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Analysis on the Molecular Species and Concentration of Circulating ADAMTS13 in Blood

Kenji Soejima*, Hitomi Nakamura, Masaki Hirashima, Wataru Morikawa, Chikateru Nozaki and Tomohiro Nakagaki

Research Department 1, The Chemo-Sero-Therapeutic Research Institute, Kyokushikawabe, Kikuchi, Kumamoto 869-1298

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ADAMTS13 is the metalloprotease responsible for the proteolytic degradation of von Willebrand factor (VWF). A severe deficiency of this VWF-cleaving protease activity causes thrombotic thrombocytopenic purpura. This protease, comprising 1,427 amino acid residues, is composed of multiple domains, *i.e.*, a preproregion, a metalloprotease domain, a disintegrin-like domain, a thrombospondin type-1 motif (Tsp1), a cysteine-rich domain, a spacer domain, seven Tsp1 repeats, and two CUB domains. We prepared one polyclonal and seven monoclonal antibodies recognizing distinct epitopes spanning the entire ADAMTS13 molecule. Of these antibodies, two of the monoclonal ones, which recognize the disintegrin-like and cysteine-rich/spacer domains, respectively, abolished the hydrolytic activity of ADAMTS13 toward both a synthetic substrate, FRETS-VWF73, and the natural substrate, VWF. In addition, these antibodies blocked the binding of ADAMTS13 to VWF. These results revealed that the region between the disintegrin-like and cysteine-rich/spacer domains interacts with VWF. Employing these established polyclonal and monoclonal antibodies, we examined the molecular species of ADAMTS13 circulating in the blood by immunoprecipitation followed by Western blot analysis, and estimated the plasma concentration of ADAMTS13 by enzyme-linked immunosorbent assay. These studies indicated that the major fraction of ADAMTS13 in blood plasma consisted of the full-length form. The concentration of ADAMTS13 in normal plasma was approximately 0.5–1 μg/ml.

Key words: ADAMTS13, antibody, cysteine-rich/spacer domains, TTP, VWF.

ADAMTS13 was originally identified and purified as a von Willebrand factor (VWF)-cleaving protease (VWF-CP). This protease cleaves the peptide bond between amino acid residues Tyr1605 and Met1606 within the VWF A2 domain (*1–3*, reviewed in Ref. *4*). The gene encoding this protease, *ADAMTS13*, was cloned on the basis of an isolated NH_2-terminal amino acid sequence (*3, 5*). This enzyme is involved in the pathogenesis of thrombotic thrombocytopenic purpura (TTP), which is diagnosed on the basis of Moschowitz' pentad (*6*) of thrombocytopenia, microangiopathic hemolytic anemia, fever, renal failure, and neurological dysfunction. Positional cloning of the genes responsible for congenital TTP identified the *ADAMTS13* gene (*7*). Expression of recombinant ADAMTS13 confirmed that ADAMTS13 cleaves VWF directly (*8*, *9*); mutations of *ADAMTS13* result in decreases or the complete loss of VWF-CP activity (*8*).

ADAMTS13 is a 1,427–amino acid protein belonging to the ADAMTS (A disintegrin and metalloprotease with thrombospondin type I motif) family (*10*, *11*). The protein comprises a preproregion, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type-1 motif (Tsp1), a cysteine-rich domain, a spacer domain, seven Tsp1 repeats, and two CUB domains. The molecule has 10 potential *N*-linked glycosylation sites. As yet, VWF is the only natural substrate identified for ADAMTS13.

The ADAMTS13 preproregion is significantly shorter than that in other members of the ADAMTS family. This domain, which is not required for folding or secretion, does not perform the common function of maintaining enzyme latency (*12*).

Analyses of sequential C-terminal truncations of ADAMTS13 revealed that the region spanning from the metalloprotease domain to the spacer domain was essential for VWF cleavage. The C-terminal Tsp1 motifs and CUB domains appear to be dispensable for VWF cleavage *in vitro* (*13*, *14*). In addition, C-terminal truncations of the region between the spacer and CUB domains do not change the specificity of the VWF cleavage site (*13*). The cysteine-rich and spacer domains are the major epitopes targeted by neutralizing autoantibodies isolated from patients with acquired TTP (*13*, *15*, *16*), which suggests that these regions are likely to be involved in VWF recognition. Furthermore, a number of inbred mouse strains have a short-type ADAMTS13 consisting of 1,037 amino acids with a C-terminal portion truncated after the seventh Tsp1 motif (*17*). It has been reported that ADAMTS13 cleaves VWF on the endothelial cell surface, interacting with VWF via the first CUB domain, under flow conditions (*18–20*). Conflicting reports, however, suggested that, even under flow conditions, the metalloprotease to spacer domains might be the minimum essential domains (*21*).

