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ADAMTS13 unbound to larger von Willebrand factor multimers in cryosupernatant: Implications for selection of plasma preparations for TTP treatment

Short title:

ADAMTS13 unbound to larger VWF multimers in cryosupernatant

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Abstract

BACKGROUND: Thrombotic thrombocytopenic purpura (TTP) is characterized by deficient ADAMTS13 activity. Treatment involves plasma exchange (PE). Both fresh frozen plasma (FFP) and cryosupernatant (CSP) are used, but it remains to be determined which is more effective.

STUDY DESIGN AND METHODS: To analyze the interaction between von Willebrand factor (VWF) and ADAMTS13, we used large-pore isoelectric focusing (IEF) analysis followed by detection with anti-ADAMTS13 monoclonal antibody. FFP, CSP, cryoprecipitate (CP), and purified ADAMTS13 were analyzed for their effects on high shear stress–induced platelet aggregation (H-SIPA).

RESULTS: IEF analysis of normal plasma revealed three groups of ADAMTS13 bands with pI of 4.9–5.6, 5.8–6.7, and 7.0/7.5. Two band groups (pI 4.9–5.6 and 5.8–6.7) were found in plasma of a patient with type 3 von Willebrand disease, in which VWF is absent, whereas no bands were found in plasma of a patient with congenital ADAMTS13 deficiency. Mixing these plasmas generated the bands at pI 7.0/7.5, representing the VWF-ADAMTS13 complex; these bands were absent in CSP. FFP and purified ADAMTS13 down-regulated H-SIPA in a dose-dependent manner. However, CP did not inhibit H-SIPA in the initial phase, and the degree of inhibition at the endpoint was almost indistinguishable from those of the other two plasma products.

CONCLUSION: Both plasma products (FFP, and CSP) were effective for PE in TTP patients. However, CSP may be more favorable, because it has lower levels of VWF, and almost normal ADAMTS13 activity, but lower levels of ADAMTS13 in complex with larger VWF multimers.

Key words: IEF, VWF-ADAMTS13 complex, VWF, ADAMTS13

ABBREVIATIONS: ADAMTS13=a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13, VWF=von Willebrand factor, UL-VWFMs=unusually large VWF multimers, CP=cryoprecipitate, CSP=cryosupernatant, IEF: isoelectric focusing, H-SIPA= high shear stress induced platelet aggregation

von Willebrand factor (VWF), a multimeric hemostatic glycoprotein, is secreted from vascular endothelial cells into circulation as unusually large VWF multimers (UL-VWFMs).[1] The UL-VWFM is the most biologically active form with regard to platelet adhesion properties.[2] Under conditions of high shear stress, UL-VWFMs cause enhanced platelet aggregation and gives rise to VWF-rich thrombi in the microvasculature. ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13) down-regulates the function of UL-VWFMs by cleaving the VWF A2 domain at the Tyr1605–Met1606 bond, yielding a series of smaller molecular forms.[3-5] The proteolytic activity of ADAMTS13 is located in its amino-terminal metalloprotease domain, but optimal enzyme activity requires cooperative interactions with other domains of the ADAMTS13 molecule. [6]

Deficiency of ADAMTS13 activity causes a life-threatening generalized disease, thrombotic thrombocytopenic purpura (TTP), which can be caused either by mutation of the ADAMTS13 gene (Upshaw-Schulman syndrome, USS) or by acquisition of autoantibodies against the ADAMTS13 enzyme.[7] USS is often treated by prophylactic infusions of fresh frozen plasma (FFP) as a source of ADAMTS13, but cryoprecipitate (CP) has also been effective.[8,9] On the other hand, for patients with acquired TTP, plasma exchange (PE) is the first-line treatment.[10] For PE treatment, cryosupernatant (CSP) is preferentially used in Canada, but FFP is used in many other countries, including Japan. However, it has not been firmly established which material is more favorable for PE.[11] In this context, immunoprecipitation using anti-VWF antibodies revealed that approximately ~3% of ADAMTS13 in plasma is bound to VWF, with a

stoichiometry of one ADAMTS13 molecule to 250 VWF monomeric subunits.[12] However, the characteristics of the VWF-ADAMTS13 complex in the plasma milieu remain unclear, as does the physiological relevance of functional differences, if any, between the bound and unbound forms of ADAMTS13.

To address these issues and analyze the VWF-ADAMTS13 complex in the plasma milieu, we employed isoelectric focusing (IEF) analysis using a large-pore agarose-acrylamide composite gel. Using this method, we were able to visualize the VWF-ADAMTS13 complex. We found that in the plasma milieu, ADAMTS13 forms a complex with larger VWFMs, but is less likely to do so with smaller VWFMs (dimers and tetramers); the complex can be separated from its unbound counterpart by cryoprecipitation. Based on these observations, we hypothesize that the bound and unbound forms of ADAMTS13 possess functional differences with respect to the microvascular condition of the patient. Furthermore, we evaluated the functional differences between ADAMTS13 in CSP and CP, by testing their inhibitory effects on high shear stress–induced platelet aggregation (H-SIPA).

MATERIALS and METHODS

Plasma samples

Anti-coagulated blood containing one-tenth volume of 3.8% sodium citrate was collected from normal individuals and from patient with either type 3 von Willebrand

disease (T3-VWD) or Upshaw-Schulman syndrome (USS: patient USS-EE4). The citrated plasmas were then separated by centrifugation and stored at −80°C until use. T3-VWD plasma had less than 3% of the normal control levels of both VWF antigen and ristocetin cofactor. The USS-EE4 patient had plasma levels of both ADAMTS13 activity and antigen less than 0.5% and 0.1% of the normal control, respectively; the ADAMTS13 gene mutation was identified as 2259delA/2259delA.[8] Informed consent was obtained from all subjects.

Preparation of cryosupernatant (CSP) and cryoprecipitate (CP)

FFP was prepared at Nara Red Cross Blood Center and stored in inventory at −30°C. However, FFP preparations beyond one year for the inventory were kindly provided to us. These FFP preparations were then kept frozen at −80°C in our institution. Outdated FFP was then thawed overnight at 4°C, followed by centrifugation at 7,000×*g* for 30 min at 4°C. After centrifugation, the CSP was separated and kept frozen in aliquots at −80°C. For analysis of ADAMTS13 activity and VWF antigen, the CP was dissolved in one-fifth volume of 20 mM Tris-buffered saline (TBS, pH 7.4) without rinsing.

For H-SIPA, the CP was rinsed with cold TBS containing 0.38% Na_3-citrate, 2 mM benzamidine-HCl, 20 mM 6-amino-*n*-caproic acid, and 0.02% NaN_3, and then centrifuged at 4°C. This procedure was repeated twice. Ultimately, the CP was dissolved in one-tenth volume of TBS containing 0.38% Na_3-citrate, and then stored in aliquots at −80°C.

Purified plasma VWF and ADAMTS13

Purification of plasma VWF was performed essentially as previously described[13]: cryoprecipitation of outdated pooled FFP collected from normal volunteers, removal of fibronectin by gelatin-agarose affinity chromatography, precipitation with 40% saturated $(NH_4)_2SO_4$, and finally purification by size-exclusion chromatography in Sepharose 4B gel. Fractions eluted in the anterior half of the void volume of the Sepharose 4B column was pooled; the resulting protein consisted of higher VWF multimers and migrated as a single 250 kD band on a SDS-5% polyacrylamide gel under reducing conditions.[13] After dialysis against TBS, the purified plasma-derived (pd)-VWF was kept frozen in aliquots at −80°C until use.

