Reduced von Willebrand factor survival in type Vicenza von Willebrand disease

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Type Vicenza variant of von Willebrand disease (VWD) is characterized by a low plasma von Willebrand factor (VWF) level and supranormal VWF multimers. Two candidate mutations, G2470A and G3864A at exons 17 and 27, respectively, of the VWF gene were recently reported to be present in this disorder. Four additional families, originating from northeast Italy, with both mutations of type Vicenza VWD are now described. Like the original type Vicenza subjects, they showed a mild bleeding tendency and a significant decrease in plasma VWF antigen level and ristocetin cofactor activity but normal platelet VWF content. Unlike the original patients, ristocetin-induced platelet aggregation was found to be normal. Larger than normal VWF multimers were also demonstrated in the plasma. Desmopressin (DDAVP) administration increased factor VIII (FVIII) and VWF plasma levels, with the appearance of even larger multimers. However, these forms, and all VWF oligomers, disappeared rapidly from the circulation. The half-life of VWF antigen release and of elimination was significantly shorter than that in healthy counterparts, so that at 4 hours after DDAVP administration, VWF antigen levels were close to baseline. Similar behavior was demonstrated by VWF ristocetin cofactor activity and FVIII. According to these findings, it is presumed that the low plasma VWF levels of type Vicenza VWD are mainly attributed to reduced survival of the VWF molecule, which, on the other hand, is normally synthesized. In addition, because normal VWF-platelet GPIb interaction was observed before or after DDAVP administration, it is proposed that type Vicenza VWD not be considered a 2M subtype. (Blood. 2002;99:180-184)

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HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Introduction

With type 2 von Willebrand disease (VWD), we refer to structural and functional abnormalities of von Willebrand factor (VWF). A defect in the A1 domain of VWF causes type 2B or 2M VWD, as manifested by an enhanced or a decreased VWF affinity for platelet GPIb, respectively. Even when large VWF multimers are present (type 2M) or absent (type 2B), platelet plug formation at the site of vascular injury is compromised. Type Vicenza VWD described in patients originating from the Vicenza area in Italy, is characterized by autosomal dominant inheritance, low plasma VWF levels, and normal platelet VWF content; its peculiarity is the presence of larger than normal (supranormal) VWF multimers that usually are not present in the plasma but are observed after the infusion of desmopressin (DDAVP; Emosint, Sclavo, Italy). From a hemostatic perspective, high-molecular-weight multimers are known to be more efficient because of their capacity to induce multiple binding sites at the subendothelial matrix. However, the larger forms displayed by type Vicenza VWF are not characterized by increased hemostatic function; indeed, their functional activity is said to be decreased, which means that type Vicenza, which was originally included in the type 1 group, is now classified as a 2M subtype. Nonetheless, it is unclear whether the decreased function in type Vicenza VWF is a quantitative problem or a true functional abnormality. After the first demonstration that the molecular defect is linked to the VWF gene, 2 candidate mutations were identified: the first one in exon 27 (G3864A; R1205H) and the other in exon 17 (G2470A; M740I). We here report 4 more families with type 2 Vicenza VWD with the same hemostatic profile as the patients originally described.

Patients, materials, and methods

Patients

Seven patients from 4 unrelated families were studied. Two families (families 1 and 2), which include 3 patients, are from the Padua province, and one family (2 patients) was previously classified as type 1 Padua VWD. Family 3 comes from the Vicenza province and family 4 from the Venice province. All 7 patients had a lifelong history of mild bleeding, with typical mucosal bleeding, which required a referral to the Coagulation Center at the University of Padua Medical School for investigation. As control, DDAVP was administered to 5 healthy subjects (physicians and students from our department).

