Variable content of von Willebrand factor mutant monomer drives the phenotypic variability in a family with von Willebrand disease

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Key Points

- VWD is characterized by variable expressivity, even within families with the same VWF mutation.
- The content of mutant monomers in the final multimeric structure may explain the observed variability.

Introduction

Von Willebrand disease (VWD) is the most common congenital bleeding disorder of humans and is caused by quantitative or qualitative defects in von Willebrand factor (VWF), a large plasma glycoprotein required for effective platelet adhesion at sites of blood vessel injury. The VWF gene spans approximately 178 kb and maps to chromosome 22 and contains several nonsense and splice-site mutations, exons 18-52 encode the mature VWF polypeptide and 3' untranslated region. A pseudogene with homology to exons 23 to 34 of VWF maps to chromosome 22 and contains several nonsense and splice-site mutations, suggesting that it is unable to generate functional transcripts.5

VWF is synthesized by endothelial cells and megakaryocytes and either secreted constitutively or stored in endothelial Weibel-Palade bodies and platelet α-granules, from which it is released during activation.6,7 During synthesis, VWF monomers first homodimerize and undergo several posttranslational modifications in the endoplasmic reticulum, then form large multimers through polymerization in the trans-Golgi, with mature multimers ranging in mass from 500 to 20,000 kDa.8,9

The current clinical classification of VWD characterizes mild and severe quantitative defects of VWF as VWD types 1 and 3, respectively, whereas qualitative defects of VWF are classified as VWD type 2.11 VWD type 2 can be the result of abnormal multimerization, impaired secretion of high-molecular-weight multimers, increased ADAMTS13 proteolysis after secretion, enhanced or decreased interaction of VWF with the platelet receptor glycoprotein (GP) Ib-IX-V, or defective binding to collagen, or coagulation factor VIII.12

The complexity of VWD is underscored by the variability observed in both the clinical phenotype (mucocutaneous bleeding) and the biochemical phenotype (represented by VWF levels and activity). Although considered a monogenic disease, VWD is characterized by incomplete penetrance and variable expressivity, even in families with a single causative VWF mutation.13,14

We have identified a 24-member family with 11 individuals affected with VWD caused by a mutation in exon 28 of the VWF gene. Among the affected members, there was considerable phenotypic variability: in bleeding manifestations, VWF levels, and VWF activity, but most remarkably in multimer distribution. We thoroughly investigated the potential genetic and molecular mechanisms involved in this variability and demonstrated variable content of the mutant subunit in plasma VWF among affected individuals as a potential explanation.


J.C. and J.D.H. contributed equally to this study.

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Methods and materials

Participants

Twenty-four members of a single European-American multigenerational pedigree (11 VWD patients and 13 control relatives) were recruited. The study received institutional review board approval from the University of Iowa and the University of Colorado Anschutz Medical Campus, and informed consent was obtained for all participants, in accordance with the Declaration of Helsinki. Further details regarding diagnosis and treatment of this family can be found in the supplemental data on the Blood Web site.

VWF analysis

Assays to examine VWF antigen, multimers, collagen, llama nanobody AU/VWFa-11 and platelet binding, VWF propeptide antigen, factor VIII coagulant activity, ADAMTS13 activity, recombinant VWF expression, and VWF-storage granules in mammalian cells are described in the supplemental data.

Bleeding scores

To determine the bleeding phenotypes of pedigree members, we employed 2 different bleedings scores. One is a modified bleeding questionnaire that was originally designed and validated by the Epidemiology Branch of the Centers for Disease Control and Prevention to screen women with VWD (supplemental data).5 The other is the current International Society on Thrombosis and Haemostasis/Scientific and Standardization Committee bleeding score that was administered to 7 of the 11 affected members.16

Isolation of genomic DNA and VWF sequencing

Genomic DNA was isolated from whole blood using a commercially available kit (Qiagen). Because VWF ristocetin cofactor (VWF:RCo) and VWF antigen (VWF:Ag) in several pedigree members suggested the diagnosis of VWD type 2, the sequencing strategy for VWF focused on exon 28, which harbors the majority of type 2 mutations. Polymerase chain reaction (PCR) and sequencing primers were designed to discriminate and selectively amplify VWF vs its corresponding pseudogene.5 PCR was performed on genomic DNA by standard methods, and Sanger sequencing was performed using BigDye V3.1 on an ABI3730xl sequencer (Life Technologies). The sequencing data were analyzed using Sequencher V4.9 software (Gene Codes Corporation). To rule out the presence of additional mutations in the VWF gene, we sequenced all exons and introns for VWF in 3 affected members and 1 control relative at the Partners Healthcare Center for Genetics and Genomics (Harvard Medical School, Boston, MA).