Purification of plasma ADAMTS13 was achieved using anti-ADAMTS13 monoclonal antibody A10 (IgG2b-k)–coupled beads as recently described.[14] The epitope of A10 resides on the disintegrin-like domain of ADAMTS13.[15] Briefly, the CSP was prepared essentially as described above, from outdated FFP in the presence of two protease inhibitors (2 mM benzamidine-HCl and 20 mM 6-amino-n-caproic acid) and 0.02% NaN_3. The CSP was then applied to an A10-coupled column at 4°C and washed extensively. The ADAMTS13 bound to the column was eluted in two steps, first with 10% dimethylsulfoxide (DMSO), and then with 40% DMSO. The ADAMTS13 eluted with 40% DMSO was pooled and concentrated, and then purified by size exclusion chromatography on a Superdex HR10 column. The purified pd-ADAMTS13 migrated on a SDS-5% polyacrylamide gel as a single 170 kD band before reduction, and a single 190 kD band after reduction; specific activity was 300 unit/mg.[14] One unit

of ADAMTS13 activity was defined as the amount contained in 1 mL of pooled normal plasma.

Assays for ADAMTS13 and VWF

The ADAMTS13 activity and antigen were measured with a chromogenic ADAMTS13-act-ELISA,[16] and an in-house sandwich ELISA using two monoclonal antibodies, respectively.[17] The VWF antigen was determined with a sandwich ELISA using a rabbit polyclonal anti-human VWF antibody (Dako Cytomation, Kyoto, Japan).[18] A value of one hundred percent of the ADAMTS13 activity and antigen were defined as the amount in the pooled normal human plasmas, which were prepared from a total of 40 normal volunteers, consisted of each 10 individuals with different ABO blood groups.

Preparation of ADAMTS13-depleted plasma

The ADAMTS13-depleted (dp) plasma was prepared from the whole FFP using an A10-agarose column equilibrated with TBS at room temperature. Flowthrough fractions were monitored with ADAMTS13 activity and antigen, both the values indicated less than 0.5% and 0.1% of the normal, respectively, and were pooled and stored in aliquots at -80 ºC.

IEF using an agarose-acrylamide composite gel

IEF gel plate was assembled with two glass plates and 1mm thick plastic spacers.

Four grams sucrose and 0.3 g agarose (final 0.75%, Agarose IEF, GE Healthcare Bio-Science AB, Sweden) were mixed with 34.2 mL distilled water. The mixture was dissolved by microwave oven and kept at 56 ºC. Then, 1.67 mL 30% acrylamide-bisacrylamide (final 1.25%), 1.67 mL distilled water, 2.5 mL 40% Pharmalyte ™ 3-10 (GE Healthcare Bio-Science AB, Sweden), 0.27 mL ammonium peroxodisulfate (22.8 mg/mL) and 0.01 mL N,N,N',N'-tetramethylethylenediamine were added to this mixture. The mixture was poured into the IEF gel plate quickly and left it for more than 1 hour at room temperature followed by 4 °C overnight.

The IEF gel was placed on the Multiphor apparatus (GE Healthcare Bio-Science AB, Sweden) equilibrated at 10°C. The electrode strips were prepared using 0.5 M acetic acid at the anode and 0.5 M sodium hydroxide at the cathode. The electrical conditions used for IEF were the first 30 min at a maximum of 100 V, 5 mA and 15 W: then 60 min at a maximum of 200 V, 10 mA and 6 W: and finally 90 min at a maximum of 1500 V, 15 mA and 6 W. After IEF, the isolated proteins were electrophoretically transferred to nitrocellulose membrane.

Iodoacetamide effect on complex of ADAMTS13 and VWF in plasma milieu

Recent studies have indicated that free thiols exposed in ADAMTS13 play an important role to regulate thiol-disulfide exchange of VWF under a high shear stress. Furthermore, blocking these active thiols decreases ADAMTS13 activity in cleaving UL-VWFM under flow conditions.[19] We evaluated the effect of iodoacetamide (IAA), which blocks the free thiols and prevents the formation of a covalent complex through

disulfide bonds. For this experiment, each reagent of ADAMTS13-dp plasma, purified pd-ADAMTS13, and pd-VWF was separately treated with or without 100 mM IAA before mixing for 30 min at room temperature. The mixture of these 3 reagents was exposed to a high shear stress generated by a vortex mixer at 3200 rpm for 5 min. The final concentration of each reagent in this mixture (a total of 130 μL) was 60 μg/mL for pd-VWF, 2.3 μg/mL for purified pd-ADAMTS13, and 65 μL for ADAMTS13-dp plasma.

Two-dimensional gel electrophoresis using either PAGE or agarose

In some experiments, after IEF the two-dimensional gel electrophoresis was performed using either SDS-5% PAGE under reducing conditions or SDS-0.9% agarose gel electrophoresis under non-reducing conditions. The former was used for an analysis of ADAMTS13 antigen and the latter for VWF multimer patterns. In both the instances, the separated proteins were electrophoretically transferred to PVDF membrane or nitrocellulose membrane, and then the blot proteins were immuno-reacted with anti-ADAMTS13 mAb (WH2-11-1, an epitope residing on the 4th thrombospondin type-1 domain of ADAMTS13)[20] or rabbit polyclonal anti-human VWF antibody, and then were visualized by chemiluminescent detection kits (Perkin-Elmer Life Science, Inc., Boston, MA).

H-SIPA in the absence of ADAMTS13

To reproduce platelet aggregation assumed to be occurring in TTP patients, H-SIPA

at a constant shear rate of 108 dynes/cm^2 was measured with an argon laser-assisted cone platelet aggregometer (Toray Medical, Tokyo),[21] using a mixture of normal washed platelets (300x10^9/L, final), ADAMTS13-dp plasma (29% v/v, final), and the purified pd-VWF (250 % of the normal plasma, final).

For this assay, normal washed platelets were prepared and suspended in a Hepes-Tyrode buffer (pH 7.3) containing 1.8 mmol/L $CaCl_2$.[22] The mixture with a total volume of 400 μL was preincubated at 37 ºC for 5 min, and then H-SIPA was measured for 6 min. The maximum platelet aggregation was seen in the absence of any additives, and the minimum or nonspecific platelet aggregation was determined in the presence of anti-VWF monoclonal antibody NMC-4 (10 μg IgG/mL, final), which totally blocks the VWF binding to platelet GPIb.[13]

For assessment of the inhibitory effect of various forms of pd-ADAMTS13 to H-SIPA, they were spiked into the abovementioned assay mixtures and incubated for 5 min at 37 ºC before measurement. H-SIPA was measured at room temperature and completed within 2.5 h after blood collection. The inhibition rate of H-SIPA was calculated in the following formula: Inhibition rate (%) = [1- (% light transmittance of tested sample / % light transmittance of control)] x100. These data were expressed as the mean±SD. We calculated the inhibition rate in two points at 140 seconds and 340 seconds after the initiation of H-SIPA. Comparison between these 2 points were tested for statistical significance using paired t-test using StatView (SAS Institute Inc, Cary, NC, USA). A p-value <0.05 was considered significant.