*To whom correspondence should be addressed. Tel: +81-968-37-3100, Fax: +81-968-37-3616, E-mail: soejima@kaketsuken.or.jp

To explain these contradictory data, a working model in which ADAMTS13 is in equilibrium between a closed state, in which the second VWF-binding domain (possibly the spacer domain) is cryptic, and an open state, in which both the primary (the first CUB domain) and the secondary binding sites are available for interacting with VWF, has been proposed (*20*).

SDS-PAGE revealed that the ADAMTS13 purified from human plasma (*1*), cryoprecipitates (*2*), or Cohn fraction-I precipitates (*3*) is composed of several distinct molecular weight species ranging from 110 to 150 kDa under non-reducing conditions and from 120 to 190 kDa under reducing conditions. The cloned cDNA sequence also suggested the possibility of alternatively spliced transcripts (*3*, *5*, *7*).

In this study, we established one polyclonal and seven monoclonal antibodies recognizing distinct epitopes across the entire ADAMTS13 protein. Employing these antibodies, we studied the site on ADAMTS13 interacting with VWF in further detail and examined the circulating forms of ADAMTS13 in blood.

MATERIALS AND METHODS

Construction of Expression Plasmids of ADAMTS13 Mutants—Expression vectors encoding ADAMTS13 fragments under the control of a strong promoter, CAG (*8*, *13*, *22–25*), were constructed by PCR-based mutagenesis using the full-length wild-type (WT) ADAMTS13 cDNA as a template (*3*).

Stable Expression and Cell Culture of Recombinant ADAMTS13 Protein—An expression vector encoding full-length WT ADAMTS13 was transfected into HEK293 cells using the polyamine transfection reagent TransIT^{TM}-LT1 (Mirus Corporation, Wisconsin), according to the manufacturer's instructions. Briefly, HEK293 cells were cultured in α-MEM (Sigma) supplemented with 10% fetal bovine serum. The expression plasmid (2 μg) was transfected into 40–70% confluent cells in 35-mm wells. To obtain stably transfected HEK293 cells expressing ADAMTS13, cells were transferred into α-MEM medium containing 10% fetal bovine serum, 1 mg/ml G418, 10 units/ml penicillin G, and 100 μg/ml streptomycin. After 3 weeks, the cells were cloned by limiting dilution. The clone expressing the highest level of ADAMTS13 was selected, cultured, and expanded. The culture medium was replaced with serum-free medium. After harvesting, the conditioned medium was stored at –80°C until analyzed.

Preparation of Recombinant ADAMTS13—Conditioned medium from cultures of cells expressing WT ADAMTS13 was processed by affinity purification using an immobilized anti-ADAMTS13 antibody. The bound protein was eluted with 0.1 M glycine-HCl containing 0.1 M NaCl (pH 2.8). The eluted fraction was pooled and neutralized with a one-tenth volume of 1 M Tris-HCl (pH 8.0). After concentration by centrifugation using Centricon^{TM} YM-30 (Millipore, Massachusetts), the pooled fractions were applied to a HiLoad Superdex^{TM} 200 gel filtration column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl (TBS). The protein concentration was determined with Coomassie plus^{TM} protein assay reagent (PIERCE, Rockford, IL), using bovine serum albumin (BIO-RAD) as a standard, and also by amino acid analysis. The molecular masses of recombinant ADAMTS13 species were determined by MALDI-TOF/MS (BIFLEX III, Bruker Daltonics, Billerica, MA) using protein calibration standard II (Trypsinogen ([M+H]+ = 23,982), Protein A ([M+H]+ = 44,613), and BSA ([M+H]+ = 66,431) (Bruker Daltonics) as standards.

Preparation of Polyclonal and Monoclonal Antibodies against ADAMTS13—We raised a polyclonal antibody (PoAb) against whole molecule of ADAMTS13 in a rabbit by DNA-based immunization. Briefly, a rabbit was immunized by intradermal injection of 1 mg of expression plasmid encoding WT ADAMTS13 without a FLAG-tag at 20 sites. Three electronic pulses of 50 V each were then delivered with an electroporator (CUY21; BEX Co. Ltd., Tokyo) with a 100 msec pulse length after the plasmid DNA injection. The process was repeated 3 weeks later.

Monoclonal antibodies (MoAbs) were generated by immunization of mice with a peptide fragment (amino acid residues 1169–1190), purified recombinant WT, or W688X C-terminal truncated mutant protein (Fig. 1) (*13*), according to standard protocols.

Epitope Mapping of the PoAb and MoAbs against ADAMTS13—Epitope mapping of the established MoAbs was performed by Western blot analyses using WT protein and nine C-terminally truncated mutants with FLAG-tags (Fig. 1) that were transiently expressed in HeLa cells under non-reducing conditions as described previously (*13*).

