Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain

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Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain

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Abstract

Background
The majority of patients diagnosed with thrombotic thrombocytopenic purpura have autoantibodies directed towards the spacer domain of ADAMTS13.

Design and Methods
In this study we explored the epitope specificity and immunoglobulin class and immunoglobulin G subclass distribution of anti-ADAMTS13 antibodies. The epitope specificity of anti-spacer domain antibodies was examined using plasma from 48 patients with acute acquired thrombotic thrombocytopenic purpura by means of immunoprecipitation of ADAMTS13 variants containing single or multiple alanine substitutions. Using similar methods, we also determined the presence of anti-TSP2-8 and CUB1-2 domain antibodies in this cohort of patients.

Results
Antibody profiling revealed that anti-ADAMTS13 immunoglobulin G1 and immunoglobulin G4 predominate in plasma of patients with acquired thrombotic thrombocytopenic purpura. Analysis of anti-spacer domain antibodies revealed that Arg568 and Phe592, in addition to residues Arg660, Tyr661, and Tyr665, also contribute to an antigenic surface in the spacer domain. The majority of patients (90%) lost reactivity towards the spacer domain following introduction of multiple alanine substitutions at Arg568, Phe592, Arg660, Tyr661 and Tyr665. Anti-TSP2-8 and anti-CUB1-2 domain-directed antibodies were present in, respectively, 17% and 35% of the patients’ samples analyzed.

Conclusions
Immunoglobulin G directed towards a single antigenic surface comprising residues Arg568, Phe592, Arg660, Tyr661 and Tyr665 predominates in the plasma of patients with acquired thrombotic thrombocytopenic purpura.

Key words: ADAMTS13, spacer domain, thrombotic thrombocytopenic purpura, antibodies, epitope.


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Introduction

Acquired thrombotic thrombocytopenic purpura (TTP) is a rare and life-threatening autoimmune disease characterized by the presence of autoantibodies directed towards ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Most autoantibodies directed towards ADAMTS13 are of the immunoglobulin (Ig) G class, although IgM and IgA have also be detected. Subclass analysis revealed that IgG4 antibodies are more frequent than IgG1 antibodies, which dominantly respond to TTP. ADAMTS13 regulates the accumulation of ultra-large or unusually-large von Willebrand factor (VWF) multimers on the surface of endothelial cells. The persistence of ultra-large VWF multimers promotes platelet aggregation resulting in obstruction of the microvasculature. VWF multimers are quickly cleaved by ADAMTS13 at the Tyr1605-Met1606 scissile bond in the A2 domain of VWF. Shear stress induces unfolding of VWF multimers, thereby exposing the scissile bond in the A2 domain for cleavage by ADAMTS13. It has been postulated that multiple exoxites within the disintegrin-like/TSP1/cysteine-rich/spacer (DTCS) domains interact with unfolded A2 domain. For example, Arg349 within the disintegrin domain has been shown to interact with residue Asn1614 of VWF whereas spacer domain residues Arg660, Tyr661 and Tyr665 interact with residues Glu1660-Arg1668 in the carboxy-terminal alpha-helical region of the TSP2-8 and CUB1-2 constructs in order to increase ADAMTS13 specificity. Sequences of the sense and antisense oligonucleotide primers used for construction of these variants are available upon request. The availability of a large cohort of patients allowed us to simultaneously address whether antibodies binding to the TSP2-8 and CUB1-2 domains are present in our cohort of patients with acquired TTP.

Design and Methods

Patients

Plasma samples from a panel of 48 patients with acquired TTP containing high titers of anti-ADAMTS13 antibodies were included in this study. The study protocol was approved by the Medical Ethical Committee of the University Medical Center Utrecht in accordance with the Declaration of Helsinki. ADAMTS13 activity levels in all plasma samples were 10% or less as measured using the fluorogenic FRETs-VWF73 substrate assay kit (Peptides International, Louisville, KY, USA). Inhibitor titers were measured with the TechnoZym ADAMTS13 inhibitor enzyme-linked immunosorbent assay (ELISA; Technoclone, Vienna, Austria) or with an ELISA developed in-house. All patients included had a history or were at presentation with primary acquired TTP with hemolytic anemia with fragmented erythrocytes and thrombocytopenia. ADAMTS13 inhibitor levels of plasma samples included in this study were greater than 50 U/mL as measured by the TechnoZym ADAMTS13 inhibitor ELISA.