RESULTS

ADAMTS13 and VWF on IEF agarose-acrylamide composite gels

We detected pd-ADAMTS13 (15 ng) as one band at pI 4.9–5.6 (median 5.4) using anti-ADAMTS13 monoclonal antibody (WH2-11-1) on IEF agarose-acrylamide composite gels (Fig 1, left panel). Next, we analyzed various amounts (1–10 μL) of normal citrated plasma, and found that ADAMTS13 antigen in the plasma milieu could also be detected as a major band at pI 4.9–5.6, as in the case of purified pd-ADAMTS13. In plasma, however, two additional bands of ADAMTS13 antigen were also detected: one was composed of a cluster of blurred bands at pI 5.8–6.7, and the other consisted of two clear bands at pI 7.0/7.5. In T3-VWD plasma, two groups of ADAMTS13 bands, pI 4.9–5.6 and 5.8–6.7, were detected, but the bands at pI 7.0/7.5 were totally absent (Fig.1 right panel). T3-VWD plasma lacks VWF antigen; therefore, the two groups of bands at pI 4.9–5.6 and 5.8–6.7 appear to exist independently of the presence of plasma VWF. Conversely, we assumed that the bands at pI 7.0/7.5 represented a complex with VWF that exists within the plasma milieu. The bands at pI 7.0/7.5 were also detected after mixing FFP with 1 M NaCl (final), excluding the possibility that the complex is formed by an ionic linkage (data not shown).

Generation of the pI 7.0/7.5 band of ADAMTS13 complex with VWF

Next, we performed the mixing experiments shown in Fig. 2A. T3-VWD plasma

spiked with purified pd-VWF yielded a new band at pI 7.5. USS-EE4 plasma initially lacked three groups of ADAMTS13 bands (pI 4.9–5.6, 5.8–6.7, and 7.0/7.5), but once that plasma was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When T3-VWD and USS-EE4 plasma samples were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTS13.

ADAMTS13 (pI 7.5) is a non-covalent complex with VWF in the plasma milieu

We next evaluated the effects of IAA, which blocks free thiols and prevents the formation of disulfide bond–mediated covalent complexes, under high shear stress in a vortex mixer. As shown in Fig. 2B, the band at pI 7.5, representing the VWF-ADAMTS13 complex, was generated irrespective of the presence of IAA. When pd-VWF was spiked into this mixture, the density of the band at pI 7.5 increased. These results indicate that in our experiments, formation of the VWF-ADAMTS13 complex does not depend upon disulfide bond bridges.

ADAMTS13 is present in plasma in complex with a large VWFM

As shown in Fig. 3, IEF gel analysis of normal plasma revealed ADAMTS13 as three groups of bands (pI 4.9–5.6, 5.8–6.7, and 7.0/7.5) (also see Fig. 1), and VWF antigen was largely separated into four series of bands: pI 4.8–5.6 (trace), 6.0–6.7, 7.1–7.8, and 7.9–8.4 (Fig. 3, middle).

Since two ADAMTS13 bands with pI 4.9–5.6 and 5.8–6.7 were seen in T3-VWD

plasma (Fig. 1), both bands appeared to be present in plasma irrespective of the presence of VWF. Further, two-dimensional analysis of normal plasma (IEF gel followed by SDS-0.9% agarose gel electrophoresis) confirmed that ADAMTS13 forms a complex with a larger VWFM with pI 7.1-7.8, but less likely with a smaller VWFM (dimers and tetramers) with pI 4.8-5.6 (Fig. 3 lower panel).

Amounts of ADAMTS13 and VWF in CP and CSP

Gill et al.[23] reported that the level of VWF antigen in plasmas from normal individuals with blood group O is significantly lower than that in plasmas with non-O blood groups. Further, Feys et al.[12] indicated that ADAMTS13 is bound to VWF with a stoichiometry of one ADAMTS13 molecule to 250 VWF monomeric subunits. Taken these two reports together, it is conceivable that the amount of a complex of ADAMTS13 and VWF in CP could be influenced by the ABO blood groups.

We analyzed ADAMTS13 activity and VWF antigen in FFP, CSP, and CP from 120 normal volunteers, with 30 individuals of each ABO blood type (A, B, O, and AB). The recovery rates of ADAMTS13 activity and VWF antigen in CP or CSP were expressed as the level in CP or CSP divided by the sum of the levels in both (CP + CSP). As summarized in Table 1, an average of 7.3% (range, 4.8–8.8%) of plasma ADAMTS13 was recovered in CP, whereas an average of 92.7% (range, 91.2–95.2%) remained in CSP. The amounts of ADAMTS13 remaining in CP from A, O, B, and AB plasmas were 5.4±2.4%, 3.5±1.6%, 6.5±2.9%, and 6.9±2.8%, respectively; the amount of ADAMTS13 in CP was significantly lower in blood group O than in other blood

groups. On the other hand, an average of 86.0% (range, 85.4–87.3%) of plasma VWF antigen was recovered in CP, whereas an average of 14.0% (range, 12.7–14.6 %) remained in CSP. The amounts of VWF in FFP, CP, and CSP from blood group O were significant lower than in samples from other blood groups. The recovery rate of VWF antigen in CP was significantly higher in blood group A than in other blood groups.

As shown in Fig. 4, we analyzed the correlation between the levels of ADAMTS13 activity and VWF antigen in three plasma preparations with the Spearman rank test. We did not find a significant correlation between VWF antigen in FFP and ADAMTS13 activity in either FFP or CSP. In contrast, we did observe a significant correlation between ADAMTS13 activity in CP and VWF antigen in FFP ($r=0.646$, $p<0.01$) (Fig. 4, right). These results indicate that the decreased level of ADAMTS13 in CP of blood group O was correlated with the low level of VWF.

Cryoprecipitation efficiently removes the VWF-ADAMTS13 complex from plasma

To determine whether cryoprecipitation can remove the VWF-ADAMTS13 complex from plasma, we performed two-dimensional analysis (IEF followed by SDS-5% PAGE) under reducing conditions. As shown in Fig. 5A, all three groups of ADAMTS13 bands (pI 4.9–5.6, 5.8–6.7, and 7.0/7.5) in normal plasma migrated mainly as a 190 kD band in SDS-5% PAGE, indicating that all three groups of bands included ADAMTS13. In CSP, however, the pI 7.0/7.5 band of ADAMTS13 was totally absent, almost indistinguishable from the case of T3-VWD plasma (Fig. 5, B and D). Furthermore, when CSP was spiked with purified pd-VWF, a new band with pI 7.5 was

generated (data not shown), indicating that ADAMTS13 in CSP can bind to higher molecular weight VWFMs and form a complex, as is the case in FFP.

In CP, we observed two faint bands with pI ranges of 4.9–5.6 and 5.8–6.7, and also several strong bands with pI greater than 7.0 (Fig. 5C).