Assay of Neutralization of VWF-CP Activity by the Antibodies—MoAbs were evaluated for the ability to

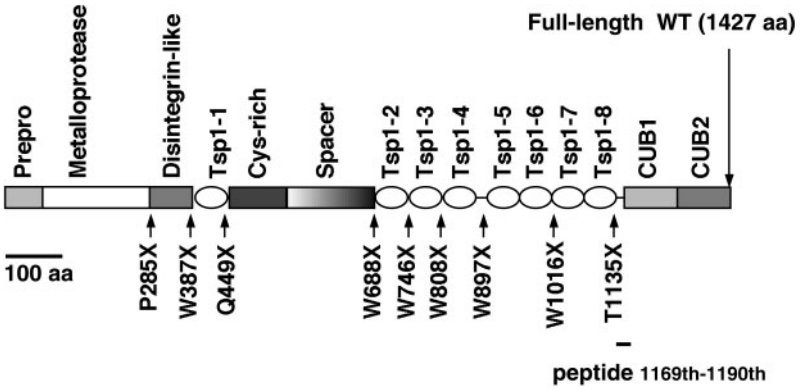

Fig. 1. **Schematic representation of ADAMTS13 mutants and an immunization peptide.** The series of nine domain-deleted mutants and WT ADAMTS13 used for epitope-mapping are shown schematically.

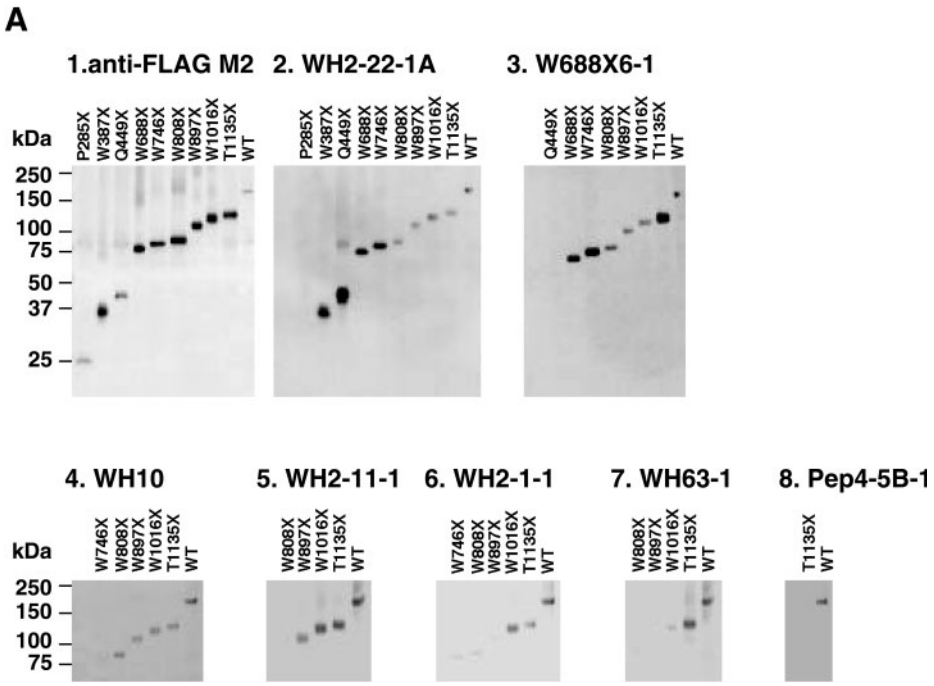

Fig. 2. **Epitope mapping of the established MoAbs and PoAb against ADAMTS13.** Samples of the culture medium of each of the series of nine domain-deleted mutants and WT ADAMTS13 were subjected to SDS-PAGE under non-reducing conditions, followed by Western blotting. Each of the MoAbs (A1-8) and the PoAb (B1-2) were used as a primary antibody. A1, anti-FLAG M2 MoAb (as a positive control); A2, WH2-22-1A; A3, W688X6-1; A4, WH10; A5, WH2-11-1; A6, WH2-1-1; A7, WH63-1; and A8, Pep4-5B-1. B1, pre-immunized rabbit IgG; and B2, the PoAb specific for ADAMTS13 generated by DNA-based immunization.

neutralize ADAMTS13-mediated hydrolysis of a fluorogenic substrate, FRETS-VWF73 (*26*, *27*) (Peptide Institute, Osaka). Briefly, each MoAb was preincubated with an aliquot of normal human plasma. The hydrolysis of FRETS-VWF73 by the mixture was then measured according to the manufacturer's instructions.

