RESEARCH ARTICLE

Mechanisms of the Interaction Between Two ADAMTS13 Gene Mutations Leading to Severe Deficiency of Enzymatic Activity

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The inherited deficiency of the von Willebrand factor-cleaving protease ADAMTS13 is associated with rare forms of thrombotic thrombocytopenic purpura (TTP). We investigated a woman with a family history of chronic recurrent TTP and undetectable plasma levels of ADAMTS13 activity. Genetic analysis revealed two missense mutations in the heterozygous state: p.Val88Met substitution in the metalloprotease domain and p.Gly1239Val substitution in the first CUB domain of ADAMTS13. To explore the mechanism of ADAMTS13 deficiency in this patient, the wild type (WT; ADAMTS13WT) and each mutant construct (ADAMTS13Val88Met, ADAMTS13Gly1239Val) were transiently expressed in HEK 293 and COS-7 cells. To recapitulate the compound heterozygous state of the patient, both mutant ADAMTS13 proteins were also expressed together. The p.Val88Met mutation led to a secretion defect causing intracellular accumulation of the protease. The mechanistic effects of the mutations were further explored by means of differential immunofluorescence, that demonstrated an homogeneous distribution of ADAMTS13WT in both compartments, while ADAMTS13Gly1239Val failed to reach the Cis-Golgi compartment and remained in the ER. Hum Mutat 27(4), 330–336, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: ADAMTS13; thrombotic thrombocytopenic purpura (TTP); mutations expression study

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is an inherited or acquired disorder characterized by the massive formation of platelet thrombi in the microcirculation accompanied by hemolytic anemia, thrombocytopenia, and clinical and laboratory signs of renal and neurological failure. In many cases the trigger of TTP is the presence in plasma of ultralarge multimers of the adhesive glycoprotein von Willebrand factor (ULVWF; MIM# 193400), due to the inherited or acquired deficiency of the plasma metalloprotease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin 1 repeats; MIM# 604134) that physiologically reduces the size of VWF multimers [Moake, 2002]. Unlike other members of the ADAMTS family of proteases, ADAMTS13 consists of a relatively short propeptide, of several thrombospondin-1 (TSP1) repeats and 2 CUB (complement components C1r/C1s, urinary Epidermal Growth factor and bone morphogenetic protein-1) domains at the C-terminus. The enzymatic activity of ADAMTS13 depends on divalent metal ions in the following order of potency: Zn2+ < Ca2+ < Sr2+ < Ba2+ ions [Furlan et al., 1996; Zheng et al., 2001].

Inherited TTP is a rare autosomal recessive disorder due to homozygous or double heterozygous mutations in the ADAMTS13 gene [Assink et al., 2003; Bestetti et al., 2003; Kokame and Miyata, 2004; Licht et al., 2004; Studt et al., 2005; Uchida et al., 2004; Veyradier et al., 2004]. As many as 53 mutations causing inherited ADAMTS13 deficiency have been identified so far in regions of the gene encoding different domains [Assink et al., 2003; Bestetti et al., 2003; Kokame and Miyata, 2004; Licht et al., 2004; Studt et al., 2005; Uchida et al., 2004; Veyradier et al., 2004], with only a few of them characterized by in vitro expression studies [Kokame and Miyata, 2004; Uchida et al., 2004]. We carried out a molecular investigation in an Italian woman from a previously reported family with chronic recurrent TTP [Noris et al., 2005], who had her first episode of TTP at the age of 23 years. DNA analysis identified two missense mutations, a G to A substitution of nt 262 (c.262G>T, p.Val88Met), located in the metalloprotease domain [Bestetti et al., 2003] and a G to T substitution of nt 3717 (c.3717G>T, p.Gly1239Val), located in the first CUB domain of ADAMTS13. The metalloprotease

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domain is known to have an important role for Zn$^{2+}$ binding and protease activity whereas the physiological role of the CUB domains located at the C-terminus of ADAMTS13 is less certain. In order to explain the patient’s phenotype of severe ADAMTS13 deficiency, we chose to explore the mechanistic effect of each separate mutation and of their combination by means of expression studies in mammalian cells.

