Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: Molecular and Clinical Markers for the Diagnosis and Management of Type 1 VWD (MCMDM-1VWD)

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The decreased survival of von Willebrand factor (VWF) in plasma has been implicated as a mechanism in a subset of type 1 von Willebrand disease (VWD) patients. We have previously reported that the ratio of plasma levels of VWF and its propeptide (VWFpp) can be used to identify patients with reduced VWF survival. In this study, we report the assay of VWFpp and VWF:Ag in 19 individuals recruited from 6 European centers within the MCMDM-1VWD study. Eight individuals had a VWF:Ag level less than 30 IU/dL. Seven of these patients had a robust desmopressin response and significantly reduced VWF half-life that was predicted by a markedly increased steady-state plasma VWFpp/VWF:Ag ratio. VWF mutations previously associated with reduced VWF survival were identified in each of the 7 individuals. Thus, a substantially increased ratio of steady-state VWFpp/VWF:Ag predicted a reduced VWF half-life in patients with markedly decreased VWF:Ag levels. These data indicate that a reduced VWF survival is found in a subpopulation of patients with type 1 VWD. The systematic assay of both plasma VWF and the VWF propeptide in moderately severe type 1 VWD patients may identify patients with a reduced VWF survival phenotype.

Introduction

von Willebrand factor (VWF) is a multimeric adhesive protein that promotes platelet binding at the site of vascular injury, promotes platelet aggregation and platelet-platelet interactions at high-shear rate, and serves as the carrier protein for coagulation factor VIII.1 VWF is synthesized in endothelial cells and megakaryocytes/platelets where it undergoes extensive intracellular modifications.2,3 In the Golgi, proteolytic processing yields the large propeptide (VWFpp) and mature VWF multimers.4 The VWFpp and VWF proteins remain noncovalently associated and are stored in α-granules in megakaryocytes/platelets or Weibel-Palade bodies in endothelial cells for regulated release.5,6 In plasma, VWFpp and mature VWF multimers dissociate and circulate independently.7 VWFpp circulates in plasma as a homodimer with a half-life of 2 to 3 hours, while mature VWF circulates with a half-life of 8 to 12 hours.7,9

Defects or reduced levels of VWF result in von Willebrand disease (VWD).10 Type 1 VWD is characterized by a partial quantitative deficiency of VWF and is the most common type of VWD. Provisional diagnostic criteria for type 1 VWD have been published in a report of the subcommittee on VWF.10 Patients with type 1 VWD have normal VWF functional activity but may have circulating mutant subunits, and the proportion of large VWF multimers is not significantly decreased. Until recently, there have been very few VWF mutations identified in type 1 VWD patients.11-20 Several mutations that have been identified cause intracellular retention and decreased secretion of VWF;11,13,15,21 Additional mutations have recently been identified that are associated with the reduced plasma survival of VWF.22-24 We have recently reported that type 1 VWD patients with either a W1144G or S2179F VWF mutation and increased plasma VWF clearance phenotype can be identified by determining the ratio of steady-state VWFpp to VWF:Ag ratio.25 This VWFpp to VWF:Ag ratio was also found to be predictive of increased clearance by Schooten et al.23 Concentrations of VWFpp and VWF in plasma are expressed in units, and 1 mL normal human plasma by definition contains one unit of each. The ratio of VWFpp to VWF:Ag in plasma is expected to equal 1. Individuals with an increased VWF clearance phenotype...
are expected to have normal VWF and VWFpp secretion, decreased plasma levels of VWF, and normal plasma levels of VWFp, resulting in an increased ratio of VWFp to VWF:Ag.