The ability of each antibody to neutralize ADAMTS13-mediated cleavage of its natural macromolecular substrate, VWF, was assessed as the change in the VWF multimeric pattern. Briefly, VWF was purified from pooled human plasma cryoprecipitates as described (*3*). Five-fold dilutions of plasma were then mixed with an equal volume of a 1 mg/ml antibody solution. Each mixture was incubated for 15 min at room temperature. After the addition of the purified VWF, the mixture was incubated overnight in the presence of 1.5 M urea at 37°C. The multimeric pattern of VWF was analyzed by agarose electrophoresis.

Assay of Binding to Immobilized Plasma VWF—Binding assays were performed as described (*28*) with the following modifications. Briefly, plasma VWF purified as described above (7.5 μg/ml, 100 μl/well) was immobilized in Maxisorp™ 96-well microtiter plates (NalgeNunc). Then, the wells were blocked by the addition of 200 μl of 25% Block Ace™ (Dainippon Pharmaceuticals, Osaka) in PBS containing 0.1% Tween 20 (PBST). After washing with PBST, the wells were incubated with 100 μl of a mixture of polyclonal or monoclonal antibody preincubated with plasma for 15 min, followed by the addition of 5 mM EDTA, and then bound plasma ADAMTS13 was detected with biotinylated anti-ADAMTS13 PoAb and peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA).

Immunoprecipitation and Western Blotting Analysis of ADAMTS13 from Normal Human Plasma—The polyclonal anti-ADAMTS13 antibody was directly coupled to Magna-Bind™ Magnetic Beads (PIERCE), according to the manufacturer's instructions. An aliquot of the antibody-coupled magnetic beads was added to 100 μl of normal human plasma, followed by incubation for 1 h at room temperature. The beads were then isolated by magnetic separation, and the separated beads were washed three times in TBS containing 0.05% Tween-80. Bound proteins were eluted with 0.1 M glycine-HCl (pH 2.8) containing 4 M urea and 0.1 M NaCl. After neutralization with a one-tenth volume of 1 M Tris-HCl (pH 8.0), the eluate was resolved by 5–20% gradient SDS-PAGE and then transferred to an Immobilon™ polyvinylidene-difluoride membrane (Millipore). ADAMTS13 was detected using biotinylated polyclonal or monoclonal antibodies followed by streptavidin-peroxidase conjugate. Immunopositive bands were visualized with BCIP/NBT (KPL, Gaithersburg, MD).

Measurement of ADAMTS13 Antigen Level in Normal Human Plasma—The level of ADAMTS13 antigen was measured by enzyme-linked immunosorbent assay (ELISA) using recombinant ADAMTS13 as a standard. Diluted plasma samples (100 μl) were incubated in

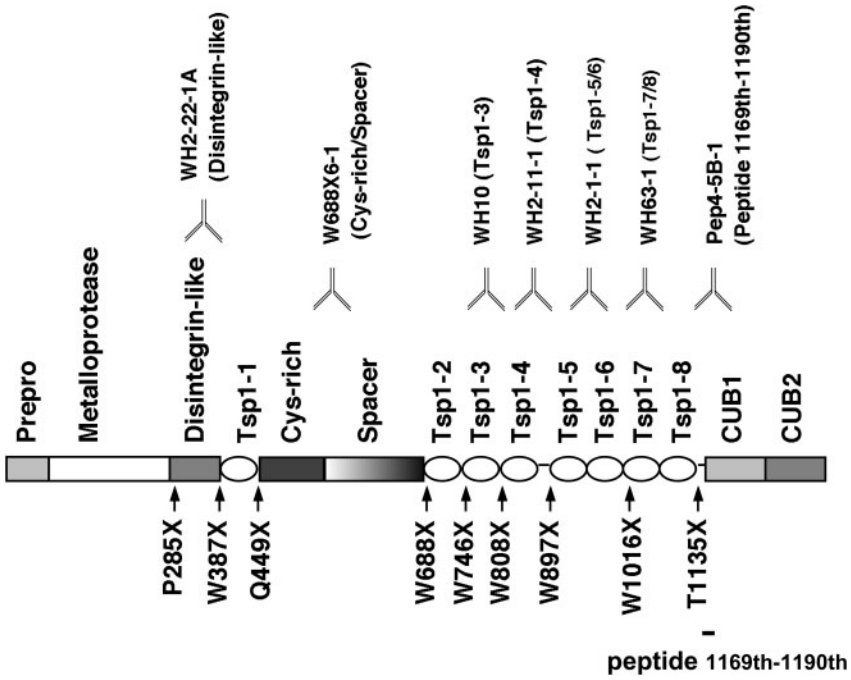

Fig. 3. **Schematic representation of the epitope of each MoAb.** The clone names of the MoAbs established here (with the deduced epitope indicated in parenthesis) are shown in the estimated epitope region in the ADAMTS13 molecule. Seven MoAbs recognizing independent epitopes were established.

