CaCl_2 , and 0.005% Tween 20, pH 6.0 containing 2 mM of Pefabloc) in the wells of a white 96-well plate. Then, 100 μ l of rF-VWF73 (final concentration 2 μ M) diluted in the same assay buffer was then added^{34,35}. The cleavage of rF-VWF73 was monitored at 37°C every 2 minutes for 60 min on a fluorescent reader (Molecular Device, CA) (excitation/emission: 485/535 nm). The initial rate of fluorescence generation was obtained to determine the relative activity compared with the same amount of NHP. The activity in NHP was defined as 100%. For the inhibitor assay, 10 μ l of NHP was mixed with 10 μ l of heat-inactivated normal or patient plasma at 37 °C for 60 min. The residual activity in the 6 μ l of mixed plasma (3 μ l NHP) was determined by rF-VWF73 exactly the same way as described for plasma activity assay. The inhibitors were positive if the proteolytic activity of the NHP was reduced by 30% compared with the control NHP being mixed with heat-inactivated NHP.

Plasma anti-ADAMTS13 IgG by ELISA

Plasma anti-ADAMTS13 IgG was determined by an ELISA kit according to the manufacture's protocol (Technoclone, Vienna, Austria).

Plasma ADAMTS13 activity by vortex-based assay

Purified plasma VWF (37.5 μ g/ml or 150 nM) was incubated at 25°C for 3 min with different amounts (0-4 μ l) of diluted or undiluted plasma (final concentrations of ADAMTS13, 0-1.0 nM) or recombinant ADAMTS13 (0 ~200 nM) in 20 mM HEPES, 5 mM CaCl_2 , and 1 mg/ml of BSA, pH 7.5 in the absence or the presence of EDTA (10 mM). A protease inhibitor pefabloc (final concentration 2 mM) was added to each reaction mixture. The reaction mixture (total volume, 20 μ l in 0.2 ml PCR tube) was then subjected to vortexing at the rotation rate of 2,500 rpm for 0~60 min on a MixMate® PCR mixer (Eppendorf, Hauppauge, NY). Under this condition, approximately 32 dynes/cm² of fluid shear stress was generated based on calculation and experimental determination with comparison with that on cone-plate viscometer¹³. The reaction was quenched by heating the samples at 60°C for 20 min in 2 $\times$ sample buffer (70 mM Tris, 2.4% SDS, 0.67 M urea, 4 mM EDTA, and 0.01% bromophenol blue, pH 6.5). The samples were then loaded onto a SeaKem HGT agarose gel (1.5% and 2.5%, wt/vol) and separated by electrophoresis at 100 mA for 60 min. After transfer onto a nitrocellulose membrane, the membrane was blocked with 1% casein (wt/vol) in 20 mM Tris-HCl, 0.15 M NaCl, and 0.05% Tween-20 (TBST) and then incubated at room temperature with rabbit anti-VWF IgG (A0082, DAKO) (1:5,000) in 1% casein TBST for 3 h. After washing 3 times with TBST, the membrane was incubated for 1h with IRDye 800CW-labeled goat anti-rabbit IgG (LI-COR Bioscience) in 1% casein TBST. After a 15 min wash with TBST and a 5 min wash with PBS, the

fluorescent signal was obtained using an Odyssey Infrared Imaging System (LI-COR Bioscience) and quantified by densitometry using ImageJ software¹³.

Plasma ADAMTS13 activity by a denatured assay

Purified VWF (1.5 μ M) was denatured with 1.5 M guanidine-HCl at 37°C for 1 h²³. The denatured VWF was diluted 1:10 and incubated with plasma ADAMTS13 (0 ~1 nM) or recombinant ADAMTS13 (0 ~200 nM) at 25°C for 1 h in the absence or the presence of EDTA (10 mM) in the assay buffer. The proteolytic cleavage products were determined by 2.5% agarose gel electrophoresis and Western blot as described in the assay for ADAMTS13 under the vortexing conditions above.