Here we report the assay of VWFp and VWF:Ag in 19 individuals recruited from 6 European centers within the MCMDM-1VWD (Molecular and Clinical Markers for the Diagnosis and Management of type 1 von Willebrand disease) study.17,26,27 The half-lives of VWFp and VWF:Ag were determined after desmopressin administration. These patients were included in the prospective trial to determine the biologic response to desmopressin. We identified 8 patients with VWF:Ag levels of less than 30 IU/dL. Seven of these individuals had a markedly reduced VWF half-life that was predicted by a significantly increased VWFp/VWF:Ag ratio. Eleven patients had slightly or moderately reduced VWF:Ag and a VWFp/VWF:Ag ratio within the normal range. These data indicate that a reduced VWF survival phenotype is found in a subpopulation of patients with type 1 VWD. The systematic assay of both plasma VWFp and VWF:Ag in moderately severe type 1 VWD patients may identify these individuals.

Methods

Patients

The MCMDM-1VWD study is a multicenter, European Union (EU)–funded study on type 1 VWD.17,26,27 The aim of the study is to determine the value of clinical, phenotypic, and molecular markers for the diagnosis of type 1 VWD. The local ethical review committees of all centers approved the study protocol. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The design of this study has been described in more detail elsewhere.17,26,27 All participating centers planned to recruit 10 to 15 families with type 1 VWD, with the intent to recruit a full spectrum of type 1 VWD families, including milder phenotypes. This study did not set a cutoff for baseline VWF:Ag; instead, patients had been historically diagnosed with type 1 VWD by the referring center and had a historic low VWF level. Some centers were able to monitor response to desmopressin only when required for treatment, and some centers could not obtain or did not seek ethical approval for detailed desmopressin trials. We studied a subset of 19 individuals recruited from 6 European centers within the MCMDM-1VWD study. Patients in this subset were selected based on (1) availability of a detailed desmopressin trial with samples obtained at multiple time points and (2) the type and location of mutations, to ensure that both missense and other mutation types throughout the VWF gene were represented.

Antibodies

The following antibodies were produced by our laboratory: (1) anti-VWF monoclonal antibody AVW-1; (2) anti-VWFp monoclonal antibodies 239.2 and 239.3; and (3) a polyclonal rabbit anti-VWFp, “Mango IgG.” An additional rabbit polyclonal antihuman VWF was purchased from DAKO (Carpinteria, CA).

ELISA assays

The concentrations of VWF:Ag and VWFp in patient plasma were determined using standard antigen-capture enzyme-linked immunosorbent assay (ELISA).25 Samples were referenced against pooled normal human plasma. VWF was captured using the monoclonal antibody, AVW-1, and detected using a rabbit-antihuman-VWF polyclonal antibody. VWFp was captured using monoclonal antibodies, 239.2 and 239.3, and detected using a rabbit–anti-VWFp polyclonal antibody, Mango. Immune complexes were detected using biotin-conjugated goat anti–rabbit IgG (Pierce, Rockford, IL), avidin-horseradish peroxidase, and o-phenylenediamine substrate (Pierce).

Infusion of desmopressin

Desmopressin was infused intravenously at a dose of 0.3 μg/kg. Venous blood samples were taken prior to the start of the desmopressin infusion (time = 0), and 1, 2, and 4 hours after infusion, according to a standard protocol.24 Whole venous blood was collected and platelet-poor plasma prepared and stored at −80°C until assayed as described.26 Frozen samples were warmed at 37°C and thoroughly mixed prior to assay to prevent cryoprecipitation of VWF.

Multimer analysis and mutation analysis

Multimer analysis was performed by one central laboratory as previously detailed.17,26 Multidistribution and structural analysis were determined using low-resolution (1.2% agarose) and medium-resolution (1.6% agarose) gels. Patient samples were compared with VWF from normal pooled human plasma.29 Multimer classification was based upon the 2006 report of the Subcommittee on VWF for classification of VWD.10 Mutations in the VWF gene of each patient were identified as previously described.17 Novel sequence changes were determined to be mutations by examining the corresponding sequence in 100 healthy controls recruited from the same center to exclude previously unreported single-nucleotide polymorphisms.

