A Patient With Type 2N von Willebrand Disease Is Heterozygous for a New Mutation: Gly22Glu. Demonstration of a Defective Expression of the Second Allele by the Use of Monoclonal Antibodies

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We report the case of a Chinese patient who has subnormal von Willebrand factor (vWF) level and normal vWF multimeric pattern, but a lack of vWF capacity to bind factor VIII (FVIII). Exons 18 to 20 of the patient’s vWF gene were analyzed by DGGE and a G2354 → A substitution which changes the encoded amino acid sequence from Gly22 to Gln was identified. The patient is heterozygous for this substitution, creating a unique Sac I restriction site. Recombinant vWF (rvWF) containing the candidate mutation was transiently expressed in COS-7 cells. It was processed and secreted normally but failed to bind FVIII. FVIII binding ability of hybrid rvWF, obtained by cotransfection of normal and mutated expression vectors and corresponding to a heterozygous genotype, was moderately decreased. To explain this functional discrepancy between patient’s plasma vWF and hybrid rvWF, we used anti-vWF monoclonal antibodies (MoAbs) as capture in an enzyme-linked immunosorbent assay test. MoAb 32B12 recognized both wild-type and mutated rvWFs whereas MoAb 418 did not recognize mutated rvWF. Because MoAb 418 also failed to capture the plasma vWF from propositus, it means that his second nonmutated allele is not expressed or expressed at a very low level.

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MATERIALS AND METHODS

Case report. The propositus (II-3) whose family tree is shown in Fig 1A is a 38-year-old man with a lifelong history of mild bleeding (epistaxis and easy bruising). He has a bleeding time of 9 minutes (normal, <10 minutes) and a plasma vWF antigen (vWF:Ag) level of 49 U/dL (normal range, 50 to 150 U/dL). Ristocetin-induced platelet aggregation (RIPA) with 1.2 mg/mL ristocetin is normal. Plasma FVIII activity (FVIII:C) is decreased to 12 U/dL (normal range, 50 to 150 U/dL). The vWF:Ag levels of the family members are indicated in Fig 1A.

FVIII-vWF binding assay. FVIII binding to immobilized vWF was performed as previously described10 with minor modifications. Briefly, microtiter wells were coated with 100 µL of polyclonal anti-vWF antibodies for 16 hours at 4°C. After washing with TBS (50 mmol/L Tris-HCl, 150 mmol/L NaCl) pH 8.0 containing 0.1% human serum albumin (HSA), 100 µL of various dilutions of plasma or conditioned medium in TBS-3% HSA plus 0.05% Tween 20 was added and incubated overnight at 4°C. Endogenous FVIII was removed from captured plasma vWF by incubating with of 0.35 mol/L CaCl2 twice for 30 minutes. After washing, 100 µL of recombinant FVIII (0.1 U rFVIII:C/ml in washing buffer plus 10 mmol/L CaCl2) was incubated for 2 hours at 37°C. The amount of bound

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Fig 1. (A) Family of type 2 N vWD with G22E mutation. Family tree. vWF antigen levels are given below the symbols for the family members. Arrow indicates the propositus (II-3). Open symbols (○, □) indicate normal individuals and half-filled symbols (●, △) indicate the family members who are heterozygous for the mutation. NT, for not tested individuals. (B) Binding of recombinant FVIII to plasma vWF. Serial dilutions of plasma samples were incubated into microtiter plates coated with anti-vWF polyclonal antibodies. A constant amount of recombinant FVIII (0.1 U/mL) was then added and the activity of bound FVIII was determined using a chromogenic assay. The vWF was measured by reaction with a peroxidase-conjugated MoAb as described in Materials and Methods. Plasma samples: (●), pool of normal plasmas; (□), propositus (II-3); (○), sister (II-7); (●), mother (I-2); (△), brother (II-1).

FVIII was then quantified by adding the reagents of a chromogenic assay of FVIII:C (Stachrom VIII:C; Diagnostica Stago, Asnières, France). Dilutions of a calibrated pool of normal plasmas, deposited buffer (40 mmol/L Tris-acetate, pH 7.4, 1 mmol/L EDTA) with a linear denaturing gradient (100% denaturant: 7 mol/L urea and 40% formamide) at 160 V for 2 hours. TAE buffer was maintained at a constant temperature of 60°C and recirculated by a peristaltic pump. After electrophoresis, gels were stained in ethidium bromide, washed in water and viewed with a UV light source.