Results

Characterization of purified plasma VWF substrate and optimization of agarose gel concentrations for detection

To determine proteolytic cleavage of VWF by plasma or recombinant ADAMTS13, we purified VWF substrate from pooled normal human plasma using the methods established in the laboratory¹³. The purified VWF was assessed by Coomassie blue staining and Western blotting analyses. As shown, purified VWF exhibited a single band at 250 kDa on SDS-PAGE under denatured and reduced conditions (Fig. 1A, lane 2) and at least 10-13 distinct bands on a 1% agarose gel (Fig. 1B, lane 1). In comparison with the VWF released from histamine-stimulated endothelial cells (Fig. 1B, lane 2) or in the conditioned medium (Fig. 1B, lane 3) of cultured human umbilical vein endothelial cells, the purified plasma VWF was devoid of ultra-large VWF and contained predominantly the intermediate size of multimers (Fig. 1B, lane 1). When recombinant ADAMTS13 (25 nM) was added into the purified VWF (75 nM) in the presence of 10 mM EDTA (Fig. 1C, lane 1) or in the absence of EDTA (Fig. 1C, lane 2) and vortexed at 2,500 rpm for 30 min, the proteolytic cleavage of VWF could be readily detected with 1% agarose gel electrophoresis and Western blotting. However, when patient plasma (1.0 μ l) was added into the similar reactions in the presence of 10 mM EDTA (Fig. 1D, lane 1) or in the absence of EDTA (Fig. 1D, lane 2), the newly formed proteolytic cleavage products were obscured by the preexisting degradation products in plasma samples (Fig. 1D, lane 2), which made the visualization and quantification of the VWF cleavage less reliable. This problem was circumvented by the fractionation of the reaction mixture in the agarose gel with higher concentration (i.e. 2.5%). As shown, the modified multimer analysis technique allowed the separation of the newly formed cleavage product from the other preexisting bands from the plasma samples (Fig. 1E, lane 2, “e”). These results suggest that the high resolution (2.5%) of mini-agarose gel can be used to detect the proteolytic activity of plasma ADAMTS13 under vortex-induced shear stress conditions.

Time-dependent cleavage of VWF by plasma ADAMTS13 under vortex-based shear stress

To determine the optimal vortex time required for generation of detectable cleavage products with a limited volume of plasma, we incubated purified VWF (150 nM) with 1 μ l of NHP (the final concentration of ADAMTS13, 0.25 nM) under constant vortexing at 2,500 rpm for 0~120 min in the absence or in the presence of EDTA (10mM). The proteolytic cleavage product was detected 2.5% agarose gel electrophoresis and Western blotting. We showed that the proteolytic cleavage product increased as a function of vortex time with the maximal formation of the cleavage product at 60 minutes (Fig. 2A and 2B). Further incubation under constant vortex for up to 120 minutes did not increase the formation of the cleavage product (Fig. 2A and 2B). In the presence of 10 mM EDTA, very little proteolytic cleavage product was detected, indicating that vortex alone up to 120 minutes does not cause spontaneous proteolysis of VWF substrate in the absence of ADAMTS13 activity.

Concentration-dependent cleavage of VWF by plasma and recombinant ADAMTS13 under shear stress or denaturing conditions