Materials and methods

Patients and healthy volunteers were studied after we received their written informed consent in accordance with the Declaration of Helsinki. Blood was drawn from the antecubital vein and was anticoagulated using sodium citrate (1:10, vol/vol); samples for washed platelet preparations also contained 50 mM EDTA, 50 IU/mL Trasylol, 10 mM leupeptin, and 60 mM N-Ethylmaleimide as protease inhibitors. The methods to prepare platelet-rich plasma and platelet-poor plasma and to determine bleeding time, platelet count, and ristocetin-induced platelet aggregation (RIPA) were previously described. Platelets for VWF antigen (VWF:Ag) measurement...
were prepared by differential centrifugation in phosphate-buffered saline (PBS) buffer containing 3% EDTA and were resuspended at a final concentration of 10^9/µL in PBS containing protease inhibitors.16

VWF ristocetin cofactor activity (VWF:RCo) was measured with normal washed, formalin-fixed platelets and 1 mg/mL ristocetin, as described.17 VWF:Ag was determined by enzyme-linked immunosorbent assay (ELISA).18 Factor VIII (FVIII) coagulant (FVIII:C) was measured by a one-stage method, using cephaloplatin as activated cephalin, as reported elsewhere.19 Platelet VWF:Ag was evaluated by ELISA.

VWF collagen binding activity (VWF:CBA) was evaluated by ELISA using type I and type 3 collagen diluted in acetic acid solution (95% and 5%, respectively), as described.19 Briefly, after overnight coating with collagen, microtiter plates were incubated with plasma VWF for 1 hour at room temperature; bound VWF was evaluated with an anti–horse-radish peroxidase-conjugated VWF antibody (DAKO, The Netherlands).

VWF multimer analysis was performed on high-telling temperature agarose containing 0.1% sodium dodecyl sulfate, using 1.2% or 2.2% agarose to obtain low- or high-resolution conditions, respectively.20 After reaction with a purified sodium iodide I 125-labeled anti-VWF antibody, VWF multimers were detected by autoradiography. Autoradiographs were analyzed by densitometer scanner (LKB, Uppsala, Sweden).

DDAVP was administered subcutaneously, at a dose of 0.4 µg/kg. Patient and normal blood samples were collected before and 30, 60, 120, 180, 240, 480 minutes, and 24 hours after DDAVP administration. Time courses of factor VWF and FVIII plasma concentrations after DDAVP administration were analyzed according to a one-compartment model with first-order input and output kinetics,21 in which baseline concentrations, B, were also incorporated, as follows: plasma concentration = \( A \times (e^{-Kre \times t} - e^{-Kre \times t}) + B \), where A is the y-axis intercept, Kre is the release rate constant, Kel is the elimination rate constant, and t is time. The model was fitted to each set of combination-time data by means of the Prism statistical package (GraphPad, San Diego, CA). Goodness of fit was evaluated by \( r^2 \). Area under the concentration-time curve (AUC), release (t1/2 re), and elimination (t1/2 el) half-lives were calculated with standard formulas: AUC = \( AKre + \frac{Kre}{Kre} \); t1/2 re = 0.693/Kre; t1/2 el = 0.693/Kel.

Nucleotides of the complementary DNA are numbered from the major transcription cap site (+1), located 250 nucleotides upstream of the first nucleotide in the ATG initiation codon. Amino acid residues are numbered from the ATG initiation codon (residue 1) of the pre-pro-VWF.

Genomic DNA was extracted from peripheral blood leukocytes using the Easy DNA extraction kit (Invitrogen, Carlsbad, CA). Exons 17 and 27 of the VWF gene were amplified from 100 ng genomic DNA by polymerase chain reaction with AmpliTaq polymerase (Perkin Elmer) in a thermal cycler (2400 Perkin Elmer). Primer sequences for amplification and sequencing of exon 17 were GGTGAGGCACCAGGTATAG for 17A and CGTGAAGATTCTGGCCAGG for 17B. Primer sequences of exon 27 AGAGGAGGTGGCTTCTAGG for 27A and AAGATTCATACCTTAGGACAC for 27B.