**MATERIALS AND METHODS**

**Patient**

An Italian woman had her first episode of TTP at the age of 23 years during her first pregnancy. Ten additional episodes subsequently occurred, usually in association with such precipitating events as pregnancy, first-trimester miscarriages, or infections. She was successfully treated for each episode with plasma infusions, antiplatelet agents, and anticoagulants. The patient’s sister died at the age of 55 years because of a cerebrovascular event during a severe episode of TTP; her brother has had no clinical manifestation of TTP so far in spite of ADAMTS13 deficiency at the same degree of severity of the propositus [Noris et al., 2005]. This study was conducted with the patient’s consent and approval by the Institutional Review Board, IRCCS Maggiore Hospital, Mangiagalli and Regina Elena Foundation.

**Measurement of ADAMTS13 Activity, Anti-ADAMTS13 Activity, and VWF Multimers in Plasma**

Venous blood was collected into 0.1 vol 0.129 M trisodium citrate during a remission period. Platelet-poor plasma was obtained by centrifugation at 3,000g for 20 min, snap frozen, and stored at −80°C until tested. ADAMTS13 activity was measured in plasma with the collagen binding assay (CBA) [Gerritsen et al., 1999]. Antibodies neutralizing ADAMTS13 were detected using a previously described method and residual ADAMTS13 activity was measured by CBA [Peyvandi et al., 2004a]. Plasma ADAMTS13 activity was also measured with a modification [Allford et al., 2000] of an immunoblotting assay [Furlan et al., 1998], tailored to be more sensitive to very low levels of protease activity. Plasma was diluted in 10 mM Tris, 150 mM NaCl, 1 mM Pefabloc (pH 7.4), and incubated at 37°C for 5 min with 10 mM barium chloride (Sigma-Aldrich, St. Louis, MO, www.sigma-aldrich.com). A total of 100 μl of the incubation mixture was added to 50 μl of a 5 U/ml solution of recombinant VWF (kindly provided by Dr. Turencek, Baxter Bioscience, Vienna, Austria). The reaction mixture was overlaid on a circular dialysis membrane (Millipore VSWP, diameter 7.2–7.4, and lysed with 1 ml of buffer (1% NONIDET NP40, 150 mM NaCl, 5 mM Tris). To recapitulate the compound heterozygous state of the patient, both mutant constructs were also transiently transfected together (ADAMTS13Val88Met+Gly1239Val).

The VWF multimeric pattern was analyzed in platelet poor-plasma by discontinuous low-resolution SDS-agarose gel electrophoresis (0.9% LGT-agarose) and autoradiography [Ruggeri and Zimmerman, 1981].

**Genetic Analysis**

DNA was extracted from peripheral blood leukocytes and all the exons and intron–exon boundaries of the ADAMTS13 gene (NT_017539) were amplified by PCR and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Monza, Italy; www.appliedbiosystems.com). The primer sequences and amplification parameters are available on request. The secondary structure prediction of the effect of each mutation on ADAMTS13 structure was obtained using the predict protein program available on line (www.aber.ac.uk/compsi/research/biol/ds/prof/ or http://us.expasy.org).

**Transient Transfection of Wild-Type (WT) and Mutant ADAMTS13 Expression Vectors**

A pCDNA 3.1 mammalian expression vector (kindly provided by Dr. F. Scheiflinger, Baxter Bioscience, Vienna, Austria) containing the entire coding region and a small part of the 3’ untranslated region of the ADAMTS13 gene (pCDNA 3.1-ADAMTS13[WT]) was used as a template for site directed mutagenesis. The p.Val88Met and p.Gly1239Val mutations were introduced into pCDNA3.1-ADAMTS13[WT] using the Quick-Change Site-directed Mutagenesis Kit on an ABI Prism 310 Genetic Analyzer (Stratagene, La Jolla, CA; www.stratagene.com). A forward primer (5'-CTGAGAATGCTGTCGAGCCATGGGCCCCGATGTCTTG-3') and a reverse primer (5'-GGAGAAACATCCGGGCCCCATTGCCACCGCACTGCCAG-3') were used to introduce c.262G>C (signed as A) in human ADAMTS13 cDNA (NM_017587), to obtain pCDNA 3.1-ADAMTS13Val88Met. A forward primer (5'-CTCATCTGCACTTGGGCGTGGACATTGTGTCGCTTTCCGCTTTCCGCGCTG-3') and a reverse primer (5’-CCCAACGCAACATGAAGCTGGCCACCACCGCACTGCCAGTGGAGAACAGA-3’) were used to introduce c.3717G>A (signed as T) in human ADAMTS13 cDNA, to obtain pCDNA 3.1-ADAMTS13Gly1239Val.