The conformational changes of multimeric VWF can be induced *in vitro* by denaturation^{1,2} or fluid shear stress^{5,31}. To determine the amount of plasma or recombinant ADAMTS13 required for the detection of proteolytic cleavage product under the vortexing conditions in comparison with that under the denaturing conditions, we incubated purified native or pre-denatured VWF (150 nM) with various amount of normal plasma (0–4 μ l) or various concentrations of purified recombinant ADAMTS13 (0–200 nM) under constant vortex at 2,500 rpm (Fig. 3A and 3D) or in the presence of 0.15 M guanidine-HCl (Fig. 3B and 3E) for 60 minutes. The proteolytic cleavage product was determined by 2.5% agarose gel and Western blotting. We showed that under the vortexing conditions the proteolytic cleavage product increased in a concentration-dependent manner with the maximal product formation at 0.25 nM of ADAMTS13, which corresponds to 1 μ l of undiluted plasma in a total of 20 μ l of reaction mixture. The formation of proteolytic cleavage product was reduced when plasma volume increased beyond 2 μ l (in a total of 20 μ l reaction mixture) (Fig. 3A and 3C). In comparison, under denaturing conditions, the proteolytic cleavage product was not detectable until plasma ADAMTS13 concentration reached 0.25 nM, corresponding to 1 μ l of undiluted plasma in the reaction (Fig. 3B and 3C). At the optimal amount of plasma (1 μ l in a total 20 μ l of reaction), the cleavage product formation was much greater with the vortexing assay than with the guanidine-denaturing assay when plasma was used as a source of enzyme (Fig. 3C). Interestingly, when recombinant ADAMTS13 was used as an enzyme, the rate of cleavage product formation under both assay conditions (shear vs. denaturant) was quite similar (Fig. 3D, 3E, and 3F). These results suggest that the vortex assay is more sensitive analytically than the denatured assay to detect low levels of plasma ADAMTS13 activity, but appears to perform equivalently as compared with the denatured assay for assessing the proteolytic activity of recombinant ADAMTS13. To determine the reasons why an increased plasma volume beyond 1.0 μ l in a total of 20 μ l reaction mixture in the vortex-based assay reduced the formation of proteolytic cleavage product, a heat-inactivated or unheated plasma (0.25 μ l) was added into the reaction mixture containing a fixed concentration of VWF (150 nM) and various concentration of recombinant ADAMTS13 (0–100 nM). We showed that the addition of small amount (0.25 μ l) of plasma increased the formation of cleavage product (Fig. 4B). The heat-inactivated plasma exhibited a reduced efficacy accelerating VWF proteolysis by recombinant ADAMTS13 (Fig. 4C) as compared with unheated plasma (Fig. 4D). These results suggest that certain protein cofactors may accelerate, rather than inhibit the proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress. Alternatively, when various volumes of plasma (either heated or unheated) were added into a reaction mixture consisting of purified VWF (150 nM) and recombinant ADAMTS13 (25 nM), the proteolytic cleavage product was initially increased and then dramatically decreased after more than 1 μ l of heat-inactivated plasma (Fig. 4C) or unheated plasma (Fig. 4D) was added in a total of 20 μ l reaction. These results suggest that certain ion such as Cl^- and citrate in plasma may inhibit proteolytic cleavage of VWF by recombinant ADAMTS13 under fluid shear stress. Thus, the amount of plasma to be used in the assay should be limited to 1 μ l in a total of 20 μ l assay volume.

Effect of zinc ion on ADAMTS13 activity under shear stress

The metalloprotease domain of ADAMTS13 contains a HEXHXXGXXHD motif that coordinates Ca^{2+} and Zn^{2+} ion binding³⁷. To determine the requirement of Zn^{2+} ion for activation of plasma ADAMTS13, purified VWF (150 nM) was incubated with NHP (1 μ l) in the presence of a fixed concentration of CaCl_2 (5 mM) and various concentrations of ZnCl_2 (0–500 μ M) for 60 minutes under constant vortexing at 2,500 rpm. The control reactions included EDTA (10 mM) or omitted ADAMTS13. Under these conditions, low

concentrations of Zn^{2+} ion between 0.17-17 μM appeared to have little effect on plasma ADAMTS13 activity (Fig. 5A and 5B), but slightly increased recombinant ADAMTS13 activity (Fig. 5C and 5D). However, further increases in Zn^{2+} concentrations in the reaction beyond 17 μM resulted in a dramatic inhibition of both plasma (Fig. 5A and 5B) and recombinant (Fig. 5C and 5D) ADAMTS13 activities in a concentration-dependent manner. At a concentration of 500 μM , Zn^{2+} completely blocked the proteolytic cleavage of VWF by either plasma or recombinant ADAMTS13 under fluid shear stress (Fig. 5A-D). These data suggest the exogenous zinc ion may be not required for the activation of plasma ADAMTS13 *in vitro* under fluid shear stress.