Construction and expression of recombinant ADAMTS13 and truncated variants

Construction and expression of wild-type pcDNA3.1-propeptide/metalloprotease/disintegrin-like/TSP1/cysteine-rich/spacer-V5-HIS fragment (Invitrogen, Carlsbad, CA, USA) (PMDTCS13-V5-HIS, but termed MDTCS here); a TSP2-8-V5-HIS fragment (TSP2-8) and a CUB1-2-V5-HIS domain fragment (CUB1-2) were produced in stably transfected HEK293 cells as described previously. An Ig-kappa signal peptide was cloned in front of the coding region of the TSP2-8 and CUB1-2 constructs in order to increase the levels of expression of these variants. Primer sequences are available upon request. Cells were grown in Optimem supplemented with glutamax medium (Invitrogen). The medium was harvested after 4 days and expression of ADAMTS13 was confirmed by western blotting or immunoprecipitation using a monoclonal, horseradish peroxidase (HRP)-labeled anti-V5 antibody (Invitrogen), a mouse anti-TSP5-8 monoclonal antibody (20A5)35, a mouse anti-CUB1-2 domain monoclonal antibody (20D2) and a rabbit anti-metalloprotease polyclonal antibody (Abcam, Cambridge, UK). Monoclonal antibody 20D2 was prepared essentially as described previously. A sheep-anti-mouse-HRP-labeled antibody (in conjunction with 20A5 and 20D2) and a donkey anti-rabbit-HRP-labeled antibody (in conjunction with the rabbit anti-metalloprotease antibody), both obtained from GE Healthcare (Bio-Sciences AB, Uppsala, Sweden), were used for chemiluminescence detection (Roche Applied Science, Almere, the Netherlands).

Single alanine (R568A and F592A) and double alanine mutants (R568A/F592A) were introduced into the wild-type vector (pcDNA3.1-PMDTCS13-V5-HIS) using Quick-Change PCR (Stratagene, Amsterdam, the Netherlands). The same mutants were introduced into the previously described plasmid pcDNA3.1-PMDTCS13-R660A/Y661A/Y665A-V5-HIS (termed MDTCS-RYY) resulting in a total of eight variants including the wild-type. Sequences of the sense and antisense oligonucleotide primers used for construction of these variants are available upon request. All vectors were verified by sequencing. All ADAMTS13 variants were transiently expressed essentially as described previously.

Epitope mapping of anti-ADAMTS13 antibodies

Recombinant antibodies or antibodies in plasma of the patients were bound to protein G sepharose (GE Healthcare) in 50 mM Tris pH 7.6 (Invitrogen), 500 mM NaCl (Merck), 1% (w/v) bovine serum albumin (BSA; Merck) 1% (w/v) Triton X-100 (Merck) and 0.1% (v/v) Tween-20 (Sigma Aldrich, Zwijndrecht, the Netherlands). As a negative control an anti-pneumococcal monoclonal antibody was included. The two human monoclonal anti-spacer domain antibodies I-9 and II-1 have been described previously. Monoclonal anti-V5 antibody (Invitrogen) coupled to protein G sepharose (GE Healthcare) was used as a positive control. Normal human pooled plasma from 47 healthy donors was included as a negative control. Thirty microliters of patients' plasma were incubated with protein G sepharose for 1 h at room temperature. Cell culture medium containing 1 µg of recombinant ADAMTS13 fragments was added to antibody-loaded sepharose.
and incubated in 50 mM Tris pH 7.6, 150 mM NaCl, 1% BSA, 1% Triton X-100 and 0.1% Tween-20 for at least 14 h. After washing two to three times with buffer of the same composition, and a third to fourth time with 20 mM Tris pH 7.6, bound proteins were eluted with 100 mM glycine pH 2.0 and applied to either 7.5% or 10% sodium dodecylsulfate-polyacrylamide gels depending on the size of the fragments. Immunoblots were prepared on Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, USA) and stained with HRP-labeled anti-V5 monoclonal antibody (Invitrogen). For detection of chemiluminescence substrate (Roche Applied Science) and CL-Xposure films (Perbio Science, Etten-Leur, the Netherlands) were used. The composition of the immunoprecipitation buffer was adjusted for the experiments performed with the TSP-2 and CUB1-2 variants by lowering the amount of Triton X-100 from 1% to 0.5% and Tween-20 from 0.1% to 0.05% in order to facilitate antibody binding towards these carboxy-terminal fragments.