Maxisorp™ 96-well microtiter plates precoated with polyclonal or monoclonal antibodies against ADAMTS13. The wells were blocked with 25% Block Ace in TBS. After incubation for 2 h at 37°C, the microtiter plates were incubated for 1 h at 37°C with a second biotinylated polyclonal or monoclonal antibody against ADAMTS13. Horseradish peroxidase-conjugated streptavidin (Vector Laboratories) was added to each well for 1 h at 37°C. Between each step, the microtiter plates were washed three times with TBS containing 0.05% Tween-20. The chromogenic reaction was developed by the addition of 100 μl of TMB peroxidase substrate (KPL). Reactions were quenched by adding 100 μl of 0.5 M H_2SO_4. The absorbance at 450 nm was measured using a spectrophotometer with a reference filter at 650 nm (Molecular Devices, Sunnyvale, CA).

RESULTS AND DISCUSSION

Epitope Mapping of the MoAbs and PoAb against ADAMTS13—To raise MoAbs in mice, we used purified recombinant WT and W688X proteins as immunogens. The culture medium from established hybridoma clones or the purified PoAb IgG fraction was used for epitope mapping (Fig. 2, A and B). While an anti-FLAG M2 MoAb reacted with all of the mutants and the WT protein, clone WH2-22-1A reacted with W387X, but not P285X (Fig. 2A). This demonstrated that WH2-22-1A recognizes the disintegrin-like domain. The epitopes recognized by each clone, as determined by Western blotting analyses, are given for each antibody in parentheses: WH2-22-1A (disintegrin-like), W688X6-1 (cysteine-rich/spacer), WH10 (Tsp1-3), WH2-11-1 (Tsp1-4), WH2-1-1 (Tsp1-5 or 6), WH63-1 (Tsp1-7 or 8), and Pep4-5B-1 (amino acid residues 1169–1190) (Fig. 2A). WH2-1-1 and WH63-1 exhibited cross-reactivity with multiple Tsp1 motifs; there were faint but significant positive bands for shorter mutants bearing the Tsp1 motifs. The PoAb generated here reacted with all of the mutants except P285X (Fig. 2B). Furthermore, a binding ELISA competing PoAb with each MoAb to immobilized WT ADAMTS13 revealed that the epitopes recognized by the PoAb at least ranged from the disintegrin-like domain to the C-terminal Tsp1 repeats of ADAMTS13 (data not shown). The epitopes of the MoAbs described here are summarized in Fig. 3.

Neutralizing Activities of the MoAbs and PoAb against ADAMTS13—We assessed the neutralizing activity of each MoAb and the generated PoAb by examining the inhibition of the hydrolysis of a fluorogenic substrate, FRETS-VWF73. WH2-22-1A, W688X6-1, and the PoAb exhibited clear, dose-dependent inhibitory activity toward ADAMTS13-mediated hydrolysis (Fig. 4A). As WH2-22-1A and W688X6-1 are thought to recognize the disintegrin-like and cysteine-rich/spacer domains, respectively (Figs. 2A and 3), it was thought that the region between the disintegrin-like and cysteine-rich/spacer domains could interact directly with FRETS-VWF73 (Asp1596 to Arg1668). Therefore, the interaction between the disintegrin-like to cysteine-rich/spacer domains of ADAMTS13 and Asp1596 to Arg1668 of VWF would be minimally essential interaction for the expression of enzymatic activity under *in vitro* assay conditions. Similar results were obtained from experiments using plasma VWF. VWF multimer degradation was clearly inhibited by the addition of WH2-22-1A and W688X6-1 to plasma (Fig. 4B). The finding that W688X6-1 recognizing the cysteine-rich/spacer domains had neutralizing activity is consistent with the results of epitope mapping studies of IgG samples purified from plasma of patients with acquired TTP (*13*). Furthermore, these two MoAbs clearly inhibited the binding of ADAMTS13 to VWF in a dose-dependent manner (Fig. 4C). Taken together, our data confirmed that regions of the protein in close proximity to the disintegrin and cysteine-rich/spacer domains are involved