Before sequencing, polymerase chain reaction products were purified through Microcon filters (Amicon) to remove any remaining deoxyribonucleotide triphosphates and primers. Sequencing of the amplified fragments was performed by the dyeoxy method using the Big Dye terminator sequencing kit (Perkin Elmer). Products were precipitated with ethanol and sodium acetate to remove excess dye terminator and were analyzed in the ABI PRISM 310 Genetic analyzer. We also amplified and sequenced exons 18 to 26 and exon 28 using the method described above, adapted to a panel of published primers.

Results

The main hemostatic findings in the patients with VWD studied are reported in Table 1. With the exception of one patient (III-2), all had normal or almost normal bleeding times: all had significant decreases in plasma VWF:Ag and VWF:RCo levels and less pronounced decreases in FVIII. The VWF:RCo/VWF:Ag ratio was normal (0.99 ± 0.40; normal range, 0.8-1.2). Despite the low VWF levels, RIPA was always normal, as were the platelet VWF:Ag content and the platelet count whose values ranged from 154 000/µL to 294 000/µL (normal range, 150 000-350 000/µL). Analysis of plasma VWF multimers by means of a low-resolution gel (1.2% agarose) demonstrated a decrease of all oligomers, with the presence of supranormal VWF multimers in all patients studied (Figure 1A). Using high-resolution gel (2.2% agarose), which resolves each single oligomer into 3 discrete bands, the VWF multimer pattern of the patients appeared characterized by the presence of doubles instead of the triplets observed in the normal counterparts (Figure 2). More precisely, a dark-stained band migrating as the central component of the normal triplet was evident, as was a second band that ran like the fast moving band of normal VWF. A slow migrating band, instead, was much less represented. This pattern was demonstrable in all patients investigated. Platelet VWF displayed a normal VWF multimer pattern (Figure 1B). Because of the presence of these supranormal components, the capability of patient VWF to bind collagen was investigated by VWF:CBA. This activity was decreased when it was expressed as an absolute value (mean, 13.06 ± 6.35 U/dL; normal range, 70-140 U/dL), but it was normal when expressed as a ratio (mean 0.91 ± 0.26; normal range, 0.8-1.3) in all patients studied. These findings suggested that the activity of the supranormal VWF multimers was not increased as far as collagen binding function was concerned.

DDAVP infusion

To better characterize the type of VWD or to prepare the patients for surgical procedures, DDAVP was administered to 5 of the 7 patients. Time courses of FVIII, VWF:Ag, and VWF:RCo after DDAVP administration in patients and healthy subjects were expressed as the best-fit curve. Data pertaining to patient II-1 and a healthy subject are reported in Figure 3. At the peak FVIII, VWF:Ag, and VWF:RCo levels reached normal values, but starting at 120 minutes after DDAVP, their levels decreased at a

<table>
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<th>Family</th>
<th>Subject</th>
<th>BT (min)</th>
<th>RIPA (%)</th>
<th>VWF:Ag (U/dL)</th>
<th>VWF:RCo (U/dL)</th>
<th>FVIII (U/dL)</th>
<th>Plat. VWF:Ag (U/dL)</th>
<th>VWF:CBA (U/dL)</th>
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<td>3.23</td>
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<td>72.5</td>
<td>12.4</td>
<td>7.1</td>
<td>18.0</td>
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<td>81.1</td>
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Normal ranges: <4 58-84 60-160 60-130 60-160 70-140 70-140