Effect of chloride ion on ADAMTS13 activity under shear stress

Previous studies have shown that chloride ion (Cl^-) inhibits proteolytic cleavage of VWF and FRTES-VWF by ADAMTS13³⁸. To assess whether the Cl^- affects the cleavage of VWF by ADAMTS13 under shear stress, purified VWF (150 nM) was incubated with plasma (1 μl) or recombinant ADAMTS13 (25 nM) for 60 minutes under constant vortexing at 2,500 rpm in the presence of various concentrations of NaCl (0~300 mM) or EDTA (10 mM). We showed that the formation of proteolytic cleavage product was dramatically inhibited by added NaCl in a concentration-dependent manner (Fig. 6A-D). The concentrations of NaCl that resulted in the half-maximal inhibition of proteolytic cleavage of VWF (IC_{50}) by plasma ADAMTS13 (Fig. 6A and 6B) and recombinant ADAMTS13 (Fig. 6C and 6D) were ~50 mM and ~150 mM, respectively. These data suggest that ADAMTS13 activity can be modulated by chloride ion under these conditions. Therefore, the vortex-based assay was performed in the absence of added sodium chloride in the buffer to maximize the analytical sensitivity for the detection of proteolytic cleavage products.

Plasma ADAMTS13 activity in patients with acquired TTP

To determine whether the vortex-based assay could be used to assess plasma ADAMTS13 activity in patient samples, we assessed ADAMTS13 activity in plasma samples from 22 randomly selected patients with acquired TTP. Of these, 14 were idiopathic TTP and 6 were hematopoietic progenitor cell transplantation (HPCT)-related TTP. The plasma ADAMTS13 activity in each patient was also determined by the cleavage of fluorogenic peptide, rF-VWF73, for a comparison. We showed that plasma from 12 of 16 patients with idiopathic TTP (except for patients 29, 42, 60 and 83) (Table 1) exhibited severely reduced VWF cleaving activity (less than 10% of normal) using the vortex assay (Fig. 7) despite many patients received plasma exchange therapy at the time of sample collection (Table 1, column 5). No severe deficiency of plasma ADAMTS13 activity was identified in patients with HPCT-associated TTP except for patients 107 and 110, in whom the preexisting excessive cleavage bands interfered with accurate assessment of plasma ADAMTS13 activity by the vortex technique (Fig. 7). Overall, there was a good correlation between ADAMTS13 activity obtained from the vortex assay and that from rF-VWF73 assay (Pearson's correlation coefficient, $r=0.769$) ($p<0.001$) (Fig. 9). These results suggest that the vortex assay may be useful for assessing plasma ADAMTS13 activity in patients with acquired, particularly idiopathic TTP.

Plasma anti-ADAMTS13 inhibitors in patients with acquired TTP

Most of acquired TTP are caused by autoantibodies against ADAMTS13^{23,39}. To determine if the vortex assay was able to detect inhibitors against ADAMTS13, we incubated normal human plasma with heat-inactivated patient plasma (50:50 mixing) for 60 minutes at 25°C. The residual ADAMTS13 activity in the normal human plasma was determined by the vortex assay. We showed that most of patient plasmas from idiopathic TTP (except for patients 12, 29, 42, 60, and 83) had demonstrable autoantibodies which inhibited ADAMTS13 activity by $\geq 30\%$ in normal human plasma (Fig. 8). The patients who showed

no inhibition by the vortex-based assay had no inhibitory activity by the rF-VWF73 assay or anti-ADAMTS13 IgG by ELISA (Table 1). These results suggest that the vortex-based shear assay may also be useful to detect plasma autoantibodies against ADAMTS13 in patients with acquired, particularly idiopathic TTP.