ABO genotyping

ABO genotyping was performed by amplifying and sequencing exon 6 and part of exon 7 of the ABO gene using primer pairs 1 and 3 as described by Mizuno et al.17

Molecular modeling

The crystal structure of the wild-type VWF A1 (Protein Data Bank: 1AUQ) was used as the starting point for the molecular dynamics (MD) simulations. The mutant A1 structure was obtained by replacing the side chain of methionine with that of arginine at position 1304. Coordinates for the mutated side chain were constructed and minimized with 100 steps of steepest descent using the program CHARMM.18 The MD simulations were performed with the program NAMD28 using the CHARMM all-hydrogen force field (PARAM22)18 and the TIP3P model of water. The details are described in the supplemental data.

Allele-specific mRNA assay

RNA was isolated from platelets of the studied family members and complementary DNA (cDNA) was synthesized from 200 ng of RNA for each individual. Standard locus-specific PCR using VWF exon 28 primers (5-GAGCCCCACC ACTCTGTATG-3 and 5-TGCCCGCATACCTCACCCT-3) was performed (details can be found in the supplemental data). Ratios of allele-specific messenger RNA (mRNA) were calculated as previously described.20

Results

Pedigree of a family with VWD

The family we studied contains 24 members from 3 generations, with 11 individuals carrying the diagnosis of VWD based on low VWF levels (Figure 1A). The disease was transmitted in autosomal-dominant fashion. The affected individuals exhibited low VWF antigen levels (Figure 1B), low ratios of ristocetin cofactor to VWF antigen (≤0.7), and decreased VWF binding to recombinant GPIba and collagens type III and VI (Figures 1F-I). All but 2 affected individuals had ratios of VWF propeptide to VWF antigen lower than 3, indicating normal VWF clearance (Figure 1E). All affected individuals had decreased factor VIII activity (Figure 1C), but normal ADAMTS13 activity (Figure 1D). Demographic parameters and VWF measurements are compiled in supplemental Table 1.

Phenotypic variability among affected pedigree members

The bleeding scores, obtained using 2 clinical bleeding assessment tools, differed significantly among affected family members (supplemental Table 1). VWF multimer patterns also varied significantly among affected family members, with some individuals having severely reduced quantities of high-molecular-weight multimers, and others exhibiting multimer patterns almost indistinguishable from those of the unaffected members (Figure 2B). The multimer patterns remained similar over time for most of the affected members (supplemental Figure 4), and no increases in satellite bands were detected on a high-resolution gel, indicating that the loss of high-molecular-weight multimers is not due to excessive ADAMTS13 cleavage (supplemental Figure 5). The distribution of multimers did not correlate with the bleeding scores (Figure 2). We then measured the binding of nanobody AU/VWFa-11 to plasma VWF from the affected individuals. The nanobody detects an epitope within the A1 domain exposed when the A1 domain is decrypted or in a platelet-binding conformation.21,22 In addition, the nanobody binds at elevated levels to type 2B19 and type 2M VWF.23 We found that AU/VWFa-11 binds at elevated levels to VWF from 9 patients. Nanobody binding was heterogeneous among the different family members and did not correlate with the bleeding scores (Figure 2A).

VWD in this family is caused by a mutation (M1304R) in VWF

Sequencing of exon 28 revealed a T>G substitution at position 3911 of the VWF gene that predicts a change of methionine to arginine at position 1304 (M1304R) in the A1 domain of VWF. The mutation segregates with the VWD phenotype (defined by VWF:RCo < 20 U/dL, supplemental Table 1) in all affected members and is not present in the unaffected individuals. Complete sequencing of VWF in 3 affected individuals in the pedigree (individuals I.1, III.4, and III.6) revealed 5 sequence variations in addition to the M1304R mutation (supplemental Table 2). All are previously reported single nucleotide polymorphisms. Interestingly, individuals I.1 and III.4 were heterozygous for the D1472H variant in exon 28, which has been associated with lower values for VWF:RCo. This finding is of no clinical significance because it is due to an artifact related to the assay. All exonic nonsynonymous single nucleotide polymorphisms found in these individuals were then
genotyped in all members of the pedigree. All but 2 affected individuals (III.6 and III.7) were heterozygotes for the D1472H variant. None of the remaining variants segregated with the disease phenotype. ABO blood group status, a known modifier of VWF levels, did not significantly influence levels in individuals affected by the mutation (supplemental Table 1). Although the putative causative mutation, M1304R, has not been previously reported, mutations in the same region are known to cause type 2B VWD.24