Sequence analysis. The bands relating to both the normal and the mutant allele were excised from the gel, reamplified, purified using the Qiagen PCR purification kit (Qiagen Inc, Chatsworth, CA), and directly sequenced by the dideoxy-termination procedure using Polymerase chain reaction (PCR) amplification of genomic DNA. Genomic DNA was extracted from peripheral blood leukocytes and exons 18, 19, and 20 of the vWF gene were amplified from 500 ng of genomic DNA by the PCR as previously described.17,18 Three sets of primers were used: for exon 18, primers p18A (5′-GC-TTGCTT-TTCTACCTTCGAG-3′) and p18B (5′-GCAGGACCAGCTCTGTG-3′); for exon 19, p19A (5′-GC-TGAGGACTTTTGGATTC-3′) and p19B (5′-GC-GTGCACCCTCACTCCA-3′); and for exon 20, p20A (5′-GC-ACTTGTCATCTCTCCA-3′) and p20B (5′-CCCCTTCTAGAAAAGAAACAGCA-3′). GC indicates a 50G/C nucleotide-rich region added to the 5′ end of the primer for DGGE. DGGE. The melting behavior of exons 18, 19, and 20 of the vWF gene was calculated using the MELT87 and SQHTX computer programs kindly provided by L. Lerman (M.I.T., Cambridge, MA). DGGE was performed as described.19 Ten microliters of amplified fragment was mixed with 5 μL loading buffer (80% glycerol, 10 mmol/L Tris HCl, pH 8.25 mmol/L EDTA, 0.01 % bromophenol blue) and electrophoresed on a 6.5% polyacrylamide gel in TAE buffer (40 mmol/L Tris-acetate, pH 7.4, 1 mmol/L EDTA) with a linear denaturing gradient (100% denaturant: 7 mol/L urea and 40% formamide) at 60°C and recirculated by a peristaltic pump. After electrophoresis, gels were stained in ethidium bromide, washed in water and viewed with a UV light source.

Restriction enzyme analysis. The PCR product obtained with p18B and an internal primer p18C (5′-CATGGTCAAGCTGGTG-TGTGTC-3′) was digested with Sac I (Boehringer-Mannheim, Meylan, France) according to the manufacturer’s instructions. After 2 hours of incubation, the digest was electrophoresed on a 10% polyacrylamide gel and the restriction enzyme pattern visualized after staining the gel with ethidium bromide. The transition G2354rA was introduced into pSVvWF using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). The oligonucleotides used for mutagenesis were the following:
5'-CGGGCTGAAGAGTCGAGTG-3' (vWF nt 2344-2364; substitution is underlined) and 5'-AGTTCCAGCTTTTATTTTGATG-3' (vWF nt 5101-5125). The first primer was used to introduce the desired mutation into pSVvWF used as template and the second primer to destroy the unique Nhe I restriction site of the plasmid for the purpose of selection. Clones containing the desired mutation were identified by specific-allele PCR and the nt substitution was confirmed by DNA sequence analysis.

**Transient expression of vWF.** vWF expression plasmids, pSVvWF and pSVG22E, were transfected into COS-7 cells in the presence of diethylaminoethyl (DEAE)-dextran as previously described. The transfected cells were grown for 72 hours in serum-free Dulbecco's modified Eagle's medium. vWF:Ag in conditioned media was measured with polyclonal anti-vWF antibodies by ELISA.

**Comparative recognition of vWF by two MoAbs.** MoAbs 418 and 32B12 have been selected among a panel of MoAbs directed toward the N-terminal part of the vWF mature subunit. The epitope of MoAb 418, which is sensitive to the conformation change of vWF, has been previously localized between Leu2 and Arg53. The epitope of MoAb 32B12, which recognizes reduced vWF, has been precisely mapped to aa residues 51 to 60.

MoAb 32B12 or MoAb 418 diluted in carbonate buffer (50 mmol/L NaHCO3; pH 9.6) at 2 μg/mL was coated by incubating 100 μL of the solution into wells of Maxisorp Nunc-Immuno plates (A/S Nunc, Roskilde, Denmark) overnight at 4°C. After washing with PBS-T (150 mmol/L NaCl, 10 mmol/L phosphate buffer pH 7.4, 0.05% Tween-20), 100 μL of plasma or conditioned media, tested in duplicate and diluted in PBS-T containing 1% HSA at 10 mU/mL vWF:Ag, as previously determined by ELISA with polyclonal antibodies, was added and incubated for 1 hour at 37°C. After washing with PBS-T, peroxidase-conjugated rabbit anti-vWF polyclonal antibodies were incubated for 1 hour at 37°C. After washing, 100 μL of O-phenylene diamine dihydrochloride (0.4 mg/mL in 20 mmol/L citrate-phosphate buffer, pH 5.2) containing 0.015% H2O2 were added and the reaction was stopped 3 minutes later with 100 μL of 1 mol/L H2SO4. The plates were read photometrically at 492 nm.