Down-regulation of H-SIPA with purified pd-ADAMTS13, CP, and CSP

In H-SIPA using a mixture of normal washed platelets, ADAMTS13-dp plasma, and purified pd-VWF, maximum platelet aggregation (~70% light transmittance) was achieved in the absence of ADAMTS13 (Fig. 6A). Under this condition, purified pd-ADAMTS13 spiked into the mixture inhibited H-SIPA in a dose-dependent manner at ranges of 5–20% of ADAMTS13 activity, but this effect reached a plateau (~20% light transmittance) at the ranges from 50% to 500% of ADAMTS13 activity (Fig. 6A). Addition of NMC-4 almost totally blocked the platelet aggregation (~3% light transmittance).

Further, ADAMTS13 in both FFP and CSP from normal individuals inhibited H-SIPA in a dose-dependent manner at the ranges of 5–20% of ADAMTS13 activity (Fig. 6 B and C), comparable to the effect of purified pd-ADAMTS13.

On the other hand, ADAMTS13 in CP did not clearly inhibit H-SIPA at the initial phase before 140 seconds, even at 20% of ADAMTS13 activity. However, at the later phase of H-SIPA, the aggregation curves were uniformly reversed at a final concentration of 5–20% of ADAMTS13 activity. Consequently, the maximum platelet aggregation at the endpoint at 340 seconds was almost indistinguishable from that of

FFP or CSP. Thus, the inhibition rates (%) in CP at two time points (140 and 340 seconds) with two different final concentrations (5 and 20%) of ADAMTS13 activity were measured in each 3 times at the same occasion, and the results were the followings: 20.5±14.0% (at 140 seconds) versus 46.9±11.3% (at 340 seconds) ($p=0.012$) in the presence of 5% ADAMTS13 activity, and 57.7±5.9% (at 140 seconds) versus 85.7±2.7% (at 340 seconds) ($p=0.004$) in the presence of 20% ADAMTS13 activity (Figure not shown).

DISCUSSION

Using IEF analysis with a large-pore agarose-acrylamide composite gel, we have shown that ADAMTS13 in the plasma milieu is present in a complex with larger VWFMs, but is less likely to form complexes with smaller VWFMs (dimers and tetramers). Thus, cryoprecipitation followed by centrifugation could efficiently separate the two forms of ADAMTS13, with the VWF-bound ADAMTS13 in CP and the free ADAMTS13 and ADAMTS13 bound to smaller VWFMs in CSP. In support of these results regarding co-precipitation of ADAMTS13 and VWF, the ADAMTS13 activity levels we observed were closely correlated with VWF antigen levels in CP ($r=0.646$), but not in either CSP ($r=-0.055$) or FFP ($r=0.002$) (Fig. 4). In addition, we observed no difference among blood groups with respect to the recovery rate of VWF antigen in CP, but the VWF antigen level in CP was lower in blood group O than in the other blood

groups. As a result, both the ADAMTS13 activity and VWF antigen levels in CP were significantly lower in blood group O than in non-O blood groups (Table 1). Further, we determined that ~95% of the original ADAMTS13 activity in FFP is recovered after cryoprecipitation; ~93% of the recovered ADAMTS13 activity remained in CSP, whereas 7% was found in CP. This relative distribution of ADAMTS13 in FFP and CSP was consistent with previous reports.[24-26]

Evidence that the pI 7.0/7.5 band is a complex of VWF and ADAMTS13 is clearly provided by the following observations: 1) plasmas from both VWF antigen–defective T3-VWD and ADAMTS13 antigen–defective USS patients lacked the bands at pI 7.0/7.5; 2) an equal mixture of plasmas from T3-VWD and USS generated the bands at pI 7.0/7.5; and 3) CSP prepared from normal plasma lacked the bands at pI 7.0/7.5, whereas CSP spiked with purified VWF regenerated these bands. On the other hand, we assume that the proteins in the two other band groups (pI 4.9–5.6 and 5.8–6.7) are less involved in complex formation with VWF, because both band groups are present in T3-VWD plasma. Furthermore, because pd-ADAMTS13 purified from pooled normal plasmas has only one band with pI 4.9–5.6,[14] the pI 5.8-6.7 band might represent a complex with proteins other than VWF. This speculation originates from the observation that ADAMTS13 can bind *in vitro* to a soluble form of CD36[27] and Lys-plasminogen.[28]

In a previous study, immunoprecipitation method using anti-VWF antibodies was used to show that ~3% of the total in plasma ADAMTS13 is bound to VWF.[12] By contrast, in our IEF gel analysis, coupled with densitometry, we observed that the

amount of VWF-bound ADAMTS13 in plasma appeared to be much lower than the amount of unbound ADAMTS13, but greater than the 3% of total ADAMTS13 (Fig. 1).[12] This discrepancy might be attributable to differences in the experimental designs employed in these studies.

The mechanism by which ADAMTS13 binds to VWF in the plasma milieu is a critical issue that remains to be addressed. Because Fujikawa et al.[29] succeeded in purifying ADAMTS13 from a commercial concentrate of factor VIII/VWF, prepared from cryoprecipitate, such concentrates might contain the VWF-ADAMTS13 complex itself. After extensive fractionation, including fibrin-clot formation, ammonium sulfate precipitation, and sequential chromatography, the purified ADAMTS13 described in that study was free of VWF. However, in our experience, the VWF-ADAMTS13 complex in CP is not readily dissociated by size-exclusion chromatography in the presence of either 0.15 M or 1 M NaCl (data not shown). In fact, when we treated CP with 1 M NaCl for 1 h at room temperature before IEF, the bands at pI 7.0/7.5 persisted, indicating that no dissociation of VWF-ADAMTS13 complex had taken place under conditions of high ionic strength. These results may indicate that VWF binding to ADAMTS13 is independent of ionic strength. In this regard, McKinnon et al.[30] reported that the N-linked glycans of VWF exert a modulatory effect on the interaction with ADAMTS13, and that removal of the N-linked glycans from VWF increased its affinity for ADAMTS13 under static conditions. Furthermore, Yeh et al.[19] recently reported that ADAMTS13 possesses a disulfide bond–reducing activity that regulates shear-induced thiol-disulfide exchange. Therefore, one of the mechanisms underlying formation of

VWF-ADAMTS13 complexes might involve disulfide-bond formation between ADAMTS13 and VWF. To address this issue, we investigated whether IAA, a blocker of free thiols, might prevent the formation of a disulfide bond–mediated covalent complex under high shear stress. We observed, however, that VWF-ADAMTS13 complex formation was unaffected by IAA treatment, suggesting that in CP, the amount of VWF-ADAMTS13 complex formed in a thiol-dependent fashion is marginal. This finding rules out a major role for disulfide bonds, but otherwise we have not elucidated the binding mechanism of VWF and ADAMTS13; this issue remains to be addressed in future studies.

PE is a first-line treatment for acquired TTP. For this purpose, either FFP or CSP is commonly used,[31] but the results regarding CP have been controversial.[32] At least one case of congenital TTP (USS) has been successfully treated with CP.[9] Therefore, it is important to determine whether there is a functional difference between bound and unbound ADAMTS13, and whether any such difference has physiological relevance. Here we have clearly shown that CSP contains the unbound or less bound ADAMTS13, whereas CP contains much more bound ADAMTS13 and lower levels of its unbound counterpart. An authoritative determination regarding which form of ADAMTS13 more efficiently down-regulates H-SIPA will be crucial in establishing the optimal treatment modality for TTP patients.