Molecular modeling of the structural consequences of the M1304R mutation

Within the 3-dimensional crystal structure of the A1 domain, the Met1304 side chain is surrounded by multiple hydrophobic residues (Figure 3A). Substitution of methionine with arginine introduces a larger and positively charged side chain into a tightly packed hydrophobic environment that may destabilize the folding of the A1
domain. We simulated the effect of the M1304R mutation on the A1 structure using MD. We first replaced the methionine 1304 side chain with that of arginine to create a mutant A1 structure; we then performed MD simulations at room temperature for 50 ns on the structures of both the wild-type and mutant A1. Analysis of the simulation trajectories showed that the backbone α carbons (Cα) of the residues around position 1304 are more flexible in the mutant A1 than in the wild-type (Figure 3B, shaded area). When examining the time course of the structural changes, we found that the α-helix that contains residue 1304 in the mutant A1 domain deviates more from the starting configuration than does the wild-type A1 domain (represented by increased Cα root-mean-square deviations [RMSD] in Figure 3C). These results indicate that the arginine substitution at position 1304 kinetically destabilizes the A1 structure. Consistent with this, using the method of free energy perturbation, we estimated that the folding free energy of the mutant is less favorable than the wild-type by 36 kcal/mol (supplemental Figure 1), suggesting that mutant A1 is also thermodynamically less stable than the wild-type. This conformational change may affect both the interactions of the VWF A1 domain with platelet GP Ibα and nanobody AU/VWFa-11 binding.

Interestingly, the substitution of Met1304 with valine, a hydrophobic residue with a smaller side chain than methionine, results in gain of platelet-binding function, and defines a VWD type 2B phenotype (http://www.vwf.group.shef.ac.uk). To compare the differences caused by valine or arginine substitution at position 1304, we applied the same free energy perturbation protocol to examine the changes in stability of the A1 domain caused by M1304V mutation. We found that M1304V also reduces the stability of the A1 domain, albeit to a much smaller degree, ie, 5 kcal/mol (see supplemental data under free energy perturbation calculations). This is consistent with previous equilibrium refolding experiments showing that type 2B mutations are associated with a slight destabilization of the A1 domain.25 Thus, it is plausible that M1304V destabilizes the A1 domain in the vicinity of the mutated site enough to allow it to bind platelets, whereas M1304R drastically destabilizes the A1 domain, causing it to unfold or misfold, resulting in loss of function and complete abrogation of platelet binding.

**Biosynthetic defect associated with the VWF M1304R mutation**

To assess the impact of the M1304R mutation on VWF synthesis, we transfected VWF cDNAs encoding either wild-type or M1304R mutant VWF into human embryonic kidney cells (HEK293) and examined VWF secretion, granule storage, multimeric structure, and...
platelet-binding functions. The cells transfected with mutant VWF had a markedly reduced concentration of VWF in the cell supernatant compared with those transfected with the wild-type cDNA (17% of wild-type; \( P < .001 \)), but both cells had similar quantities of VWF in the cell lysates (Figure 4A). The secreted mutant VWF consisted primarily of low-molecular-weight multimers (Figure 5B, lane 1), whereas the wild-type contained the full range of multimer sizes (Figure 5B, lane 5). The mutant VWF did not bind platelets in the presence of ristocetin or botrocetin (Figure 5D-E, when %DNA of mutant is 100%).

Cells expressing the mutant displayed a diffuse fine granular VWF staining pattern with occasional, very small round granules (Figure 4B); in contrast, cells expressing wild-type VWF showed well-defined, cigar-shaped structures characteristic of Weibel-Palade bodies (Figure 4C). These data show that the M1304R mutation causes defects in intracellular VWF processing, such as secretion, multimerization, and granule storage, as well as defective platelet binding.