The WT and mutant constructs were also subcloned into the mammalian expression vector pCDNA 3.1/D/V5-His TOPO [Cheng and Shuman, 2000]. To explore the functional significance of the mutations, 50 μg of each vector were transiently transfected in COS-7 and HEK293 cells by electroporation according to the manufacturer’s instructions (EQUIBIO/Easyjett Plus; Thermo Electron Corp., Needham Heights, MA; www.thermo.com). Serum-free conditioned medium was added to equivalent numbers of confluent cells at 24 hr and the conditioned medium collected at 72 hr was clarified by centrifugation and concentrated 10-fold using AMICON Centricon® YM-30 Column (Millipore, Billerica, MA). Cells were washed with phosphate-buffered saline, pH 7.2–7.4, and lysed with 1 ml of buffer (1% NONIDET NP40, 150 mM NaCl, 5 mM Tris). To recapitulate the compound heterozygous state of the patient, both mutant constructs were also transiently transfected together (ADAMTS13Val88Met+Gly1239Val).
The transfection efficiency was evaluated by luciferase system (Renilla Luciferase Assay System; Promega, Madison, WI; www.promega.com). Recombinant ADAMTS13 enzymatic activity was measured in the cell conditioned media using the modification of the immuno blotting assay as described above. Serial dilutions of WT cell supernatants (1:10–1:640) were used for the calibration curve.

**Western Blot Analysis**

To measure the expression of WT and mutant recombinant ADAMTS13 in transfected cell lines, amounts of conditioned media and cell lysates according to the transfection efficiency were solubilized in gel-loading buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiotreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol), separated by SDS-polyacrylamide electrophoresis (7% polyacrylamide) and transferred on Pure Nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with skim milk, membranes were incubated with 1 μg/ml anti-V5 monoclonal antibody against the C-terminal tag of recombinant ADAMTS13 (Invitrogen, Carlsbad, CA; www.invitrogen.com) and then with peroxidase-labeled anti-mouse immunoglobulin G (Amersham Biosciences, Uppsala, Sweden). Detection was carried out using a chemiluminescent substrate (ECL Blotting System; Amersham Biosciences, Uppsala, Sweden) followed by exposure on autoradiographic film.

**Immunofluorescence Studies**

COS-7 cells were transfected with ADAMTS13 WT and with each mutant construct and plated in six-well cluster plates containing coverslips. At 24 hr after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for intracellular staining. Permeabilized cells were then incubated with the anti-V5 monoclonal antibody against the C-terminal tag of recombinant ADAMTS13 followed by anti-mouse IgG fluorescein isothiocyanate (FITC) conjugated antibody (BioSource International, Camarillo, CA; www.biosource.com). To detect the cellular localization of WT and mutant recombinant ADAMTS13, transfected cells were stained simultaneously with anti-V5 antibody and mouse polyclonal antibody against the proteins GM130 (a Cis-Golgi marker) and Bip-GRP78 stained with Cy3-conjugated antibody (BD Biosciences, Franklin Lakes, NJ; www.bdbiosciences.com). Recombinant ADAMTS13 stained with FITC conjugated antibody was detectable as green color, identifying the presence of the protein but not its localization. GM130 and Bip-GRP78 stained with Cy3-conjugated antibody (Chemicon International, Temecula, CA; www.chemicon.com) were detectable as red color, identifying the different cell compartments. Image acquisition using both fluorescent signals (green and red) identified the localization of the ADAMTS13 proteins in each cell compartment, visible as yellow color. Coverslips were mounted onto slides and examined with a Leica DMR epifluorescence microscope (Leica Imaging System, Cambridge, United Kingdom; www.leica.com) equipped with a CCD camera (Cohu, San Diego, CA; www.cohu-cameras.com) using a specific filter. The images were recorded using a QFISH software (Leica Imaging System, Cambridge, United Kingdom).