In most acquired TTP patients, plasma ADAMTS13 activity is less than 5% of normal. As a consequence, UL-VWFMs are not cleaved after secretion from endothelial cells, and remain anchored to the cell surface in long strings.[33] Circulating platelets

adhere to these long strings, resulting in occlusive platelet thrombi. However, smaller VWFMs do not induce this spontaneous adhesion and aggregation of platelets. Consequently, increased fluid shear stress is required to induce platelet aggregation in vitro.[34]

In order to reproduce the platelet aggregation generated in the microvasculature of ADAMTS13 activity–deficient TTP patients, here we employed an H-SIPA assay system that uses a mixture of washed normal platelets and ADAMTS13-dp plasma spiked with purified pd-VWF to mimic TTP plasmas. In this assay, the purified pd-ADAMTS13 inhibited H-SIPA in a dose-dependent manner, reaching a plateau of 20% ADAMTS13 activity at final pd-ADAMTS13 concentrations up to 500%. Under the same experimental conditions, FFP, CSP, and CP inhibited H-SIPA in a dose-dependent manner to the same extent at the end-points; in CP, however, the aggregation inhibition curves were different, and in fact no distinct inhibition was observed at the initial phase of platelet aggregation. This might be simply explained by the fact that the VWF concentration in the H-SIPA reaction mixtures was much higher than in CSP or FFP. Alternatively, the binary complex of ADAMTS13 and larger VWFMs might modulate a different phase of H-SIPA than unbound ADAMTS13, because CSP spiked with purified VWF readily generates the ADAMTS13–larger VWFM complex with pI 7.0/7.5. The larger VWFM is required in the earliest phase of platelet thrombi formation and high shear stress, but in the later phase the ADAMTS13–larger VWF complex embedded in the thrombi may play a role in regulating the size of the thrombi in order to prevent microvascular occlusion. Further

studies are required to determine the functional differences between ADAMTS13 in CSP and CP.

In conclusion, our results indicated that both plasma products of FFP and CSP are effective in treatment of TTP. However, CSP may be more favorable for PE in acquired TTP patients: relative to FFP, CSP has a lower level of VWF and a comparable ADAMTS13 activity, but lower amounts of ADAMTS13–larger VWFM complex.

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Authorship

Contribution: YH performed research, analyzed and interpreted data, and wrote the manuscript. MH, AI performed research. KS contributed vital reagent. MM analyzed data and wrote the manuscript. YF designed research, interpreted data, and wrote the manuscript.

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Figure Legends

Figure 1. Separation of plasma ADAMTS13 by IEF in healthy control and patient with T3-VWD.

Purified plasma-derived (pd)-ADAMTS13, normal plasma (NP), and type 3 VWD plasma (T3-VWD) were subjected to isoelectric focusing (IEF) and immunoblotting with anti-ADAMTS13 monoclonal antibody (WH2-11-1). Purified pd-ADAMTS13 (15 ng) was detected as one band at pI 4.9–5.6 (median, 5.4) (left panel). In various amounts (1–10 μL) of NP, ADAMTS13 antigen was detected as a major band at pI 4.9–5.6. Two additional groups of bands of ADAMTS13 antigen were also detected: pI 5.8–6.7 and pI 7.0/7.5. In T3-VWD, the ADAMTS13 band groups of pI 4.9–5.6 and 5.8–6.7 were detected, but the band of pI 7.0/7.5 was barely detectable (right panel). Arrow indicates the VWF-ADAMTS13 complex.

Figure 2. Spiking experiments and effect of iodoacetamide treatment on VWF-ADAMTS13 complex formation.

A: VWF-ADAMTS13 complex formation was analyzed by IEF and immunoblotting with anti-ADAMTS13 monoclonal antibody (WH2-11-1). Plasma of type 3 VWD (T3-VWD) spiked with the purified plasma derived (pd)-VWF generated a new band at pI 7.5. When plasma from a patient with Upshaw-Schulman syndrome (USS) was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When plasmas from T3-VWD and USS patients were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTWS13.

B: Purified pd-VWF (3 μg), pd-ADAMTS13 (200 ng), and ADAMTS13-dp plasma were treated for 30 min at room temperature with 100 mM iodoacetamide (IAA). ADAMTS13-dp plasma was mixed with purified pd-VWF (final concentration, 60 μg/mL) and/or pd-ADAMTS13 (final concentration, 2.3 μg/mL). Mixtures were exposed to high shear stress in a vortex mixer at maximum speed (3200 rpm) for 5 min. The VWF-ADAMTS13 complex, represented by the band at pI 7.5, is generated irrespective of the presence of IAA. Purified pd-VWF spiked into this mixture increased the density of this band.

Arrow indicates the VWF-ADAMTS13 complex.

Figure 3. Two-dimensional analysis of VWF in NP

Ten μl of NP was subjected to IEF and immunoblotting with anti-ADAMTS13 monoclonal antibody (WH2-11-1) (upper panel) and polyclonal anti-VWF antibody (middle panel). IEF gel of NP was subjected to a second dimension of electrophoresis on a SDS-0.9% agarose gel and immunoblotting with anti-VWF polyclonal antibody. In the lower panel, the upper arrowhead indicates the start point of two-dimensional SDS-0.9% agarose gel electrophoresis; the lower arrowhead indicates the position of the dye front at the termination of electrophoresis. VWF antigen in NP was separated into four series of pI bands (4.8–5.6, 6.0–6.7, 7.1–7.8, and 7.9–-8.4) by IEF (middle panel), whereas ADAMTS13 antigen was separated into three series of pI bands (4.9–5.6, 5.8–6.7, and 7.0/7.5; top panel). Two-dimensional analysis of normal plasma by IEF followed by SDS-0.9% agarose gel electrophoresis (lower panel) revealed that plasma

ADAMTS13 is primarily in complex with larger VWF multimers, and to a lesser extent with smaller VWF multimers.

Figure 4. Correlation between VWF antigen and ADAMTS13 activity in FFP, cryosupernatant and cryoprecipitate

The correlation between ADAMTS13 activity and VWF antigen in FFP, cryosupernatant (CSP), and cryoprecipitate (CP) were analyzed. In FFP and CSP, there is no correlation between ADAMTS13 activity and VWF antigen. On the other hand, a significant correlation was observed between the two parameters were found in CP ($r=0.646$, $p<0.01$).

Figure 5. ADAMTS13 in plasma fractions separated by IEF followed by SDS-PAGE.

Normal plasma (NP), cryosupernatant (CSP), cryoprecipitate (CP), and type 3 VWD (T3-VWD) plasmas were subjected to IEF (upper panel). ADAMTS13 separated by IEF was subjected to a second dimension of electrophoresis by SDS-5% PAGE under reducing conditions, and then to immunoblotting with anti-ADAMTS13 monoclonal antibody (WH2-11-1).

A: All three groups of ADAMTS13 bands (4.9–5.6, 5.8–6.7, and 7.0/7.5) in NP appeared as a 190-kD by SDS-5% PAGE. Arrow indicates the VWF-ADAMTS13 complex.

B, D: The ADAMTS13 band at pI 7.0/7.5 was completely absent in CSP, almost

indistinguishable to the case in T3-VWD plasma.