Discussion

The study demonstrates a novel vortex-based assay for assessing plasma ADAMTS13 activity and inhibitors in patients with TTP. Unlike the assays previously described in the literature which are performed in the extreme conditions^{19,23,24,40,41}, our current assay does not require the addition of denaturants such as 1.5 M urea and guanidine to unfold VWF substrate. The cleavage reaction can be carried out in a buffer with pH of 7.4 in the presence of physiological concentrations of calcium and zinc ions. The formation of proteolytic cleavage product of VWF by ADAMTS13 in the citrated plasma is time- (Fig. 2) and concentration- (Fig. 3) dependent with the maximal cleavage products detected with 1 μ l of plasma in a total of 20 μ l reaction volume after 60 minutes of vortex at 2,500 rpm using the MixMate PCR mixer. This vortex rotation rate in such a defined volume (20 μ l) generates approximately 32 dynes/cm² of shear stress as reported previously¹³, which mimics arterial shear stress. This amount of shear stress appears to be sufficient to resulting in the cleavage of VWF by ADAMTS13 without an addition of denaturants. Vortex on other bench top mini-vortexers may generate higher fluid shear stresses^{11,42,43}. The cleavage products can be readily detected by Western blotting following a separation on a 2.5% agarose SDS-gel. This high-resolution mini-agarose gel appears to be a key step separating the newly formed cleavage products from the lowest molecular weight of VWF (i.e. dimer) in patient plasmas. However, this method has its limitation as it may not be able to determine plasma ADAMTS13 activity when excessive pre-existing cleavage products are present in some patient samples such as those obtained from patients with HPCT-related TTP. In these patients, plasma VWF, during the acute disease burst, is excessively cleaved by ADAMTS13⁴⁴ and perhaps other leukocyte proteases³⁴. Therefore, it is critical that each sample is assayed in the absence and in the presence of EDTA to assess the degree of pre-existing cleaved products of VWF. The relative amount of cleavage product in the absence of EDTA and in the presence of EDTA was used for calculation of plasma ADAMTS13 activity using normal human plasma (100% activity) as a calibrator.

This vortex-based assay is able to detect the cleavage of VWF with only 0.25-1.0 μ l of plasma, corresponding to 0.125-0.25 nM of plasma ADAMTS13 enzyme (final concentration). In comparison with the denatured assay, our vortex assay appears to be more sensitive analytically assessing plasma ADAMTS13 activity (Fig. 3). Interestingly, a small amount of plasma (0.25 μ l) added into the reaction dramatically increased the cleavage of VWF by recombinant ADAMTS13 (Fig. 4). These results suggest that plasma may contain cofactors that accelerate VWF proteolysis under fluid shear stresses. The nature of the accelerating cofactors is yet to be identified. Moreover, when too much plasma either heat-inactivated or unheated (>1 μ l) was added into the reaction with a total of 20 μ l, the proteolytic cleavage of VWF by recombinant ADAMTS13 dramatically reduced (Fig. 4). These results suggest that certain ions such as Cl⁻ and citrate or a change of pH value may inhibit VWF proteolysis by ADAMTS13 under fluid shear conditions.

Consistent with the literature, however, plasma and recombinant ADAMTS13 protease are highly sensitive to exogenous Ca²⁺, Zn²⁺⁴⁵ and Cl⁻ ions³⁸. The optimal concentrations of CaCl₂ and ZnCl₂ in the shear-based assay are within the physiological range (i.e. 5 mM and 17 μ M, respectively) (Fig. 5). The concentration of NaCl that inhibits 50% of plasma and recombinant ADAMTS13 activity is estimated to be ~75 mM (Fig. 6). Therefore, additional NaCl is not required in the assay reaction to achieve the maximal analytical sensitivity.