Increased incorporation of wild-type monomers improves VWF synthesis and functions

To determine the impact of different levels of mutant incorporation on VWF synthesis and function, we cotransfected HEK293T cells with the mutant and wild-type VWF cDNAs in different ratios. As the percentage of wild-type cDNA increased, the amount of VWF secreted and the multimer size of the secreted VWF also increased (Figure 5A-B). Because the wild-type VWF has a myc-His tag not present in the mutant, we were able to differentially detect the 2 forms of VWF on multimer gels (Figure 5C). When expressed alone, the M1304 mutant was secreted only as small multimers (Figure 5C, M1304R: lane 1). However, in the presence of just 25% wild-type cDNA, the average multimer size of the secreted VWF markedly increased (Figure 5C, M1304R: lane 2). Both wild-type and mutant VWF were readily detected in the multimers in the presence of 25% and 50% of wild-type cDNA (Figure 5C, lanes 2 and 3). Consistent with the improvement of VWF secretion and multimerization, both ristocetin- and botrocetin-induced VWF binding to platelets increased as the percentage of the wild-type cDNA increased (Figure 5D-E). These findings indicate that the degree of wild-type monomer incorporation into VWF multimers not only impacts intracellular VWF processing, but also its ability to bind platelets.

The phenotypic variability observed in the affected family members is not due to a hypomorphic wild-type VWF allele

The previous results show that VWF secretion, multimerization, and functions were affected by the extent of incorporation of mutant and wild-type monomers into the final VWF multimers. Therefore, it is possible that phenotypic heterogeneities among the affected individuals are caused by different expression levels of mutant or normal VWF alleles. It is now recognized from large studies of families with VWD that, in addition to characterized VWD mutations, several polymorphisms modify VWF levels and may modify VWF activity.13,26-28 We therefore measured the levels of mutant and normal VWF mRNA in affected and unaffected members of the pedigree to investigate the potential contribution of a hypomorphic normal allele to the observed phenotypic variability. The levels of the normal mRNA were similar among the affected individuals carrying the M1304R variant, ranging from 73% to 78% of total VWF mRNA, ruling out a contribution of a hypomorphic allele (supplemental Figure 2).
Biochemical phenotypes of VWD in a large family in which the disease is caused by a mutation that converts Met1304 to arginine within the VWF A1 domain. The family was remarkable for displaying a wide phenotypic variability; we identified one cause of this variability as resulting from plasma VWF multimers of affected individuals displaying a wide range of mutant monomer content (Figure 2A).

In the current classification of VWD, different individuals in this family would be given dissimilar diagnoses, based on differences in their clinical laboratory data (Figure 2). For example, III.3 would be classified as type 1 (VWF:RCo/VWF:Ag ≥ 0.7 and normal multimer distribution); II.1, II.3, II.5, II.7, III.4, and III.6 would be classified as type 2A (VWF:RCo/VWF:Ag < 0.7 and loss of high-molecular-weight multimers); and I.1, I.3, and III.7 would be classified as type 2M (VWF: RCo/VWF:Ag < 0.7 and normal multimer distribution). We reviewed the medical records of 5 affected individuals and found that over the past 20 years, they have been described either as having types 1, 2A, or 2M based on their VWF levels and multimer distribution, suggesting that the variability we observed in our study persists over time.

Of interest, a potential diagnosis of type 2B VWD is also suggested by the location of the mutation. Type 2B VWD has been described as resulting from either substitution of valine or insertion of an additional methionine at position 1304, in addition to several other mutations in surrounding amino acids. In this family, type 2B VWD was ruled out by the presence of normal platelet counts in all affected individuals and a lack of an enhanced response to low-dose ristocetin (data not shown). Further, recombinant M1304R VWF did not bind platelets in the presence of ristocetin or botrocetin (Figure 5D-E), consistent with a type 2M phenotype.