Results of family members plasmas and conditioned media are expressed as the ratio of absorbance (Abs) obtained with MoAb 418 versus that obtained with MoAb 32B12 and each value is the mean of three experiments. Serial dilutions of a pool of 40 normal individual plasma samples were prepared against the 3rd international standard (National Institute for Biological Standards and Control [NIBSC], 91/666) were used to establish a reference curve for both MoAbs.

**RESULTS**

**Phenotypic analysis.** The propositus is characterized by a decreased level of FVIII:C, which is out of proportion of vWF:Ag level, resulting in a FVIII/vWF ratio of 0.24. The results of FVIII binding assay performed on plasma samples from the propositus and three relatives are shown in Fig 1B. The vWF from the propositus (II-3) and his sister (II-7) shows a total lack of ability to bind FVIII in regard to the assay performed as described in Materials and Methods. The vWF from the mother (I-2) and the brother (II-1) shows an intermediate binding defect as compared with a pool of normal plasmas.

**Characterization of mutation.** DGGE analysis of the amplified DNA fragments from the patient and controls was used to screen for mutation in exons 18, 19, and 20 of the vWF gene. DGGE of exon 18 showed an abnormal pattern with the patient’s DNA, suggesting a destabilizing mutation. Direct sequencing of the abnormal band showed a G to A substitution at nt 2354, which modifies the encoded aa residue from glycine (GGG) to glutamic acid (GAG) at position 22 of the mature vWF subunit. DGGE of exons 19 and 20 fragments showed a normal pattern. The G2354→A substitution creates a unique Sac I restriction site. The digestion pattern of PCR-amplified exon 18 fragment of the patient (II-3) displays a 164-bp band (undigested fragment) corresponding to the normal fragment and two bands of 116 and 48 bp corresponding to the mutated fragment (Fig 2). The mutation was also found at the same heterozygous state in the mother (I-2) as well as in the brother (II-1), one sister (II-7), and a niece (III-5).

**Expression and characterization of recombinant vWF.** The transition G2354→A was introduced in pSVvWF expression vector by site-directed mutagenesis. Normal and mutated plasmids were transiently expressed in COS-7 cells. The secretion levels of rvWF in conditioned media of transfected cells were similar for both expression plasmids and ranged from 0.10 to 0.14 U/mL. In addition, control experiments showed that wild-type rvWF and rvWFG22E were similar in subunit size and multimeric pattern to plasma vWF (data not shown).

The ability of the recombinant mutant and wild-type vWF to bind FVIII was tested. As shown in Fig 3, the regression line fitted to the binding data for wild-type rvWF indicated that this protein bound FVIII in a dose-dependent manner similarly to the plasma vWF. In contrast, rvWFG22E failed to bind FVIII, whereas comparable amounts of mutated rvWF were captured by the coated anti-vWF polyclonal antibodies. The hybrid rvWF obtained by cotransfection of normal and mutated plasmids gave an intermediate regression line, which represents a moderately decreased FVIII binding capacity.

**Recognition of plasma and recombinant vWF by two MoAbs.** To explain the discrepancy between phenotypic and genotypic data obtained with the patient, we performed antigen-capture ELISA with MoAbs directed to the N-terminal part of the mature vWF subunit. Among a panel of available MoAbs, MoAb 418 failed to bind rvWFG22E, whereas MoAb 32B12 recognized rvWFG22E as well as wild-type rvWF. A standard curve for both MoAbs was established using serial dilutions of a pool of 40 normal individual plasmas calibrated against the international standard. As shown in Fig 4, the reactivities of MoAbs 32B12 and 418 are equivalent. Owing to the limited volume of plasma available, the 10 mU/mL vWF:Ag concentration, which is situated in the linear portion of the standard curves, was used for all the samples analyzed (Fig 4B). The mean value of the Abs ratios obtained with MoAb 418 versus MoAb 32B12 was 1.12 ± 0.01 for normal plasma. The mean value of the Abs ratios obtained for wild-type rvWF was 1.0 ± 0.11 but was dramatically decreased to 0.05 ± 0.03 when testing rvWFG22E. The ratio obtained with hybrid rvWF was intermediate (0.69 ± 0.07). A normal ratio was also found for family members II-5, III-1, III-3, and III-4 who are not affected by vWD. In contrast, when testing the plasmas of the patient (II-3) and his sister (II-7), the ratio was decreased to 0.018 ± 0.001 and 0.023 ± 0.008, respectively, in the same range as the ratios obtained with rvWFG22E. Plasma vWF
from the mother (I-2) and the brother (II-1) gave an intermediate ratio (0.57 ± 0.09 and 0.43 ± 0.06, respectively), similar to that obtained with the hybrid rvWF.