C: In CP, two faint bands with pI ranges of 4.9–5.6 and 5.8–6.7 and several strong bands with pI beyond 7.0 were detected. Arrow indicates the VWF-ADAMTS13 complex.

Figure 6. Inhibitory effect of ADAMTS13 on H-SIPA.

A: The purified pd-ADAMTS13 inhibits H-SIPA in a dose-dependent manner at ranges of 5–20% of ADAMTS13 activity in the mixture. The inhibition reaches a plateau (~20% light transmittance) at ranges of 50–500% of ADAMTS13 activity.

B, C: ADAMTS13 in both FFP and CSP from normal individuals exhibited dose-dependent inhibition of H-SIPA at the ranges of 5–20% of ADAMTS13 activity.

D: ADAMTS13 in CP did not clearly inhibit H-SIPA at the initial phase at less than 10% of ADAMTS13 activity. The inhibition of platelet aggregation was found at the ranges of 5–20% of ADAMTS13 activity; at the later phase of H-SIPA the maximum aggregation at the endpoint was almost the same as in FFP and CSP.

Table 1. ADAMTS13 activity and VWF antigen levels in plasma products

		All (n=120)	Blood type				Over all *P*
			A (n=30)	O (n=30)	B (n=30)	AB (n=30)	
ADAMTS13 activity (%)	FFP	81±16	84±17	77±15	80±16	83±13	NS
	CSP	71±14	72±14	69±17	70±12	72±13	NS
	Recovery (%) *	92.7±3.7	92.9±3.6	95.2±2.1	91.4±3.9	91.2±3.5	<0.01[a]
	CP	5.6±2.8	5.4±2.4	3.5±1.6	6.5±2.9	6.9±2.8	< 0.01[a]
	Recovery (%) *	7.3±3.7	7.1±3.6	4.8±2.1	8.6±3.9	8.8±3.5	<0.01[a]
VWF antigen (%)	FFP	124±46	121±49	80±24	144±32	150±38	< 0.01[b]
	CSP	16±7	15±7	11±3	19±5	19±6	< 0.01[c]
	Recovery (%) *	14.0±2.6	12.7±2.5	14.6±2.2	14.6±2.9	14.2±2.4	<0.05[d]
	CP	98±35	100±37	64±18	112±22	116±32	< 0.01[e]
	Recovery (%) *	86.0±2.6	87.3±2.5	85.4±2.2	85.4±2.9	85.8±2.4	<0.05[d]

NS: no significant difference ($p \geq 0.05$)
* Recovery was calculated as the level in CP or CSP divided by the total (sum of levels in CP and CSP)
Overall *p* values were calculated using the Kruskal Wallis H-test. Significant differences between four groups (overall $P < 0.05$) were further analyzed by Mann-Whitney U-test with Bonferroni correction.
[a] $p < 0.01$ between O and B, AB, < 0.05 between O and A.
[b] $p < 0.01$ between O and A, B, AB, < 0.05 between A and B
[c] $p < 0.01$ between O and A, B, AB. < 0.01 between A and B, <0.05 between A and AB
[d] $p < 0.05$ between A and O, B, AB.
[e] $p<0.01$ between O and A, B, AB.

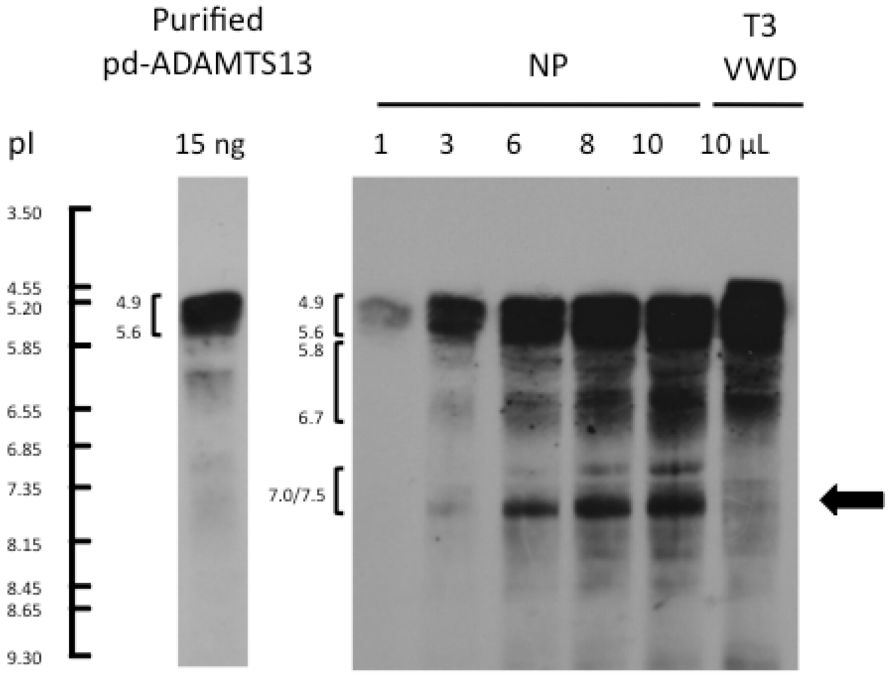
Purified
pd-ADAMTS13
NP
T3
VWD
pI
15 ng
1
3
6
8
10
10 µL
3.50
4.55
5.20
5.85
6.55
6.85
7.35
8.15
8.45
8.65
9.30
4.9
5.6
4.9
5.6
5.8
6.7
7.0/7.5