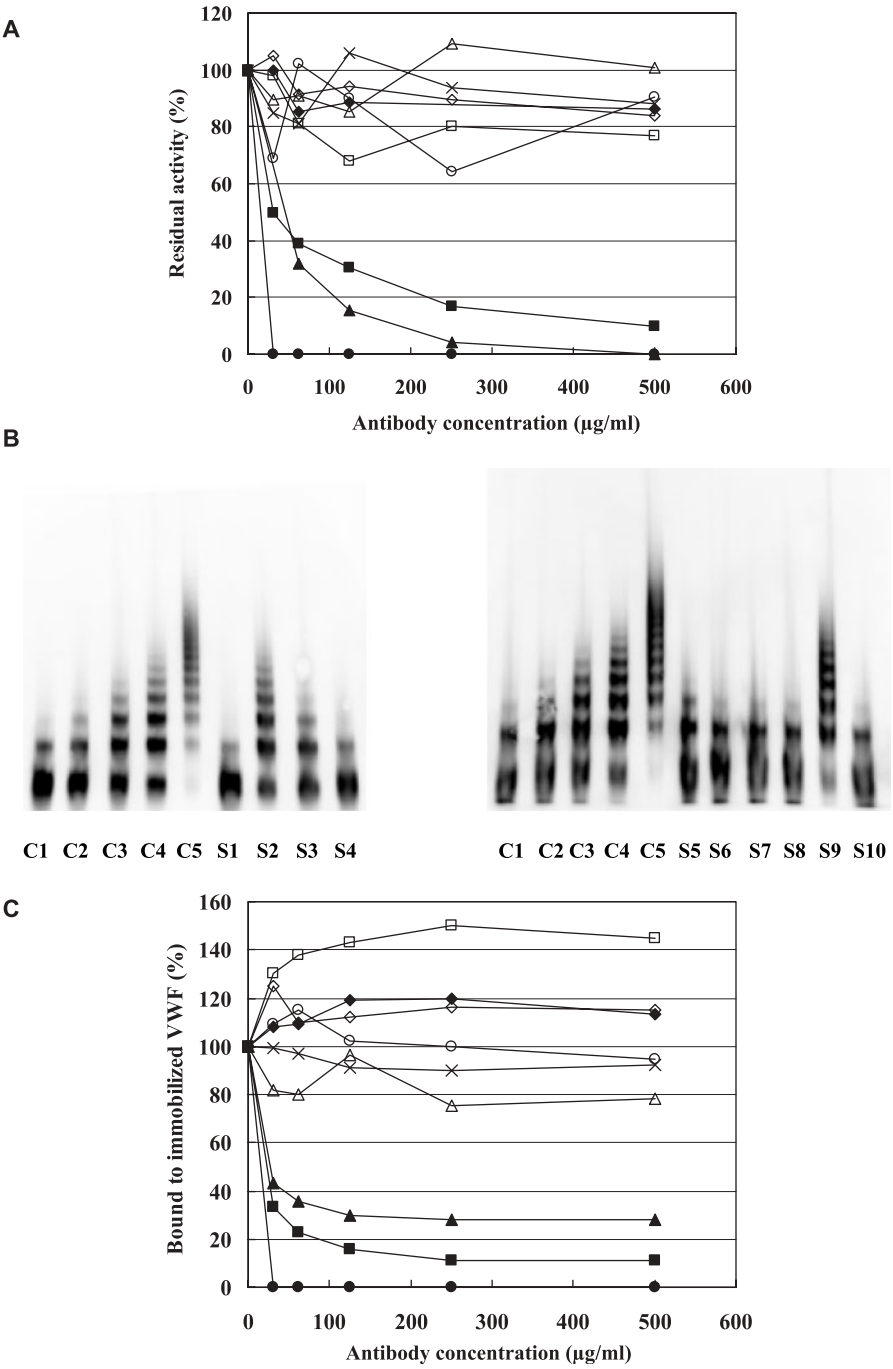

Fig. 4. **Inhibitory activity of the established MoAbs and PoAb on ADAMTS13-mediated hydrolysis.** A, inhibitory activity was evaluated using a synthetic substrate, FRETS-VWF73, as follows: 3 μl of normal human plasma and 50 μl of 0, 31.3, 62.5, 125, 250, or 500 μg/ml of each antibody solution was mixed at room temperature for 1 h. Then, 50 μl of 4 μM FRETS-VWF73 was added to the above solution (closed circles, PoAb; closed diamonds, negative control mouse IgG; closed triangles, WH2-22-1A; closed squares, WH688X6-1; open circles, WH10; open triangles, WH2-11-1; open squares, WH2-1-1; open diamonds, WH63-1; and crosses, Pep4-5B-1). The residual activity, as a percentage, was then plotted. The values represent the averages of three independent experiments. B, purified VWF from plasma was added to the plasma–antibody reaction mixture. After overnight incubation at 37°C, the multimeric pattern of VWF was analyzed by 1% agarose electrophoresis (C1-5, normal plasma control; C1, 100%; C2, 50%; C3, 25%; C4, 12.5%; and C5, 0%. S1, negative control mouse IgG; S2, WH2-22-1A; S3, W688X6-1; S4, WH10; S5, WH2-11-1; S6, WH2-1-1; S7, WH63-1; S8, Pep4-5B-1; S9, PoAb; and S10, buffer). C, three microliters of normal human plasma and 50 μl of 0, 31.3, 62.5, 125, 250, or 500 μg/ml of each antibody solution were preincubated at room temperature for 1 h. After the addition of EDTA to a final concentration of 5 mM, 100 μl of the mixture was applied to a VWF-immobilized 96-well plate. After 1-h incubation at 37°C, bound plasma ADAMTS13 was detected using biotinylated anti-ADAMTS13 PoAb and peroxidase-conjugated streptavidin (closed circles, PoAb; closed diamonds, negative control mouse IgG; closed triangles, WH2-22-1A; closed squares, WH688X6-1; open circles, WH10; open triangles, WH2-11-1; open squares, WH2-1-1; open diamonds, WH63-1; and crosses, Pep4-5B-1). The percentage of ADAMTS13 bound to immobilized VWF was plotted. The values represent the averages of three independent experiments.