**DISCUSSION**

In the present study, we report the case of a Chinese patient who has subnormal vWF:Ag level and normal vWF multimeric pattern, but low FVIII procoagulant activity level. The discrepancy between vWF and FVIII levels led to the investigation of FVIII/vWF interaction, showing the dramatically reduced capacity of his plasma vWF to bind normal FVIII. FVIII binding assay performed on vWF plasma of family members showed that one sister (II-7) displayed the same defect as the propositus, whereas the mother and the brother had an intermediate binding capacity. Molecular analysis of exons 18 to 20 of vWF gene, encoding the 132 N-terminal aa of the mature subunit, was performed by DGGE. A single nt substitution at position 2354 in exon 18, changing Gly 22 into Glu, was found. This nt substitution, creating a unique Sac I restriction site, allowed us to screen the family and to show that the patient, his mother, his brother, one of his sisters, and a niece are heterozygous for the mutation.

To show that this candidate mutation is responsible for the observed FVIII-binding defect, rvWF containing the G22E substitution was expressed by COS-7 cells. Recombinant vWF(G22E) exhibited a dramatic decrease in the capacity to bind FVIII whereas hybrid rvWF, resulting from the cotransfection with normal and mutated vWF cDNA, showed a moderately decreased binding. These data suggest that G22E is a recessive mutation, in agreement with the recessive inheritance pattern of type 2N vWD. Because the patient is
heterozygous for the mutation G22E, the behavior of his plasma vWF, which failed to bind FVIII, was not consistent with the results obtained with hybrid rvWF, except if the second allele, nonmutated in position 2354, is not expressed. Indeed, many cases of type 2N vWD associated with a quantitative defect of vWF have already been reported. Although the plasma vWF:Ag level of the propositus was subnormal (49 U/dL), we could not ascertain co-inheritance of type 1 or type 3 vWD. Since the mRNA of the propositus was not available, we used the patient’s plasma to test the hypothesis of a defective expression of the “normal” allele. A panel of MoAbs was available and used to screen the mutated rvWF protein. Among them, MoAb 418, which recognizes the unreduced, but not the reduced, N-terminal tryptic fragment of vWF and inhibits FVIII binding to vWF, was unable to recognize rvWFG22E. In the light of these data, propositus and family’s members plasmas were tested with MoAb 418. Plasma vWF from propositus (II-3) and one sister (II-7) did not bind to MoAb 418. Because they are heterozygous for the G22E mutation, this lack of binding can only be explained by the absence or very low level of normal vWF in their plasma. In contrast, plasma vWF from the mother (I-2) and brother (II-1), who are also heterozygous for the mutation, gave a moderate binding, intermediate to that of normal and propositus plasma vWF, showing the presence of normal vWF. Thus, the patient (II-3) and one sister (II-7) appear to be compound heterozygotes for two recessive vWF gene defects: one quantitative, likely inherited from their father, and the other (G22E), qualitative, inherited from their mother. MoAb 418, which fails to recognize the mutated protein allowed us, using a convenient ELISA method, to explain the phenotype of the patient and
to discriminate, in this family, the compound heterozygotes. Previous studies have shown that MoAb 418, which epitope lies within the first 53 aa residues of the mature subunit, fails to recognize the N-terminal tryptic vWF fragment of a type 2N vWD patient who is homozygous for the mutation T28M. The hypothesis of an alteration of the conformation of the FVIII binding site was put forward to explain the defect of FVIII/vWF interaction and the loss of recognition by MoAb 418 induced by the mutation T28M. In the same way, the mutation G22E may affect the secondary structure of the FVIII binding site that would be consistent with the mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the N-terminal plasmin fragments of normal rvWF and rvWFG22E, which are different before reduction but become indistinguishable after reduction (data not shown).

In conclusion, we report a new case of heterozygous type 2N patient. A novel missense mutation, G22E, was first identified on one allele, confirming the crucial role of the peptide loop between Cys 13 and 41 in the binding of FVIII. The defective expression of the other allele was then demonstrated by using an appropriate MoAb. Since the first type 2N mutation, ie, T28M, was reported, three additional substitutions (R19W, E24K, and G22E) were identified in a cluster of 10 aa residues of D’ domain of vWF. Structural studies would be necessary to discriminate the aa residues involved in the maintenance of the structure of the active site from those which participate directly in FVIII binding.

NOTE ADDED IN PROOF

Since the submission and revision of the manuscript, the suspected mutation responsible for a null allele was found in exon 7 of the propositus (II-3) vWF gene. It is a C to G substitution at position 1060 of the propolypeptide changing Thr261 for a stop codon. The propositus is thus a compound heterozygous. Family studies have shown that the stop codon is absent in the mother (I-2), the brother (II-1), and the niece III-5. It is present in the two sisters (II-5 and II-7), the son (III-3), and the nephew (III-4).

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