Data and statistical analysis

The half-life of VWF:Ag or VWFp was determined by calculation of the first-order rate constant for the elimination phase from the slope of the VWF:Ag or VWFp concentration against time.22,30 The following formula was used: \[ C(t) = C_0 - Ae^{-kt} \] where \( C(t) \) = VWF:Ag (or VWFp) as a function of time, \( C_0 \) = baseline VWF:Ag (or VWFp), \( A = y\text{-axis intercept} \), \( c = 2.71828 \), \( k_e \) = first-order rate constant for the elimination phase, and \( t = \text{time} \). Half-life, \( t_{1/2} \), was determined by the equation: \[ t_{1/2} = \frac{ln(2)}{k_e} \]. Data analysis, including calculation of means, standard deviations, quartiles, and Pearson correlation, was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA).

Results

Steady-state plasma VWF:Ag and VWFp levels in MCMDM-1VWD patient subset

The goal of this study was to determine whether type 1 VWD patients with a decreased VWF survival phenotype could be identified using the ratio of steady-state VWFp/VWF:Ag. The subset of patients was selected based upon type and location of mutations in order to determine whether mutations in particular VWF domains are associated with increased VWF clearance. Mutations are distributed throughout the entire VWF gene and were selected to include intronic mutations, type 2N mutations, and mutations in domains D1-D2, D3-D4, A1-A3, and D4-C4 (Table 1). The subset was enriched with samples from patients having either R1205H or C1130 mutations for comparison, as the shortened survival of these variant VWF proteins in plasma has already been well documented.22,23,31 All patients in the subset had undergone a detailed desmopressin trial with samples obtained at multiple time points after desmopressin administration enabling the calculation of half-lives for VWF:Ag and VWFp. The patients in our subset were also classified as having a complete response to desmopressin. Several patients in the MCMDM- VWD study had only a partial response or were unresponsive to desmopressin administration; a comprehensive evaluation of the influence of genotype and phenotype on response to desmopressin has been described by Castaman et al.32

Plasma samples were assayed for VWF:Ag and VWFp levels using ELISA assays as described. Steady-state VWF:Ag and VWFp levels for all individuals are shown in Figure 1 with the
All patients in this study were administered 1-desamino-8-D-arginine vasopressin (desmopressin). For most, desmopressin administration resulted in an increase in both VWF:Ag and VWFpp plasma protein levels. The relative increase \((t_1/t_0)\) in VWF:Ag after desmopressin is summarized in Table 1 (\(t_1/t_0(VWF:Ag)\)), and from no increase \((t_1/t_0 = 1.0)\) to a 13.5-fold increase, with an average increase of 3.9-fold. While most patients demonstrated a greater than 2-fold increase in VWF:Ag, 7 patients (nos. 1, 6, 14, 16-19) failed to achieve even a 2-fold increase in VWF:Ag (denoted with \(\ddagger\) in Table 1). In addition, several individuals had VWF:Ag more than 50 IU/dL before desmopressin administration and failed to achieve a 2-fold increase in VWF:Ag. The increase in VWFpp \((t_1/t_0(VWFpp))\) ranged from 3.3-fold to 11-fold with an average increase of 7.1-fold (Table 1). The increase in VWF:Ag in individuals was found to correlate with the increase in VWFpp \((r = 0.50, P = .03, data not shown)\). These data indicate that in most patients, both VWF and VWFpp were normally released from Weibel-Palade bodies in endothelial cells into plasma in response to desmopressin.

A detailed time course of VWF:Ag and VWFpp levels was assessed with plasma samples obtained before \((t = 0)\) and 1, 2, and 4 hours after desmopressin administration as shown in Figure 2. In some patients, VWF appeared to disappear rapidly from the circulation after desmopressin administration. In others, VWF survival appeared to be essentially normal. The half-lives of VWFpp \((t_1/2(VWFpp))\) and VWF:Ag \((t_1/2(VWF:Ag))\) were determined (Table 1). The distribution of VWFpp and VWF:Ag half-lives is shown in Figure 3. The calculated half-life of VWF:Ag in patients varied considerably, ranging from 0.3 to 12.7 hours, from significantly reduced to normal half-life. The mean VWF:Ag half-life was 3.6 plus or minus 3.6 hours, a value less than the reported normal half-life of 8 to 12 hours. The calculated half-life of VWF:RCO was found to
roughly parallel that of VWF:Ag (data not shown). The half-life of VWFpp in the patients was less variable (1.3-6.2 hours), with a mean of 3.0 plus or minus 1.4 hours, consistent with the previously reported normal range of 2 to 3 hours.8,9