RIPA was induced by 1.5 mg/mL ristocetin.
significant rate so that at 240 minutes their concentrations were near the preinfusion level. Different behavior was observed in a healthy counterpart (Figure 3). The kinetics of plasma FVIII and VWF were investigated by means of a one-compartment model (see “Patients, materials, and methods”) that satisfactorily described the time-course of post-DDAVP plasma VWF:Ag, VWF:RCo, and FVIII concentrations (r² median, 0.97; range, 0.91-0.99). Table 2 summarizes the mean values of the main kinetic parameters. It appears that patients had considerably lower AUCs for FVIII, VWF:Ag, and VWF:RCo than healthy subjects. Furthermore, both t½ re and t½ el for all parameters were shorter in patients than in healthy subjects. In particular, in type Vicenza VWD, VWF:Ag and FVIII t½ el levels were 22% and 27% of the control value (1.24 hours vs 5.51 hours and 1.04 hours vs 3.85 hours, respectively). In addition, the t½ el of VWF:RCo was reduced, though to a lesser extent (1.15 hours vs 2.61 hours). The t½ re was also reduced for VWF:Ag (10.5 minutes vs 28.8 minutes) and VWF:RCo (21.0 minutes vs 46.7 minutes), whereas it appeared to be normal for FVIII (16.6 minutes vs 19.3 minutes). Altogether these findings suggest that the release and the elimination of type Vicenza VWF are consistently different from those in healthy counterparts.

The infusion of DDAVP further evidenced the extra-large VWF multimers present at baseline and the appearance of multimers with higher molecular weights. However, these components disappeared starting at 120 minutes after infusion, together with all the other oligomers (Figure 4).

**Genetic analysis**

Genetic analysis performed in the portion of the VWF gene encoding the amino-terminal portion of the VWF molecule (exons 17-27) disclosed the presence of 2 mutations at the heterozygous level in exons 17 and 27. In exon 17, a G2470A mutation was demonstrated that changes a methionine with a histidine at position 740 of VWF molecule (M740I). In exon 27, a G3864A mutation,

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**Figure 1.** Autoradiographies of VWF multimer pattern obtained by low-resolution agarose gel (1.2%) containing 0.1% sodium dodecyl sulfate under nonreducing conditions. VWF is detected by purified 125I-labeled anti-VWF antibody. At the top are the large or extra-large VWF multimers; at the bottom are the dye front. Patient plasma (PP) (1A) and platelet (PPl) (1B) multimer patterns were compared with normal plasma (NP) and normal platelets (NPl). Patients are identified by numbers that correspond to those entered in Table 1.

**Figure 2.** Autoradiographs of plasma VWF multimer pattern obtained by high-resolution agarose gel (2.2%). For experimental conditions see Figure 1.

**Figure 3.** Time course of plasma FVIII, VWF:Ag, and VWF:RCo levels observed before and at different times after subcutaneous DDAVP. Subcutaneous DDAVP (0.4 μg/kg) in patient I-1 (Δ—Δ) compared with that in healthy subject (□—□). Similar results were observed in the other patients and healthy subjects studied.
predicting a change in the amino acid arginine at position 1205 with histidine (R1205H), was identified. These mutations were not detectable in the nonaffected members of the families studied. Sequencing from exons 18 to 28 disclosed no other nucleotide substitutions in the amplified products, with the exception of many known polymorphisms.

Discussion

We describe 7 patients from 4 unrelated families with type Vicenza VWD, characterized by hemostatic profiles similar to those of the original type Vicenza subjects, and showing the 2 candidate mutations recently associated with this variant. Indeed, besides supranormal plasma VWF multimers, plasma VWF survival in our patients was consistently reduced than in their healthy counterparts, as demonstrated by the behavior of the VWF released by DDAVP.