Fig. 1

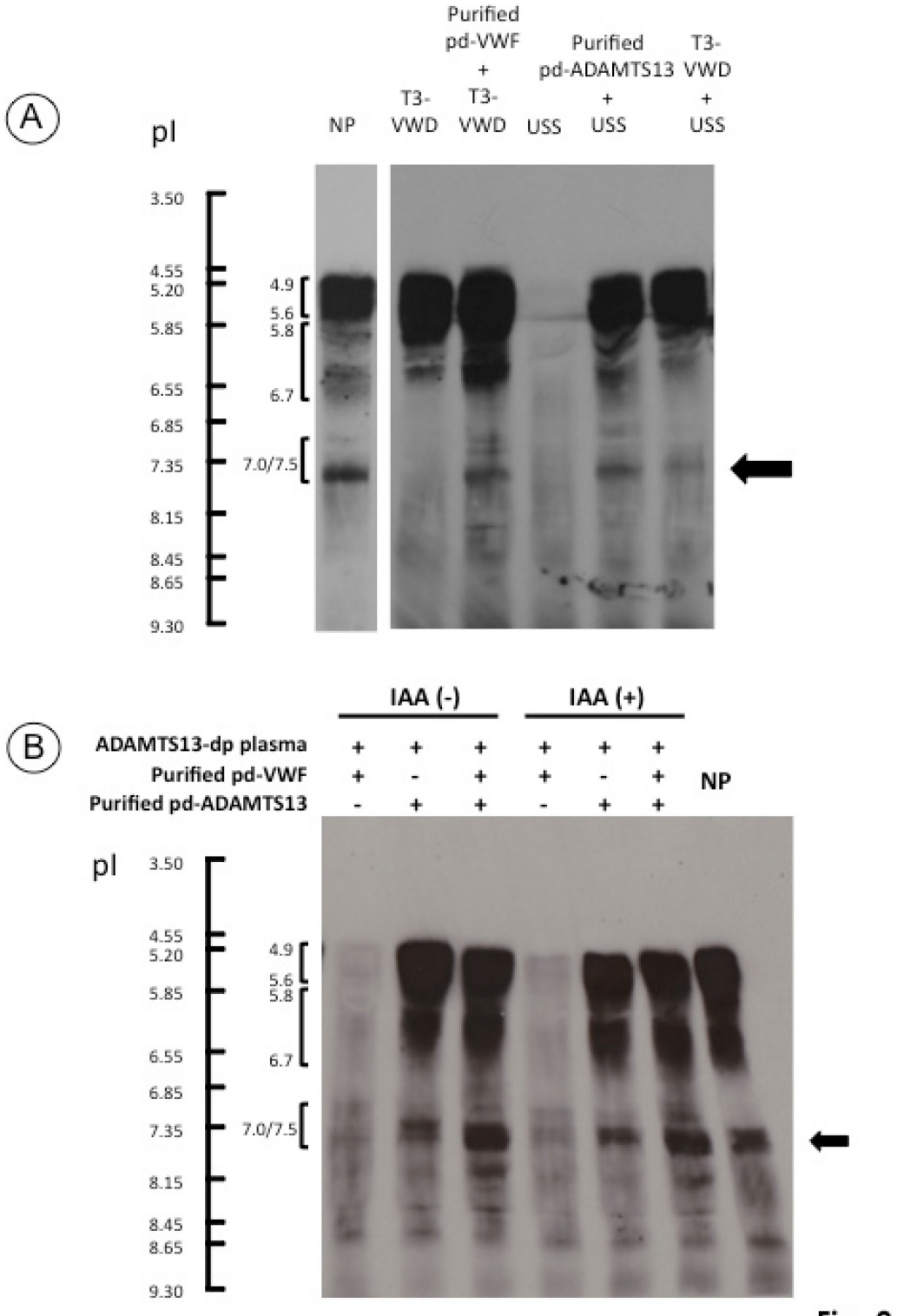
A
pI
NP
T3-VWD
Purified pd-VWF + T3-VWD
USS
Purified pd-ADAMTS13 + USS
T3-VWD + USS
3.50
4.55
5.20
5.85
6.55
6.85
7.35
8.15
8.45
8.65
9.30
4.9
5.6
5.8
6.7
7.0/7.5
B
IAA (-)
IAA (+)
ADAMTS13-dp plasma + + + + + +
Purified pd-VWF + - + + - +
Purified pd-ADAMTS13 - + + - + +
NP
pI
3.50
4.55
5.20
5.85
6.55
6.85
7.35
8.15
8.45
8.65
9.30
4.9
5.6
5.8
6.7
7.0/7.5

Fig. 2

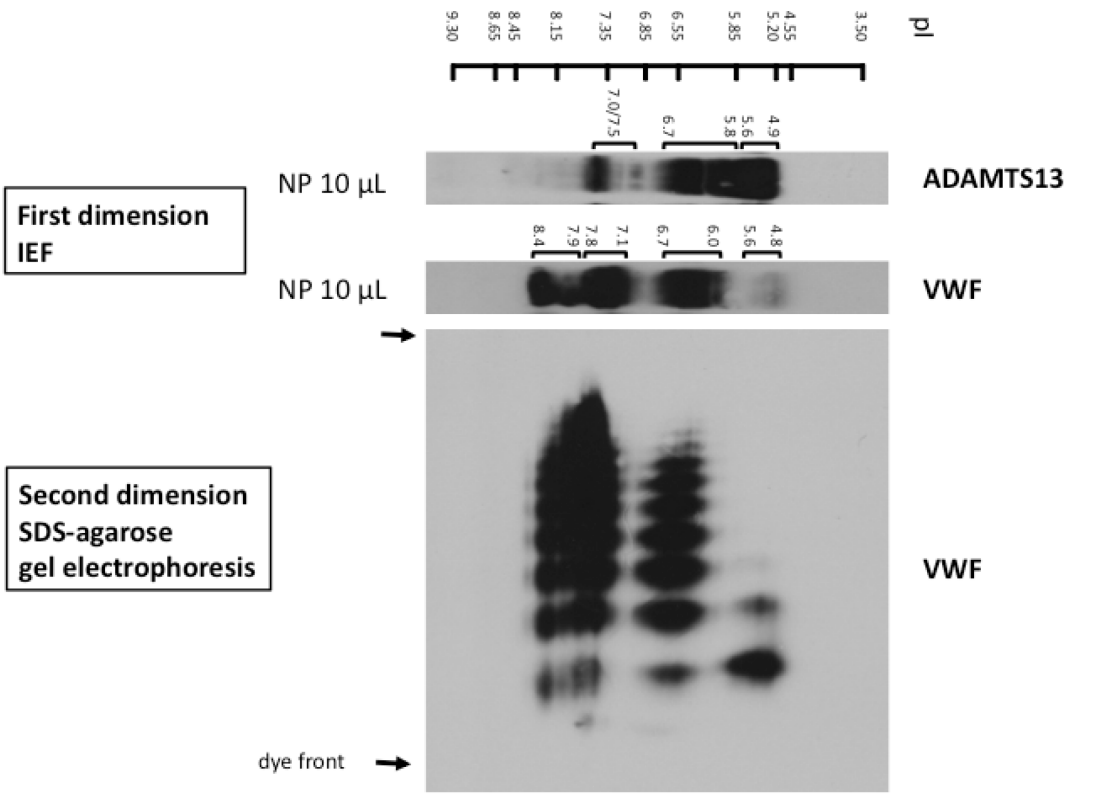
pI
9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50
7.0/7.5
6.7
5.8
5.6
4.9
NP 10 µL
ADAMTS13
First dimension
IEF
8.4
7.9
7.8
7.1
6.7
6.0
5.6
4.8
NP 10 µL
VWF
Second dimension
SDS-agarose
gel electrophoresis
VWF
dye front

Fig. 3

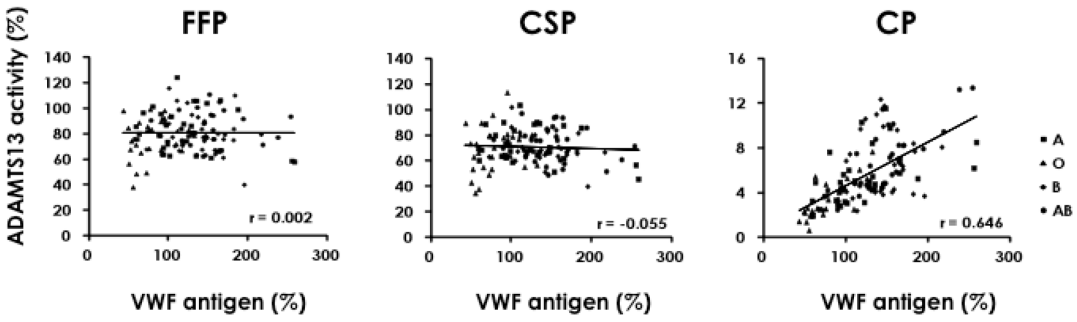
FFP
CSP
CP
ADAMTS13 activity (%)
VWF antigen (%)
VWF antigen (%)
VWF antigen (%)
r = 0.002
r = -0.055
r = 0.646
A
O
B
AB