Results

Anti-ADAMTS13 immunoglobulins in plasma of patients with acquired thrombotic thrombocytopenic purpura

Plasma samples from 48 patients with acquired TTP were analyzed for the presence of IgG, IgM and IgA as outlined in the Online Supplementary Appendix. Anti-ADAMTS13 IgM was found in five of these 48 patients (Figure 1). Anti-ADAMTS13 IgA was detected in nine out of the 48 patients (Figure 1). IgA antibodies can be further subdivided into IgA1 and IgA2. In all IgA-positive samples anti-ADAMTS13 IgA1 was found suggesting that IgA1 (and not IgA2) antibodies develop in patients with acquired TTP. IgG was present in all the patients’ samples analyzed. Levels ranged from 0.25 to 19.5 μg/mL. Analysis of the subclass of anti-ADAMTS13 IgG revealed that IgG1 was present in 55 out of 48 samples and IgG4 in 33 out of 48 samples analyzed (Figure 1). Low levels of IgG2 and IgG3 were observed in seven and four patients, respectively (Figure 1). Anti-ADAMTS13 IgG1 and IgG4 co-existed in 13 out of 46 patients. These results show that anti-ADAMTS13 IgG1 and IgG4 predominate in plasma of patients with acquired TTP.

Arg568 and Phe592 contribute to the binding of human monoclonal anti-spacer domain antibodies

Inspection of the three-dimensional structure of the spacer domain reveals that exposed residues Arg568 and Phe592 are in close proximity of Arg660, Tyr661 and Tyr665 (Figure 2A,B).12 This raises the possibility that Arg568 and Phe592 also contribute to the binding of spacer domain antibodies. We assessed the binding of two patient-derived monoclonal antibodies I-9 and II-1 to MDTCS-R568A, MDTCS-F592A and MDTCS-R568A/F592A variant (Figure 2C). As a control, we monitored the reactivity of these antibodies with the previously described R660A/Y661A/Y665A (MDTCS-RYY) variant.14 Binding to RYY variants containing R568A, F592A and R568A/F592A substitutions was also evaluated. All variants were expressed in HEK293 cells; the levels of expression of all variants were similar to that of wild-type MDTCS (data not shown). Antibody II-1 did not react with MDTCS-R568A or MDTCS-F592A (Figure 2). Antibody I-9 retained its reactivity towards MDTCS–F592A, whereas binding to both MDTCS–R568A and MDTCS–R568A/F592A was abrogated (Figure 2C). As described previously, antibodies II-1 and I-9 did not bind to MDTCS-RRY. As expected, MDTCS-RRY variants containing R568A, F592A or R568A/F592A mutations also did not interact with antibodies II-1 and I-9 (Figure 2C).

Spacer domain residues Arg568, Phe592, Arg660, Tyr661 and Tyr665 provide a major target for antibodies in patients with acquired thrombotic thrombocytopenic purpura