After the first description of type Vicenza VWD, other cases were reported in Germany and Hungary, suggesting a broader distribution of this variant. Two subtypes were identified, one characterized by normal platelet VWF content, as in the original type Vicenza patients, the other characterized by reduced platelet VWF. In so-called classic type Vicenza VWD, besides the presence of supranormal VWF multimers in plasma, the pathognomonic hemostatic aspect is the very low level of plasma VWF despite the normal level of platelet VWF. This latter finding clearly demonstrates that VWF synthesis is normal because platelet VWF content is seen as the expression of VWF synthesis by endothelial cells. Hence, type Vicenza VWF is normally synthesized, stored, and released; nevertheless, it is significantly decreased in the plasma. Schneppeneim et al. advanced an impaired constitutive VWF secretion by endothelial cells, with near-intact stimulated release. This hypothesis could explain the abnormal baseline VWF level and the normal DDAVP response. Based on the results of our study, however, we conclude that a decrease in VWF survival is the cause of the Vicenza VWD variant. This conclusion is based on the observation that, after DDAVP, the kinetics of plasma type Vicenza VWF differed from those of healthy subjects. Indeed, the AUC(s) of the DDAVP-induced VWF:Ag, VWF:RCo, and FVIII concentration time courses were consistently lower in patients with VWD than in healthy subjects. Recalling that AUC is given by the amount of molecule released (Q) divided by its plasma clearance (Cl), a reduced AUC may be attributed either to a smaller Q or a greater Cl. An increase in Cl is likely, because t½ of elimination was invariably shortened. In addition, the release process of VWF:Ag and VWF:RCo (but not of FVIII) appears to be quicker in patients than in healthy subjects. If normal VWF plasma concentration is viewed as the result of the entry of newly synthesized VWF and the removal of VWF from circulation, in type Vicenza VWF the reaction is shifted toward removal, even though more complex alterations have been taken into account—such as an abnormal VWF release process from endothelial cells. Together these findings can justify the significant decrease in baseline VWF and FVIII in type Vicenza VWD. These results differ from those of Mannucci et al., who reported, in the first description of this disorder, normal Vicenza VWF half-life. However, their conclusions were obtained by comparing Vicenza VWF half-life with type 1 VWD, not with healthy subjects as we have done. The reduced survival of type Vicenza VWF might explain, at least in part, the moderate to mild bleeding symptoms and the almost-normal bleeding times, despite the significant decreases in VWF level. Indeed, it is possible that when the integrity of the vessel wall is altered, the intact acute release of VWF might temporarily satisfy hemostatic needs, taking into account that normal platelet VWF content plays a key role in guaranteeing adequate platelet plug formation. Even though large multimers are known to be hemostatically more efficient than smaller ones, the contribution of supranormal VWF multimers of type Vicenza is unclear. Neither VWF:RCo nor VWF:CBA, which are both sensitive to large VWF multimers, were higher than the amount of VWF molecule. Indeed, VWF:RCo and VWF:CBA were normal when expressed as ratios, even though their absolute values were decreased. These findings indicate that the binding of type Vicenza VWF to platelet GPIb either is not defective or is defective in relation to plasma VWF concentration. For this reason, and because RIPA levels were normal in these patients, we propose that type Vicenza should not be classified as a type 2M VWD, as it is at present.

To date, the cause of the rapid disappearance of circulating type Vicenza VWF has not been clarified. The 2 candidate mutations in exons 17 and 27 of the VWF gene may predict abnormalities in the D’ and D2 domains, respectively, encoded by these exons; however,
their role and the underlying mechanisms are not yet described. On the other hand, it is unclear whether the presence of supranormal VWF multimers is the cause or the consequence of reduced survival or it is an unrelated finding. At any rate it is intriguing to note that, based on our results, the half-life of VWF:RCo is less compromised than that of VWF:Ag and FVIII.

That all our type Vicenza patients come from northeast Italy and have the 2 mutations, G2740A and G3864A, identified in the patients originally described7, in contrast with the patients coming from Milan and Germany,13 may indicate a founder effect. Regarding the specific role of the 2 candidate mutations, we have no explanation; the finding that patients with the G3864A mutation in exon 27 alone show the same hemostatic pattern as those with the 2 mutations, however, seems to indicate a major contribution of the G3864A mutation to the development of abnormal plasma VWF levels and multimer patterns.

Type Vicenza patients offer the first clear demonstration that a decrease in the half-life of VWF may be one of the causes of VWD. This condition differs from that observed in type 2A VWD, in which the decreased survival of circulating VWF mainly concerns the high and intermediate molecular weight multimers.26,27 In type Vicenza VWD, all multimers are instead involved, and all the components of VWF are rapidly removed from the plasma. This appears more similar to the physiological situation, where no specific multimers seem to be selected for removal at the end of VWF survival.

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