in VWF recognition. This finding is consistent with recent reports that the proximal C-terminal domains, which include the disintegrin-like to the spacer domains, determine substrate specificity (*29*).

Circulating Forms of ADAMTS13 in Blood—To identify the molecular forms of ADAMTS13 circulating in the blood, we performed immunoprecipitation using the anti-ADAMTS13 PoAb. We verified that magnetic bead-immobilized PoAb could precipitate all of the C-terminally truncated mutants, with the exception of P285X (Fig. 5A). If a transcript encoding the metalloprotease domain alone were produced, however, the product would not be detected with our method. ADAMTS13 was immunoprecipitated from the conditioned medium of cells stably expressing WT ADAMTS13 and three normal human plasma samples. All of the MoAbs established in this study were then used for the detection of ADAMTS13. The molecular weight of the major immunopositive fraction of proteins precipitated from normal plasma samples was similar to that of the recombinant full-length ADAMTS13 (Fig. 5B). The ADAMTS13 circulating in the blood was primarily composed of the full-length form with a molecular weight of 150 kDa, as estimated by SDS-PAGE analysis under non-reducing conditions. Examination of plasma samples directly by Western blotting also demonstrated that the major species of

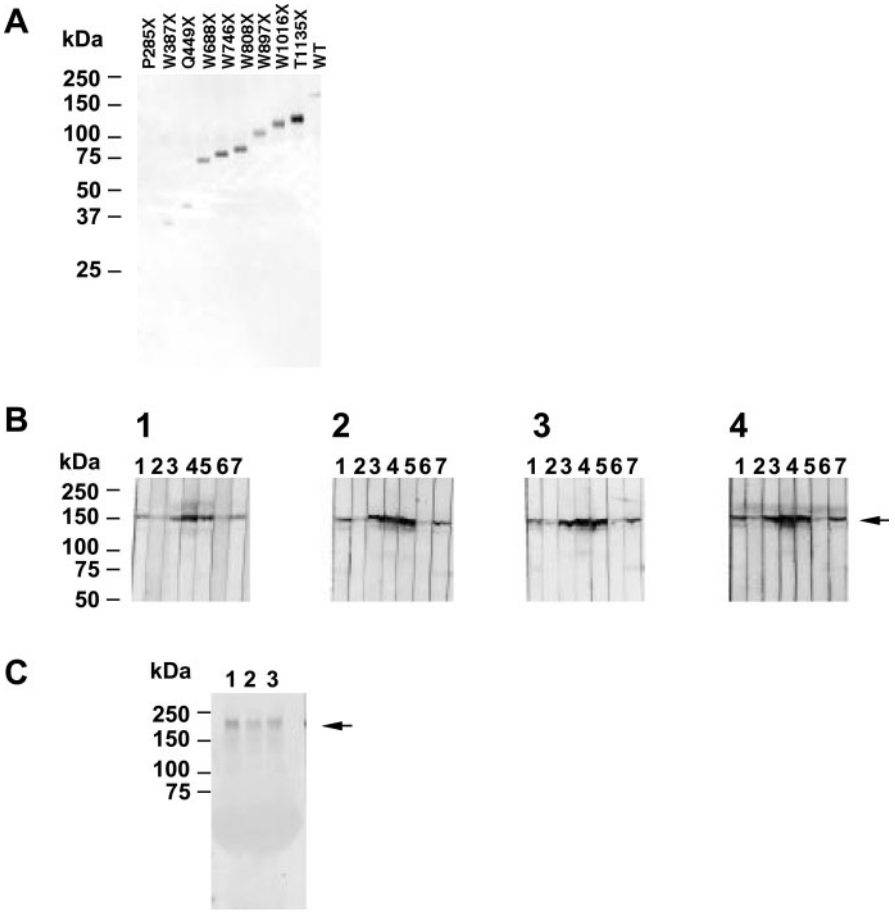

Fig. 5. **Immunoprecipitation of ADAMTS13 from normal human plasma.** ADAMTS13 was immunoprecipitated using PoAb-coupled magnetic beads. The precipitates were subjected to SDS-PAGE on a 5–20% gradient gel under non-reducing conditions, and then analyzed by Western blotting. A, the PoAb could clearly precipitate all C-terminally-truncated FLAG-tagged mutants, with the exception of P285X, from the conditioned medium of each mutant. Detection was performed by Western blotting using anti-FLAG M2 MoAb. B, immunoprecipitation was performed using the PoAb from recombinant WT ADAMTS13 conditioned medium as a positive control (B1) and three normal human plasma samples, respectively (B2-4). Detection was performed using each of the following MoAbs: Lane 1, WH2-22-1A; lane 2, W688X6-1; lane 3, WH10; lane 4, WH2-11-1; lane 5, WH2-1-1; lane 6, WH63-1; and lane 7, Pep4-5B-1. The arrow indicates the immunopositive ADAMTS13 band. W688X6-1 (lane 2) and WH63-1 (lane 6) appeared to exhibit weak reactivity with ADAMTS13. C, three normal human plasma samples were subjected separately to SDS-PAGE under reducing conditions. Electrophoresis was followed by Western blotting analysis using the WH2-11-1 MoAb. The arrow indicates the immunopositive ADAMTS13 band.