Using the conditions established in the preliminary experiment, we have determined plasma ADAMTS13 activity in patients with acquired TTP. Most plasma samples randomly selected from idiopathic TTP (75%) show severely reduced ADAMTS13 activity (less than 10%) under shear stress (Table 1 and Fig. 7). These results are in a good agreement with those obtained from the fluorogenic assay with the correlation coefficient, $r=0.769$ (Fig. 9), similar to that reported in the literature for the comparison between the VWF multimer assay and the fluorogenic assay^{46,47}. Most patients with severe deficiency of plasma ADAMTS13 activity by the rF-VWF73 assay were also severely deficient by the vortex assay (Table 1 and Fig. 7). However, there are several discrepant results observed between the two assays. For instance, patients 24 and 120 who have plasma ADAMTS13 activity of 19% and 18%, respectively, by rF-VWF73, show no detectable cleavage of VWF (less than 5%) under fluid shear stress (Table 1). The discrepancy may reflect the biological differences of plasma samples as a result of anti-ADAMTS13 IgG binding and inhibition. For example, the domains of ADAMTS13 required for cleavage of multimeric VWF either under denatured conditions³⁶ or under shear stress^{30,31} may not be necessarily the same as for cleavage of the peptide substrates. Therefore, a normal ADAMTS13 activity in TTP patients assayed by the cleavage of a peptidyl substrate should be confirmed by its ability to cleave multimeric VWF under more physiological conditions. A recent report suggests that a combination of several assay methods may help reveal underlying mechanism of TTP^{46,47}.

Our novel shear-based assay appears to be useful for assessing plasma anti-ADAMTS13 inhibitors in patients with idiopathic TTP. As shown, the presence of inhibitors assessed by the shear-based assay is in a good agreement with that obtained by the rF-VWF73 assay. In most cases, a plasma sample that is positive by the rF-vWF73 assay is also positive by the vortex-based assay with only a few exceptions (i.e. patients 103 and 110) (Table 1). These results indicate that some plasma substance that inhibits the cleavage of a fluorogenic substrate by ADAMTS13 may not necessarily block the cleavage of multimeric VWF by ADAMTS13 under shear stress. For instance, an elevated plasma bilirubin in TTP patients with ongoing hemolysis may quench fluorescent signal, resulting in falsely low ADAMTS13 activity assessed by the fluorogenic assay, but not by other assays which do not rely on the detection of fluorescence^{48,49}.

In conclusion, we have demonstrated that a vortex-based assay may be useful for the investigation of the basic biological function and regulation of ADAMTS13 activity under fluid shear stress; the assay may also be applicable for assessing plasma ADAMTS13 activity and inhibitors in patients with acquired (idiopathic) TTP. However, the sensitivity and specificity of this assay for diagnosis of TTP remain to be determined owing to the lack of a gold standard for TTP diagnosis and the inadequate surveillance of plasma ADAMTS13 activity and inhibitors in patients with hematological disorders other than TTP.

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Fig. 1. Characterization of VWF substrate and optimization of agarose gel concentration for detection

Panel A. 10% SDS-PAGE with Coomassie blue staining visualized purified plasma VWF (lane 2, arrowhead); *Panel B.* 1% agarose gel electrophoresis plus Western blotting determined the purified plasma VWF (lane 1), the UL-VWF released from washed HUVECs upon histamine stimulation (lane 2), and VWF in the conditioned medium of cultured HUVECs (lane 3); *Panel C.* Plasma VWF (150 nM) was incubated under constant vortex with 25 nM recombinant ADAMTS13 in the presence of 10 mM EDTA (lane 1) and in the absence of EDTA (lane 2). The cleavage of VWF was determined by 1% agarose gel plus Western blotting; *Panels D and E.* Plasma VWF (150 nM) was incubated under vortexing with 1 μ l of plasma (final concentration, 0.25 nM) in the presence of 10 mM EDTA (lane 1) or in the absence of EDTA (lane 2) for 60 min. The cleavage products were determined by 1.5% (*panel D*) and 2.5% (*panel E*) agarose gel and Western blotting. Band “e” in panel E-lane 2, a newly formed proteolytic cleavage product, was used for quantification of all subsequent experiments.