We analyzed the contribution of Arg660, Tyr661 and Tyr665 to binding of spacer domain antibodies in a cohort of 48 patients with acquired TTP. In addition, we determined whether residues Arg568 and Phe592 contribute to the binding of patient-derived anti-ADAMTS13 antibodies. Binding of patients’ IgG to MDTCS, MDTCS-R568A, MDTCS-F592A, MDTCS-R568A/F592A, MDTCS-R660A/Y661A/Y665A (in short MDTCS-RRY), MDTCS-RRY-R568A, MDTCS-RRY-F592A and MDTCS-RRY-R568A/F592A was evaluated. Replacement of Arg568 by an alanine had little impact on binding of patient-derived IgG; all patients’ samples bound to this variant (Figure 3A,B). Replacement of Phe592 resulted in a decline in reactivity with the MDTCS-fragment for 12 out of 48 patients (patients 1, 4, 7, 10, 12, 16, 22, 25, 28, 38, 43 and 47). Reactivity with MDTCS-R568A/F592A was absent or strongly reduced for 16 out of 48 patients’ samples analyzed (samples 1, 3, 4, 5, 7, 9, 10, 12, 16, 22, 25, 28, 38, 39, 43 and 47). Together, these findings indicate that residues Arg568 and Phe592 contribute to the binding of antibodies to the MDTCS-fragment in a significant number of patients. Next, we determined the binding of patient-derived IgG to MDTCS-RRY (Figure 3). In 33 out of 48 samples analyzed, the binding was significantly reduced compared to that with “wild-type” MDTCS. The introduction of F592A into the RYY-variant resulted in a decrease in binding in all samples analyzed (see, for example, patients 11, 14, 34 and 41 in Figure 3). In contrast, substitution of Arg568 for Ala had little impact on binding of patient-derived IgG to the MDTCS-RRY fragment (see, for example patients 8, 19 and 41 in Figure 3). Binding of patient-derived IgG to MDTCS-RRY was completely abrogated for 43 out of 48 patients’ samples analyzed when both Arg568 and Phe592 were replaced by an alanine residue (Figure 3). Residual binding to MDTCS-RRY-R568A/F592A was still observed for patients’ samples 6, 16 and 37.

Antibodies directed towards the TSP-2 and CUB1-2 domains of ADAMTS13 in patients with acquired thrombotic thrombocytopenic purpura

We determined whether antibodies directed towards TSP-2 and CUB1-2 domains were present in plasma from the 48 patients with acquired TTP included in this study. Only a few patients had anti-TSP-2-directed antibodies (Figure 4). IgG derived from one patient’s sample (patient 3) reacted very well with the TSP-2 fragment; for two other samples (from patients 4 and 16) weak signals were observed whereas five additional samples (from patients 6, 13, 18, 26 and 28) showed weak but detectable binding to TSP-2 (Figure 4). Thus, TSP-2-directed antibodies were present in 17% of the patients’ samples analyzed. Considerable amounts of antibodies directed towards the CUB1-2 domains were detected in five samples (from patients 8, 9, 24, 26 and 28) (Figure 5). In six samples (from
patients 3, 4, 10, 11, 34 and 43) lower levels of anti-CUB1-2 domain antibodies were present whereas very low levels were observed in samples from patients 1, 40, 41, 42, 44 and 45 (Figure 5). Anti-CUB1-2 domain antibodies were found in approximately 35% of the samples analyzed. Only patients 3, 4, 26 and 28 had both anti-TSP2-8 and
Figure 2. (A) Three-dimensional presentation of an ADAMTS13 fragment consisting of the DTCS-domains is depicted with the modeled metalloprotease domain (disintegrin domain [DIS] in green; the thrombospodin type repeat 1 [TSP1] in blue; the cysteine-rich domain [Cys] in pink and the spacer domain [Spacer] in light blue). The crystal structure of the metalloprotease domain of ADAMTS1, 4 and 5 were used as a template to model the metalloprotease domain (Met in light red) and was modeled using the HHpred server as described previously. Loops that were lacking in the DTCS crystal structure (323 TFARDEHLD 330 and 459 RSSPGGA 465) were modeled using the MODELLER 9v7 program. (B) Residues Arg568, Phe592, Arg660, Tyr661 and Tyr665 are highlighted in red: these residues provide an exposed surface in the spacer domain. (C) Immunoprecipitation of MDTCS alanine(-hybrid)-mutants with recombinant monoclonal anti-ADAMTS13 antibodies I-9 and II-1. The reactivity of patient-derived anti-ADAMTS13 monoclonal antibodies I-9 and II-1 was determined by immunoprecipitation. An IgG1 isotype control antibody (-) and anti-VS (+) antibody were used as controls. The first four lanes show MDTCS, MDTCS-R568A, MDTCS-F592A and the MDTCS-R568A/F592A double mutant. The second four lanes show the triple mutant R660A/Y661A/Y665A (RYY), MDTCS-RYY-R568A, MDTCS-RYY-F592A and MDTCS-RYY-R568A/F592A.