Potential explanations for the phenotypic variability that are purely genetic are either based on the presence of a hypomorphic normal allele (an allele that produces reduced quantities of its protein product) in addition to the M1304R mutant or the presence of genetic variants that, when associated with the mutation, would either reduce or increase VWF levels. A hypomorphic VWF allele was recently described as a determinant of plasma VWF levels.29 We ruled out the possibility that a hypomorphic normal allele contributed to the observed variability by demonstrating that all affected members had similar ratios of normal to mutant mRNA (supplemental Figure 2). This effect appeared to be uniform among all affected individuals. We also looked for other genetic variants that might contribute to the phenotypic variability by sequencing the entire coding regions and exon/intron boundaries of VWF in 3 affected individuals. We then sequenced the identified variants in every member of the pedigree. None of the sequence variants was found in all the affected members, although the D1472H variant occurred in all but 2. This variant was reported by Flood et al.30 to be associated with lower VWF:RCo levels, a consequence of defective binding of ristocetin to VWF in the VWF:RCo assay, but not associated with increased bleeding. In this family, however, this variant does not alter VWF:RCo in the affected or unaffected individuals. Based on these results, it is highly unlikely that the observed variability results from a combination of the rare M1304R mutation and common genetic variants in VWF.

Another possible explanation for the observed variability in laboratory and clinical phenotypes is that the composition of plasma VWF varies between individuals. The plasma VWF from the affected members displayed a wide range of binding of AU/VWFa-I1 (Figure 2A), which preferentially recognizes the mutant monomers and whose binding is not affected by VWF multimer size (supplemental Figure 3). This indicates that plasma VWF from different affected members varies in its quantity of mutant monomers. This difference could account for differences observed in the functional assays and is supported by our in vitro studies showing that when expressed alone, the mutant VWF was poorly secreted (Figure 4A) and inadequately multimerized (Figure 5B, lane 1) and did not bind to platelets (%DNA of mutant = 100% in Figures 5D-E). Each of these parameters improved as the percentage of wild-type monomer incorporated into the multimers increased (Figure 5).

Variability in mutant content may have its origins in the early stages of VWF synthesis, with 1 scenario being depicted in supplemental Figure 6. Heterozygous cells produce both normal and mutant monomers, the latter in lower quantities. The monomers can form 3 types of dimers, normal–normal, normal–mutant, and mutant–mutant, and these dimers are transported to the Golgi to form multimers. During this process, the protein quality control (QC) system in the endoplasmic reticulum monitors the quality of newly synthesized VWF monomers or dimers and removes misfolded ones.31 Some of the misfolded proteins are transported to the Golgi, where they are retained or degraded. Our data support a mechanism in which the mutant-containing dimers and multimers have a higher probability of being recognized as misfolded and are removed by the QC system. This mechanism would account for the marked reduction in the secretion of the mutant VWF when it is expressed alone (Figure 4A).

In the family we studied, the variability of mutant content in plasma VWF among affected individuals could be related to genetically determined variations in the QC system that result in more or less...
efficient removal of mutant monomers and dimers during VWF synthesis. In addition, the extent to which the mutant-containing multimers are cleared by the QC system could also vary among individuals.

Therefore, there are several potential mechanisms that can lead to the variable multimer pattern and nanobody binding observed in the family described here. An individual could have an efficient protein QC system that removes most of the mutant-containing VWF monomers and dimers, yielding plasma VWF made mostly of normal monomers with a final multimeric distribution of low-molecular-weight forms. These multimers will exhibit low nanobody binding because they contain few mutant monomers. In the current clinical laboratory classification, this individual would be classified as having VWD type 2A. In this family, this particular situation is best represented by patients II.3 and II.5, both of whom were diagnosed with VWD type 2A based on clinical laboratory data (Figure 2).

In summary, our findings suggest that phenotypic variability in 1 family afflicted with VWD results from variable incorporation of mutant monomers into VWF multimers. The mutant content of an individual’s plasma VWF will determine the bleeding and multimer phenotype of that individual’s VWD and how the disease is classified. In this family, different individuals carrying the same mutations could be classified as type 1, type 2A, or type 2M. Cellular modifiers such as molecular chaperones in the endoplasmic reticulum and Golgi that play a role in VWF biosynthesis and trafficking may explain the variable mutant incorporation observed in this family.

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Authorship

Contribution: J.C. designed and performed experiments, analyzed data, and cowrote the manuscript; J.D.H. designed and performed experiments, analyzed data, and cowrote the manuscript; S.H. and R.M. analyzed data and edited the manuscript; P.J., V.H.F., and R.W. performed experiments; G.I. designed and performed experiments, analyzed data, and edited the manuscript; D.W.C. designed experiments, interpreted data, and edited the manuscript; and J.A.L. and J.D.P. directed the project, designed experiments and interpreted data, and cowrote the manuscript.

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