Fig. 2. Vortexing times required for detecting the proteolytic cleavage of VWF by plasma ADAMTS13

Panel A. Purified plasma VWF (150nM) was incubated under constant vortexing (2,500 rpm) with 1 μ l of plasma (final concentration, 0.25 nM) for various times (0, 5, 10, 20, 30, 60 and 120 min) in the absence (-) and the presence (+) of 10 mM EDTA. The proteolytic cleavage product (arrowhead) was determined by 2.5% agarose gel electrophoresis and Western blotting; *Panel B.* The fluorescent signal that was converted into gray scale was quantified by densitometry using NIH *ImageJ* software. The results represent the means $\pm$ standard deviation of 4 independent experiments.

Fig. 3. Concentration-dependent cleavage of VWF under shear stress and denaturing conditions Purified VWF (150 nM) was incubated with various volumes of plasma (0–4 μ l) or various concentrations of recombinant ADAMTS13 (0–200 nM) under vortexing conditions (*panels A and D*) or in the presence of 0.15 M guanidine-HCl (*panels B and E*) for 60 min. The proteolytic cleavage products were determined by 2.5% agarose gel electrophoresis and Western blotting. Panel C and F: The signal of cleavage product (arrowheads indicated) was quantified by densitometry using NIH ImageJ software and plotted against the amount of plasma (μ l) (*panel C*) or the concentration of recombinant ADAMTS13 (*panel F*). The results represent the means $\pm$ standard deviation of at least 3 independent experiments.

Fig. 4. Effect of plasma on cleavage of VWF by recombinant ADAMTS13 under shear stress
Panel A. Purified VWF (150 nM) was vortexed with recombinant ADAMTS13 at various concentrations (0-100 nM) in the absence (-NHP) and presence of 0.25 μ l heat-inactivated plasma (+NHP) for 60 min at 2,500 rpm. *Panel B.* Purified VWF (150 nM) was vortexed for 60 min with recombinant ADAMTS13 (25 nM) in the presence of various amount (0-4 μ l) of heated or unheated plasma from a hereditary TTP patient. The proteolytic cleavage of VWF by ADAMTS13 (arrowheads) was determined by 2.5% agarose gel electrophoresis and Western blotting.

Fig. 5. Effects of zinc ion on cleavage of VWF by ADAMTS13 under shear stress

Purified VWF (150 nM) was incubated with 1.0 μl of plasma (final concentration, 0.25 nM) (*panels A and B*) or recombinant ADAMTS13 (*panels C and D*) in the presence of ZnCl₂ (0-500 μM) or in the absence (-) or presence (+) of 10 mM EDTA for 60 min under constant vortexing at 2,500 rpm. The proteolytic cleavage products were determined by Western blotting. The signal of cleavage product was quantified by densitometry using NIH *ImageJ* software. The results are representative of the mean and standard deviation of at least four experiments (*panels B and D*).

Fig. 6. Effects of sodium chloride on cleavage of VWF by ADAMTS13 under shear stress
Purified plasma VWF (150 nM) was incubated with 1.0 μ l of plasma (final concentration of ADAMTS13, 0.25 nM) (*panel A*) or recombinant ADAMTS13 (25 nM) (*panel C*) for 60 min under constant vortexing at 2,500 rpm in the presence of NaCl (0-300 mM) and in the absence or the presence of 10 mM EDTA. The proteolytic cleavage product was determined by 2.5% agarose gel and Western blotting and quantified by densitometry using NIH *ImageJ* software. The results indicate the means $\pm$ standard deviation of three independent experiments (*panels B* and *C*).

Fig. 7. Plasma ADAMTS13 activity in patients with acquired TTP

Purified VWF (150 nM) was incubated with 1 μ l of pooled normal human plasma (NHP) or patient plasma from idiopathic TTP in the absence (-) and the presence (+) of EDTA (10 mM) for 60 min under constant vortexing at 2,500 rpm. The proteolytic cleavage product (arrowhead) was determined by 2.5% agarose gel and Western blotting.