### RESULTS

**Laboratory and Genetic Data**

During periods of clinical and laboratory remission the patient had on several occasions less than 6% of ADAMTS13 activity as measured by CBA and less than 1.6% as measured by the more sensitive immunoblotting assay that is able to measure as little as 1.6% of protease activity. ULVWF multimers were repeatedly detected in plasma whereas no anti-ADAMTS13 neutralizing activity was detectable. Genetic analysis (nucleotides numbered from the A of the initiation Met codon) identified 2 previously reported missense mutations and a SNP, G to C substitution at nt 1342 (c.1342G>C, p.Gln448Glu) [Levy et al., 2001]. The first missense mutation was a G to A substitution at nt 262 (c.262G>A), leading to the substitution of Valine 88 to a Methionine [Bestetti et al., 2003] in the metalloprotease domain of the ADAMTS13 protein (p.Val88Met); the second missense mutation was a G to T substitution at nt 3717 in exon 27 (c.3717G>T), leading to the substitution of Glycine 1239 to a Valine in the first CUB domain (p.Gly1239Val) [Noris et al., 2005; Peyvandi et al., 2004b]. The patient was heterozygous for both mutations and for the p.Gln448Glu polymorphism, that has an allelic frequency of 32% in Italians. The novel mutation p.Gly1239Val was not found in 100 normal Italians. Secondary structure prediction indicated that there should be no significant change in the helical region encompassing Glycine 1239 on replacement with Valine, but that the p.Val88Met mutation, located on the metalloprotease domain, should enhance the conformational instability of the 80–87 region that contains a Glutamate 83 involved in a Ca2+ ion coordination.

**Western Blot Analysis**

Both the conditioned medium and lysate of cells transfected with the ADAMTS13 WT construct contained a single immunoreactive band with a molecular mass of approximately 190 kDa (Fig. 1). The band was not detectable in the medium of untransfected cells used as a negative control. In the conditioned medium ADAMTS13 Val88Met appeared as a band with the same molecular mass of the ADAMTS13 WT but chemiluminescence intensity was estimated to be approximately 40 to 50% of that of ADAMTS13 WT. In cell lysate an increased amount of the mutant protease compared with ADAMTS13 WT was seen. For ADAMTS13 Gly1239Val a secretion defect causing intracellular accumulation with no detectable protein in supernatant was observed. In the conditioned medium and the lysate of cells transfected with both the mutant constructs (ADAMTS13 Val88Met + Gly1239Val) an intermediate pattern of results was observed (Fig. 1).

**ADAMTS13 Activity in Cell-Conditioned Media**

The enzymatic activity of recombinant ADAMTS13 was measured by immunoblotting assay 72 hr after transfection in the conditioned media of cells transfected with WT and mutant constructs. The enzymatic activities of ADAMTS13 Val88Met, ADAMTS13 Gly1239Val and ADAMTS13 Val88Met + Gly1239Val were reduced on average to 24%, 6%, and 18%, respectively, in comparison with ADAMTS13 WT (Fig. 2).

**Immunofluorescence Studies**

ADAMTS13 WT was mainly localized in the perinuclear area, ADAMTS13 Val88Met was diffusely present throughout the cytoplasm with a perinuclear enhancement, and ADAMTS13 Gly1239Val was diffusely present throughout the cytoplasm with no perinuclear enhancement (Fig. 3). An anti-V5 monoclonal antibody recognizing recombinant ADAMTS13 proteins and monoclonal antibodies recognizing
markers of Cis-Golgi and ER were used to characterize the cellular localization of recombinant ADAMTS13 detected as yellow fluorescence. ADAMTS13_{Val88Met} was visualized in both compartments, but with lesser intensity than the WT protein, while ADAMTS13_{Gly1239Val} was visualized only in the ER and not in the Golgi compartment (Fig. 4).
DISCUSSION

Congenital TTP is a rare disorder characterized by undetectable or very low plasma levels of the VWF-cleaving protease ADAMTS13 associated with an array of mutations in the ADAMTS13 gene [Assink et al., 2003; Bestetti et al., 2003; Kokame and Miyata, 2004; Licht et al., 2004; Studt et al., 2005; Uchida et al., 2004; Veyradier et al., 2004]. Until now, only few of these mutations have been characterized by in vitro expression studies [Kokame and Miyata, 2004; Uchida et al., 2004] in order to explain the mechanisms underlying phenotypes. We chose to investigate a patient who was peculiar because she developed the first episode of TTP only when she was 23 years old and in comonogeneity with her first pregnancy, and because undetectable levels of ADAMTS13 enzymatic activity were associated with compound heterozygosity for two missense mutations, one located in the metalloprotease domain and the other in the first CUB domain of ADAMTS13. As previously reported by Noris et al. [2005], her sister, who developed chronic renal failure and died, also showed the heterozygous mutation p.Ser890Ile in complement factor H (MIM# 134370).