Decreased VWF survival is predicted by an increased VWFpp/VWF:Ag ratio

In a previous pilot study, we examined VWFpp/VWF:Ag ratio in healthy individuals and determined the normal range to be 0.54 to 1.98.25 We assume that the concentration of VWFpp at steady state is representative of VWF synthesis and secretion. The ratio of VWFpp to VWF:Ag was calculated for each individual (Table 1). While many of the individuals fell within our previously established normal range, several individuals (nos. 7-13) had a significantly increased VWFpp/VWF:Ag ratio. A substantially increased VWFpp/VWF:Ag ratio was found to be predictive of a significantly decreased VWF half-life in those individuals who had a greater than 2-fold desmopressin response and an initial VWF:Ag level less than 30 IU/dL. A few individuals (nos. 6, 17, and 18) had a decreased VWF half-life that was not predicted by an increased VWFpp/VWF:Ag ratio. However, these individuals had only moderately reduced VWF:Ag levels and a less than 2-fold response to desmopressin. Individuals who had a substantially increased VWFpp/VWF:Ag ratio and significantly reduced VWF:Ag level were also found to have an enhanced response to desmopressin (greater than 4-fold increase). The desmopressin response was found to correlate with the VWFpp/VWF:Ag ratio (r = 0.92, P < .001) as shown in Figure 4.

Identification of mutations and analysis of multimer structure

VWF gene mutations and plasma VWF multimer structure were determined for each individual (Table 1). Several of the identified mutations have been reported previously and are associated with a decreased VWF survival phenotype.22,23,25,33 Allelic R1205H and M740I mutations were identified in 3 individuals in this study (nos. 11-13). These individuals had a reduced VWF:Ag half-life (1.0-1.7 hours), a
markedly increased VWFpp/VWF:Ag ratio (10.1-11.5), and a multimer structure similar to that identified in other type 2M-Vicenza patients (designated 2M-V). The VWF multimer structure of 2 of these individuals (nos. 12 and 13) is shown in Figure 5. We observed a decrease in the intensity of the satellite bands in these patients. Three individuals (nos. 7, 8, and 9) had mutations at amino acid 1130, altering the cysteine to glycine, phenylalanine, or arginine, respectively. These individuals had a reduced VWF half-life (0.9-2.7 hours) and an increased VWFpp/VWF:Ag ratio (4.6-6.5). The multimer structures of individuals nos. 7 and 9 also demonstrated a decrease in satellite band intensity (Figure 5). A W1144G mutation was identified in one individual (no. 10) with a 2.5-hour VWF half-life and VWFpp/VWF:Ag ratio of 4.6. In each of these cases (nos. 7-13), the individuals had a markedly decreased initial VWF:Ag and VWF half-life, elevated VWFpp/VWF:Ag ratio, an enhanced desmopressin response, and a loss of multimer satellite bands. Together these data indicate a mechanism of reduced VWF survival in these individuals (Table 1). Mutations were identified in the VWF gene of the remaining patients who did not have an increased VWFpp/VWF:Ag ratio. Two individuals, no. 14 with a V1822G mutation and no. 17 with a C2477S mutation, had abnormal multimers (data not shown) classified as “2A (IIE)” and “2M, smeary,” respectively (U.B., J.E., A.G., R.S., K. Will, E. Drewke, G.C., F.R., A.B.F., J.B., D.M., C.M., J.G., J.I., D.H., Z.V., L.H., S.L., J.P., F.H., and I.P., manuscript submitted). The multimer abnormalities described here are subtle abnormalities, and a full molecular weight multimer spectrum was present. Interestingly, most individuals with a normal VWFpp/VWF:Ag ratio demonstrated normal multimer structure. Even though the mechanism(s) related to these mutations remain to be identified through expression studies, these results indicate that the steady-state ratio of plasma VWFpp and VWF:Ag can be used to identify type 1 VWD patients with an increased plasma VWF clearance phenotype.