Fig. 8. Plasma ADAMTS13 inhibitor in patients with acquired TTP

Pooled normal human plasma (1 μ l) was incubated with 1 μ l of heat-inactivated normal human plasma (control) or patient plasma for 60 min at room temperature. The residual activity in the normal human plasma was determined by both shear-based assay and fluorogenic rF-VWF73 assay. Greater/or equal to 50% of reduction in proteolytic activity in normal human plasma after 50:50 mixing was considered to be positive based on the relative product formation in the absence of EDTA minus that in the presence of EDTA (20 mM).

Table 1
Patient clinical characteristics, plasma ADAMTS13 activity and inhibitors

Serial #	Pt ID#	Age (Y)/Sex	TTP Diagnosis	PEX* (Times)	Outcome	A13 Activity (%)			Anti-A13 Inhibitor		
						FRETs	Vortex	ELISA (U/ml)	FRETs	Vortex	
1	12	62/F	Idiopathic	0	Died	0.0	4.5	34.2	Neg	Neg	Neg
2	13	62/F	Idiopathic	10	Rel/Rem	0.0	9.9	68.6	Pos	Pos	Pos
3	24	39/F	Idiopathic	7	Rel/Rem	19.0	1.8	75.7	Pos	Pos	Pos
4	29	46/F	Idiopathic	2	Died	12.0	27.0	24.9	Neg	Neg	Neg
5	33	52/F	Idiopathic	3	Rel/Rem	7.0	0.0	50.2	Pos	Pos	Pos
6	36	44/F	Idiopathic	>30	Rel/Rem	6.0	1.1	54.3	Pos	Pos	Pos
7	42	77/F	Idiopathic	4	Rel/Rem	62.0	113.0	32.5	Neg	Neg	Neg
8	60	42/F	Idiopathic	12	Rem	68.0	111.0	22.7	Neg	Neg	Neg
9	83	21/F	Idiopathic	3	Rem	16.0	57.5	22.4	Neg	Neg	Neg
10	86	42/F	Idiopathic	0	Rem	0.0	6.1	33	Pos	Pos	Pos
11	113	79/F	Idiopathic	6	Rel/Rem	0.0	7.5	75.5	Pos	Pos	Pos
12	120	37/F	Idiopathic	0	Rem	18.0	2.0	>100	Pos	Pos	Pos
13	155	47/F	Idiopathic	6	Rem	3.0	0.0	ND	Pos	Pos	Weak Pos
14	175	21/F	Idiopathic	0	Rem	0.0	9.4	>100	Pos	Pos	Pos
15	219	23/F	Idiopathic	0	Rem	0.0	0.0	>100	Pos	Pos	Pos
16	248	33/F	Idiopathic	0	Rem	0.0	6.7	>100	Pos	Pos	Pos
17	53	44/M	BMT	13	Rel/Rem	39.0	82.6	9.1	Neg	Neg	Neg
18	103	25/M	BMT	4	Died	18.0	14.0	ND	Pos	Neg	Neg
19	107	45/F	BMT	3	Rem	60.0	2.5	22.2	Neg	Neg	Neg
20	110	54/M	BMT	0	Rem	21.0	7.9	ND	Pos	Neg	Neg
21	181	19/M	BMT	1	Rem	86.0	67.3	ND	Neg	Neg	Neg
22	7-5	3/F	BMT	0	Rem	100.0	74.8	ND	Neg	Neg	Weak Pos

* PEX is the number of plasma treatment received at the time of sample collection; BMT, bone marrow transplantation associated TTP Rel/Rem is the relapse followed by remission; A13, ADAMTS13; IgG, immunoglobulin G; Pos, positive; Neg, negative Anti-ADAMTS13 IgG <15 U/ml is considered to be negative; 30% reduction in cleaving activity after 50:50 mix by FRETs or vortex is considered to be positive

Indicates the serial number of this table (column 1) and patient sample ID (column 2). All 22 samples were from different TTP patients.