Figure 3. (A) Immunoprecipitation of MDTCS alanine(-hybrid)-mutants with plasma samples from patients with acquired TTP. (A)(B) The reactivity of anti-ADAMTS13 antibodies present in plasma of 48 patients with acquired TTP was determined by an immunoprecipitation experiment using the MDTCS-variants described in the legend of Figure 2. Normal human pooled plasma (--) and anti-VS (+) antibody were used as controls.
anti-CUB1-2 domain-directed antibodies, although levels of anti-CUB1-2 and anti-TSP2-8 antibodies varied considerably. For instance, patient 3 had high levels of anti-TSP2-8-directed antibodies whereas levels of CUB1-2-directed antibodies in this patient were very low (Figures 4 and 5). Conversely, patients 26 and 28 had relatively high levels of anti-CUB1-2 domain antibodies but low levels of anti-TSP2-8 antibodies.

Discussion

IgG, IgM and IgA autoantibodies directed towards ADAMTS13 are found in the plasma of patients with acquired TTP. We recently showed that residues Arg660, Tyr661 and Tyr665 contribute to an antigenic surface in the spacer domain. In this study we show that Arg668 and Phe592, which are in close proximity to Arg660, Tyr661 and Tyr665 also contribute to the binding of anti-spacer domain antibodies. The antigenic surface covered by the variable domains of an antibody usually covers around 1500 Å². The surface accessibility of an arginine is approximately 211 Å², a tyrosine can cover about 104 Å² and a phenylalanine about 123 Å². The potential surface area covered by Arg568, Phe592, Arg660, Tyr661 and Tyr665 is approximately 750Å². This value was confirmed by using a surface accessibility calculator. Arg568, Phe592 and Arg660, Tyr661 and Tyr665 originate from three distinct surface loops. All patients’ samples with the exception of samples 6 and 37 displayed a strong reduction in binding to an MDTCS fragment in which Arg568, Phe592, Arg660, Tyr661 and Tyr665 were replaced by Ala (Figure 3). This observation strongly suggests that Arg568, Phe592, Arg660, Tyr661 and Tyr665 comprise an immunodominant region that is targeted by the antibodies that develop in the majority of patients with acquired TTP. It should be noted that the ADAMTS13 variants described in this study partially retained their VWF processing activity (Online Supplementary Figure S2). In view of the restricted epitope specificity of anti-ADAMTS13 antibodies this observation may provide a basis for the design of ADAMTS13 variants with reduced antigenicity.

Binding of IgG derived from patients 6 and 37 (and to a
CUB1-2 domains were present in our cohort of patients. In view of the multiple contacts between ADAMTS13 and the VWF A2 domain it can certainly not be excluded that these antibodies impair ADAMTS13 function.\textsuperscript{11,12}

In this study we also investigated whether antibodies directed towards the proximal TSP2-8 repeats and CUB1-2 domains were present in our cohort of patients. Anti-TSP2-8 antibodies were found in 17% of the patients analyzed. Weak signals were observed when the TSP2-8 fragments were precipitated with patient-derived immunoglobulins (Figure 4). Anti-TSP2-8 antibodies were found in 57% and 28% of the patients’ samples analyzed in two previous studies.\textsuperscript{16,19} Anti-CUB1-2 antibodies were observed in 35% of patients analyzed. This percentage is similar to the value of 31% reported by Zheng and co-workers.\textsuperscript{19} In an early study anti-CUB1-2 domain antibodies were found in 64% of the patients analyzed.\textsuperscript{16} The observed discrepancies are potentially explained by differences in patients’ characteristics. Alternatively, the weak signals of the precipitated TSP2-8 and CUB1-2 domains observed in our study could have been due to the relatively stringent conditions of our immunoprecipitation assay. The functional significance of anti-TSP2-8 and anti-CUB1-2 antibodies is presently unclear. Several studies have shown that the carboxy-terminal TSP2-8 and CUB1-2 domains are important for the processing of VWF by ADAMTS13.\textsuperscript{22,30,31} Zheng and co-workers showed that platelet counts on admission were lower in patients with anti-TSP2-8 and/or anti-CUB1-2 IgG.\textsuperscript{19} This observation suggests that antibodies directed towards the carboxy-terminal domains inhibit ADAMTS13 activity or alternatively enhance its clearance from the circulation. Isolation of human monoclonal antibodies directed towards the TSP2-8 and CUB1-2 domains from patients with acquired TTP will help to determine whether these antibodies also impair ADAMTS13 function.

**Authorship and Disclosures**

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