Fig. 4

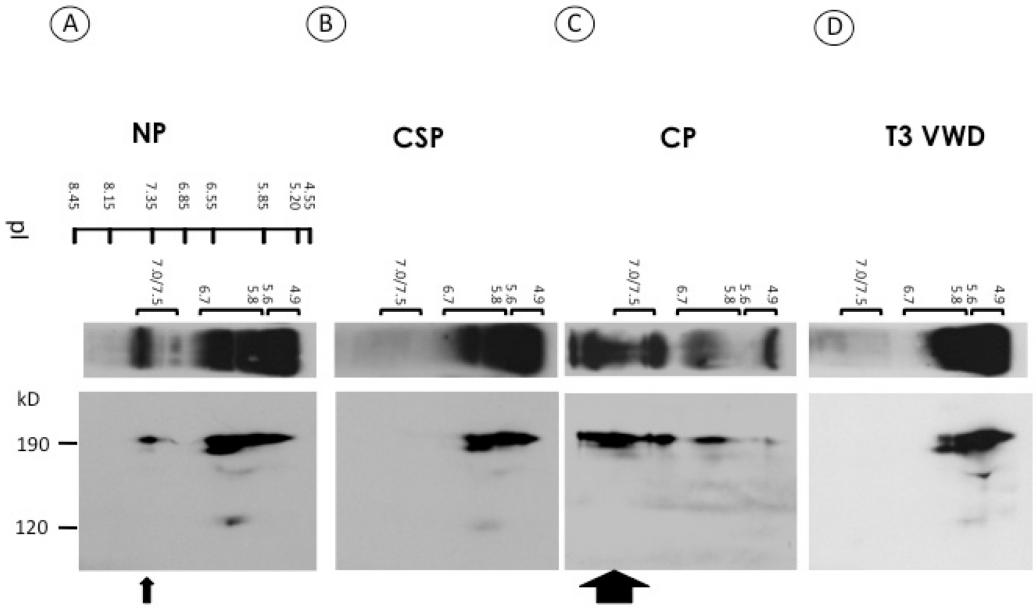
A
B
C
D
NP
CSP
CP
T3 VWD
pI
8.45
8.15
7.35
6.85
6.55
5.85
4.55
5.20
7.0/7.5
6.7
5.6
5.8
4.9
kD
190
120

Fig. 5

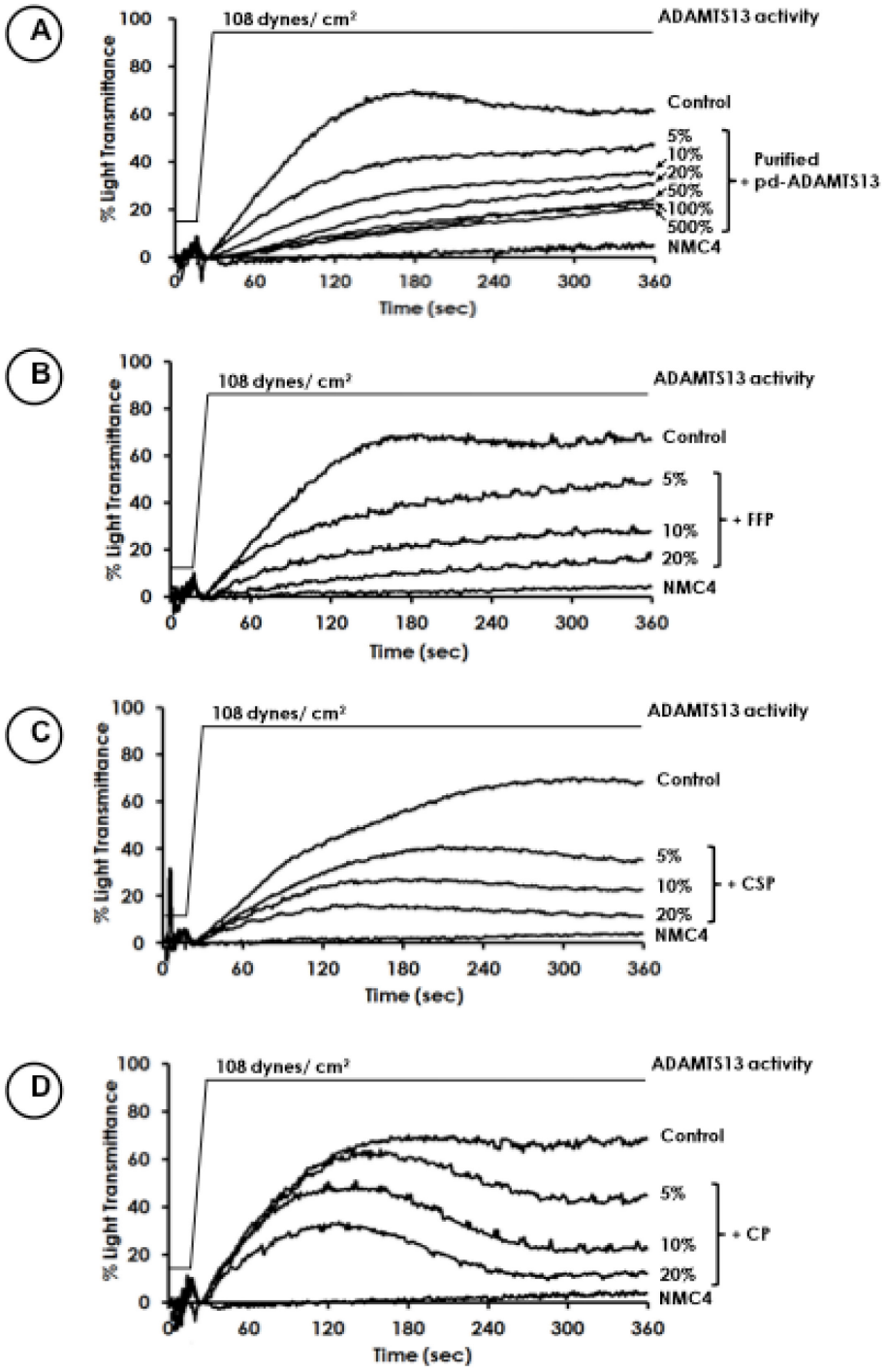
A
100
80
60
40
20
0
% Light Transmittance
108 dynes/ cm²
ADAMTS13 activity
Control
5%
10%
20%
50%
100%
500%
NMC4
Purified
+ pd-ADAMTS13
0
60
120
180
240
300
360
Time (sec)
B
100
80
60
40
20
0
% Light Transmittance
108 dynes/ cm²
ADAMTS13 activity
Control
5%
10%
20%
NMC4
+ FFP
0
60
120
180
240
300
360
Time (sec)
C
100
80
60
40
20
0
% Light Transmittance
108 dynes/ cm²
ADAMTS13 activity
Control
5%
10%
20%
NMC4
+ CSP
0
60
120
180
240
300
360
Time (sec)
D
100
80
60
40
20
0
% Light Transmittance
108 dynes/ cm²
ADAMTS13 activity
Control
5%
10%
20%
NMC4
+ CP
0
60
120
180
240
300
360
Time (sec)

Fig. 6