ADAMTS13 circulating in the plasma was of full-length (Fig. 5C).

To determine plasma ADAMTS13 concentrations, we performed ELISA using the PoAb and MoAbs. ADAMTS13-depleted plasma was prepared using an immobilized WH10 MoAb affinity column; this plasma was used as a diluent. Before using the ADAMTS13-depleted plasma, we verified that the plasma contained less than 2% of both the ADAMTS13 antigen level and activity, and less than 2.5 ng/ml of WH10, a potential product of ligand leakage from the affinity column (data not shown). Two ELISA systems, using plate-immobilized PoAb with the biotinylated PoAb and immobilized WH10 MoAb with biotinylated WH2-22-1A (*30*), respectively, were utilized. Purified recombinant ADAMTS13 was used as a standard. The purified recombinant ADAMTS13 exhibited a molecular mass peak of approximately 174 kDa by mass spectrometry. We estimated that the extinction coefficient (1 mg/ml; 1cm path) of this purified ADAMTS13 in phosphate buffered saline at pH 7.2 at 280 nm was 1.7. This value was greater than the previously reported one, 1.45 (*31*). Our amino acid analysis data used for determination of the concentration of the purified ADAMTS13 might have involved approximately 10% experimental error. Then, we measured the ADAMTS13 levels in thirty normal plasma samples by both the PoAb- and MoAb-based ELISAs. These assays gave concentrations of ADAMTS13 in plasma of (average ± 1 standard

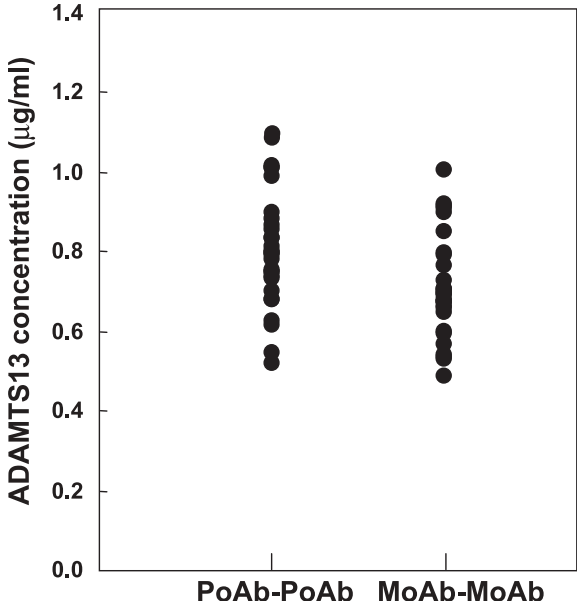

Fig. 6. **Plasma concentrations of ADAMTS13.** We performed using the PoAb and MoAbs. These assays gave concentrations of ADAMTS13 in plasma of (average ± 1 standard deviation) 0.82 ± 0.15 μg/ml (data range = 0.5–1.1 μg/ml) by PoAb-based ELISA and 0.70 ± 0.13 μg/ml (data range = 0.5–1.0 μg/ml) by MoAb-based ELISA.

deviation) 0.82 ± 0.15 μg/ml (data range = 0.5–1.1 μg/ml) by PoAb-based ELISA and 0.70 ± 0.13 μg/ml (data range = 0.5–1.0 μg/ml) by MoAb-based ELISA (Fig. 6).

In conclusion, we established seven MoAbs directed against distinct epitopes across the entire ADAMTS13 molecule. These MoAbs will be useful for clarifying the mechanisms underlying ADAMTS13 enzyme activity. ELISA and Western blotting analyses with the MoAbs and PoAb generated here will be helpful for characterizing the biosynthesis and metabolism of ADAMTS13.

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