Discussion

We have assessed VWF propeptide and VWF:Ag levels in a subset of type 1 VWD patients from the MCMDM-1 VWD study (Table 1; Figure 1). The baseline VWF:Ag levels varied considerably with a mean of 37 IU/dL. In contrast, VWFpp levels were far less variable, only 4 initial VWFpp levels were below the normal range. We also observed a wide range of VWF:RCo/VWF:Ag ratios, with only 8 of 19 individuals having a ratio more than 0.7. However, several individuals had very low baseline VWF:Ag levels, and the assay of VWF:RCo in these samples may be below the lower limit of detection. In the parent study of 154 type 1 VWD index cases, the VWF:RCo/VWF:Ag ratio was not found to be a useful tool to discriminate groups with abnormal multimers from those with normal multimers as there was considerable overlap in individual data. A detailed analysis of multimer structure in patients recruited to the MCMDM-1VWD study has been described (U.B., J.E., A.G., R.S., K. Will, E. Drewke, G.C., F.R., A.B.F., J.B., D.M., C.M., J.G., J.I., D.H., Z.V., L.H., S.L., J.P., F.H., and I.P., manuscript submitted). Multimer analysis using a highly optimized system allowed detection of very subtle deviations from normal structure. The abnormalities that were observed in 9 of our 19 individuals are very subtle and likely would not have been detected by many other centers. Using the recently updated VWD classification published in 2006, the patients with minor multimer abnormalities would still fit the criteria for type 1 VWD as there does not appear to be a decrease in high-molecular-weight multimers.

We observed considerable variability in the response to desmopressin (Table 1). Half of the individuals had only modestly decreased initial
survival including R1205H/M740I, C1130G, C1130F, C1130R, and that have been previously reported to result in decreased VWF.

Increased VWFpp/VWF:Ag ratio also had D/D-H11032 of the individuals who had a substantially decreased VWF half-life and predictive of decreased VWF half-life, particularly in patients with most cases, an increased VWFpp/VWF:Ag ratio was found to be variability in VWFpp half-lives with a mean of 3.0 plus or minus 1.4 hours, comparable with the reported normal range. The variability of data is not unexpected given the highly variable phenotype that is characteristic of type 1 VWD. Type 1 VWD may result from a decreased VWF survival in plasma, increased VWF intracellular retention/ decreased secretion, or a mixed phenotype. It is unlikely that VWF inhibitors cause the VWD in these individuals as inhibitors in type 1 and type 2 VWD have not been reported. In some individuals, VWF variation only partly accounts for phenotype, and in others, phenotypes are not linked to the VWF gene locus. We also observed variability in the ratio of VWFpp/VWF:Ag in the type 1 VWD patients, ranging from 0.6 to 11.5 (Table 1). Many individuals (nos. 1-6 and 15-19) had ratios within our previously reported normal range. Mutations were identified in individuals throughout the VWF protein (Table 1). The initial VWF:Ag levels in those with normal ratios were moderately to slightly reduced (37.4 to 78.7 IU/dL). With the exception of one individual (no. 17; C2477S), these subjects also had a normal multimer structure. Three of the individuals with a normal ratio and normal multimer structure (nos. 1, 2, and 4) with mutations G160W, N166L, and M7711 were found to have VWFpp levels below the normal range. The reduced baseline level of VWFpp (and VWF:Ag) with normal VWF multimer structure suggests a mechanism of reduced synthesis of VWF, rather than increased intracellular retention observed in dominant negative type 1 and in type 2A VWD, where only smaller VWF multimers are secreted. The mechanism(s) resulting in reduced VWF level related to the identified mutations remains to be clarified by expression studies. Several individuals had significantly elevated VWFpp/VWF:Ag ratios (> 4.0) that fell clearly outside of the normal range (nos. 7-13). In most cases, an increased VWFpp/VWF:Ag ratio was found to be predictive of decreased VWF half-life, particularly in patients with substantially decreased steady-state VWF:Ag levels (< 30 IU/dL). All 7 of the individuals who had a substantially decreased VWF half-life and increased VWFpp/VWF:Ag ratio also had D/D-3 domain mutations that have been previously reported to result in decreased VWF survival including R1205H/M740I, C1130G, C1130F, C1130R, and W1144G. In this study, the R1205H mutation was found together with M740I. Analysis of the promoter region and repeats within intron 40 of the VWF gene suggests the mutations may have arisen on a common founder allele. In previous reports, the R1205H mutation has been found individually in patients from Milan, Germany, and Hungary, and in conjunction with the M740I variation in patients residing in the province of Vicenza. It appears unlikely that the M740I variation contributes to the phenotype.