Now 15 mutations have been identified in the metalloprotease domain and nine of them have been characterized by in vitro expression studies [Kokame and Miyata, 2004; Uchida et al., 2004]. Most of them led to an alteration of the secretion pathway and/or affected the specific activity of the enzyme, sometimes resulting in the absence of enzymatic activity due to Zn$^{2+}$ binding site alterations [Uchida et al., 2004]. In this study, another mutation in the metalloprotease domain of ADAMTS13, p.Val88Met, was characterized by in vitro expression studies. Recombinant ADAMTS13Val88Met was synthesized in normal amounts in cell lysates, but its secretion was impaired by approximately 40 to 50% compared to ADAMTS13WT. This pattern was confirmed by immunofluorescence studies, showing that ADAMTS13Val88Met was visualized in both the ER and Golgi compartments, but with less intensity than the WT recombinant protease. The mutant protease also had reduced enzymatic activity, the most sensitive immunoblotting assay measuring 24% of ADAMTS13 activity in comparison with the WT protease. These data are consistent with the analysis of the secondary structure of ADAMTS13, that predicted a rearrangement of the region between residues 61–70 and a slight increase of the sheet content over residues 80–90 compared to ADAMTS13WT. We surmise that the p.Val88Met mutation alters the Ca$^{2+}$-binding site and leads to partial secretion defect due to intracellular accumulation. On the whole, these expression studies corroborate the available knowledge on the key role of the metalloprotease domain on the secretion and enzymatic activity of the protease.

The role of the CUB domains at the C-terminus of the protease, where the p.Gly1239Val mutation is located, is of uncertain physiological relevance. A few studies support the views that the seventh and eighth TSP-1 repeats and the CUB domains are dispensable for protease activity [Banno et al., 2004]. On the other hand, studies based upon peptides from the CUB domains that inhibited the VWF-cleaving protease activity under flow suggested a functional role for these domains [Bernardo et al., 2003]. Moreover Majorus et al. [2005] have recently showed that the binding of ADAMTS13 to immobilized VWF appeared to be modulated by the most C-terminal TSP-1 and CUB domains. Until now, six different mutations on the first and second CUB domains of ADAMTS13 were identified. p.Cys1213Tyr and p.Arg1219Trp, located on the first CUB domain, were associated with reduced enzymatic activity but normal secretion [Kokame and Miyata, 2004; Moro et al., 2003]. The c.4143-414insA mutation, located in the second CUB domain, had little effect on the specific activity of the enzyme but secretion was impaired, conserving only 15% of activity in comparison with the WT protease. The defect may be due to the removal of the central β-strands present in the CUB domain, resulting in the destruction of its architecture [Pimanda et al., 2004]. The missense mutation p.Gly1239Val, located in the first CUB domain, when analyzed by secondary structure prediction, was expected to produce no significant change in the helical region encompassing Glycine 1239 on replacement with Valine. However, our in vitro expression studies showed reduced enzymatic activity of recombinant ADAMTS13Gly1239Val associated with increased immunofluorescence staining throughout the cytoplasm, with no specific quantitative detection. No perinuclear enhancement and a retention of the mutant protein in the ER compartment were observed, suggesting an important role of the first CUB domain in the secretion pathway of ADAMTS13.

The constructs carrying both p.Val88Met and p.Gly1239Val and hence mimicking the compound heterozygous state of the patient were characterized by a reduction of the specific activity of the protease but failed to accurately reflect the undetectable levels measured in patient. The presence of a strong CMV promoter (human cytomegalovirus immediate-early promoter) in the expression vector used for in vitro expression studies may be
responsible for an overexpression of ADAMTS13 enzyme activity. This promoter is usually employed to permit a high level of expression of recombinant proteins in a wide range of mammalian cells [Boshart et al., 1985; Nelson et al., 1987]. It should also be considered that the patient, besides the p.Val88Met and p.Gly1239Val mutations, is also heterozygous for the common p.Gln448Glu polymorphism. We did not produce a construct containing also the p.Gln448Glu polymorphism, because previous expression studies have convincingly shown that this polymorphism does not affect ADAMTS13 activity [Kokame et al., 2002]. However, it cannot be ruled out that the mechanistic interaction between the two mutations and the polymorphism may explain the lower levels of ADAMTS13 activity measured in this patient’s plasma.

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