By examining the data presented here, together with previously published decreased plasma VWF survival studies, the characteristic elements of the increased clearance phenotype can begin to be defined. In the majority of cases, the steady-state level of VWF:Ag is significantly decreased (< 30 IU/dL) and the half-life of VWF:Ag is also markedly reduced. Whether the decreased survival of VWF causes the very low VWF:Ag level in these patients remains to be clarified. Although plasma multimers appear to be essentially normally distributed, a diminished intensity of VWF multimer satellite bands was observed (Figure 5). The decreased intensity of VWF multimer satellite bands was previously described in patients with a W1144G or S2197F mutation in the VWF gene and decreased VWF survival phenotype. This decrease in satellite band intensity could be due to rapid removal of ADAMTS13 degradation products or could be due to insufficient residence time in plasma for proteolysis. The relationship between the increased clearance of VWF and the decreased intensity of satellite bands remains unclear. The patients in this subset demonstrate a robust response to desmopressin with up to a 13.5-fold increase in VWF:Ag. Collectively, affected individuals with markedly reduced VWF:Ag and VWF half-life, a robust desmopressin response, and a VWF gene mutation affecting the D/D-3 domains had a significantly increased VWFpp/VWF:Ag ratio.

The enhanced desmopressin response could potentially identify these patients provided the postdesmopressin sample is obtained 30 minutes to 1 hour after desmopressin administration. At later time points, the released VWF may have already been cleared from circulation. These individuals could also be identified by platelet VWF:Ag level. An individual with increased VWF clearance would have platelet VWF:Ag within the normal range with a markedly reduced baseline plasma VWF:Ag. However, the platelet VWF:Ag assay requires more intensive sample preparation than the preparation of patient plasma. We have shown that patients with a reduced VWF survival phenotype can be identified by assessing the ratio of steady-state VWFpp/VWF:Ag. The advantage of the assay of plasma VWFpp/VWF:Ag is the clinical utility of an ELISA assay using only a patient plasma sample.

This study used the VWFpp/VWF:Ag assay to identify patients (nos. 7-13) with a decreased VWF survival phenotype. The sample of type 1 VWD patients presented here was heterogeneous in respect to VWF:Ag, VWF survival, and VWF half-life consistent with the variable phenotype that is characteristic of type 1 VWD. Most patients in our subset did not appear to have a reduced VWF survival phenotype. VWD in these patients may result from reduced VWF synthesis, defective VWF secretion, or other, yet to be identified mechanisms. Identification of type 1 VWD patients with a reduced VWF survival phenotype is of clinical importance as desmopressin may not be the treatment of choice in this subset of patients because of the shortened VWF half-life. Although, the R1205H mutation in VWF has been identified in a number of type 1 VWD patients in the UK Haemophilia Centre Doctors’ Organisation, MCMMD-1VWD, and Canadian Cohort studies, the relative abundance of the reduced survival phenotype has not been identified. However, studies of type 1 VWD patients in the United States, Canada, and Europe are under way to determine the prevalence of this phenotype. As more patients with this phenotype are identified, and if it proves to be a relatively frequent mechanism, a new definition for these patients as VWD type 1C (1-Clearance) should be proposed.

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