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Variability in Platelet and Collagen Binding Defects in Type 2M Von Willebrand Disease

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Abstract

Introduction—Type 2M von Willebrand disease (VWD) includes qualitative defects in von Willebrand factor (VWF) function, with normal multimer distribution but a defect in VWF activity with respect to platelet or collagen binding.

Aim—We characterized novel *VWF* gene mutations found in type 2M VWD subjects enrolled in the Zimmerman Program for the Molecular and Clinical Biology of VWD.

Methods—Subjects were enrolled based on a pre-existing diagnosis of type 2M VWD. Testing included full length gene sequencing, VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF collagen binding, and multimer distribution. Recombinant VWF variants were synthesized using site-directed mutagenesis and expressed in HEK293T cells. Platelet binding was measured by flow cytometry with fixed platelets and ELISA with recombinant GPIIb/IIIa.

Results—Four novel VWF A1 domain mutations were found in individuals with type 2M VWD: S1358N, S1387I, S1394F, and Q1402P. All subjects had a history of bleeding, VWF:RCo <40 IU/dL, VWF:RCo/VWF:Ag ratios <0.6, and normal multimer distribution. No defect in expression, secretion, or multimerization was found for any of the mutations. All showed decreased binding to intact platelets, and decreased or absent binding to a mutant GPIIb/IIIa construct with spontaneous VWF binding. S1387I had decreased binding to all collagen types tested. Q1402P had reduced binding exclusively to type VI collagen.

Conclusion—Type 2M VWD is a heterogeneous category comprised of both collagen and platelet binding defects. Understanding the precise defect for each mutation may ultimately lead to better diagnosis and treatment.

Keywords

von Willebrand disease; von Willebrand factor; bleeding; collagen; genetics

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Disclosures

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Introduction

Type 2M von Willebrand disease (VWD) represents a spectrum of mutations that affect von Willebrand factor (VWF) function. This variant presents with typical mucocutaneous bleeding, but is distinguished from other forms of VWD by laboratory findings; including normal multimer distribution but a discrepancy between VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo) [1]. Type 2M typically accounts for 5–10% of patients with VWD [2–4]. Patients with type 2M VWD are classically thought to have defects in VWF-platelet binding, but this category also encompasses collagen binding defects [1].

Most type 2M patients have point mutations in the A1 domain affecting VWF-GPIb interactions [5]. One patient has been reported with an 11 amino acid deletion, also in the A1 domain [6,7]. The A1 domain contains binding sites for platelet glycoprotein Ib [8,9] and for collagen [10,11]. A recent report from Canada suggests that mutations are more likely to be found in patients with VWF:RCo/VWF:Ag ratios less than 0.4 [12]. However, bleeding does not necessarily correlate with either ratio or mutation status. Heterogeneity in patient presentation may be due in part to heterogeneity in mutations. Unlike type 2B VWD, where a limited number of mutations account for the majority of patients, type 2M VWD includes patients with a variety of mutations spread throughout the A1 domain.

Characterization of type 2M VWD mutations has implications for treatment of affected patients. Treatment with VWF-containing concentrates may be required, as patients with type 2M VWD have a variable response to desmopressin [13]. Some sequence variations associated with a decreased VWF:RCo/VWF:Ag ratio are the result of aberrant binding to ristocetin, a non-physiologic agonist, and do not affect the interaction of VWF with platelet glycoprotein Ib (GPIb) [14,15]. Distinguishing these from true 2M VWD mutations could improve patient care. In addition, future treatments targeted at either VWF-GPIb or VWF-collagen interactions could be administered based on functional characterization of individual patient mutations.

We report here on four novel mutations found in subjects with type 2M VWD, discovered as part of the Zimmerman Program for the Molecular and Clinical Biology of VWD, a multi-center study comparing bleeding symptoms, laboratory findings, and sequence variations for subjects with VWD. Although all types were enrolled, only those subjects with type 2M VWD are included in the present study. Each mutation was expressed and characterized in order to understand its effect on interactions with crucial VWF ligands.

Materials and Methods

The Zimmerman Program is a multi-center study of patients with all types of VWD, their family members, and healthy controls, taken from treatment centers across the United States [16]. Enrollment criteria consisted of a pre-existing diagnosis of VWD of any type. Zimmerman Program subjects with an enrollment diagnosis of type 2M VWD and a novel mutation in the VWF A1 domain were evaluated for the current study. To date, 20 subjects with type 2M VWD have sequencing results available for analysis, and the four subjects reported here represent novel mutations discovered in this group. One subject was enrolled from the Indiana Hemophilia & Thrombosis Center in Indianapolis and three were enrolled from the Comprehensive Center for Bleeding Disorders in Milwaukee. Subjects enrolled in the Zimmerman program with type 2M VWD and previously characterized mutations were excluded from the present investigation. Each subject had a pre-existing diagnosis of type 2M VWD as determined by the enrolling center. The research protocol was approved by the

Institutional Review Board of each institution. Informed consent was obtained from each subject prior to enrollment.

Clinical laboratory assays of VWF function

Blood samples were collected in sodium citrate and sent to the Clinical Hemostasis Laboratory of the BloodCenter of Wisconsin for VWF testing. VWF:Ag, VWF:RCO, factor VIII activity, and multimer distribution were performed as previously described [15]. Collagen binding (VWF:CB) was also performed using type III human placental collagen [17]. VWF propeptide was measured by ELISA [18]. Full length sequencing of the *VWF* gene was performed for all index cases as previously described [19].

Synthesis of mutant VWF constructs

Recombinant VWF constructs were synthesized using the Stratagene QuikChange Lightening Kit (Agilent Technologies, Santa Clara, CA, USA) to perform site-directed mutagenesis on a full-length wild-type VWF construct [20]. Individual mutant constructs were made for each of the four mutations of interest. DNA was purified and expressed in HEK293T cells for each mutation individually, for a wild-type recombinant VWF construct, and for an empty vector to serve as a negative control. Supernatants were collected at 72 hours for use in the VWF assays described below. All experiments were performed in triplicate unless otherwise noted. Multimer distribution for each construct was assayed as previously described [16].

Platelet binding assays

Platelet binding to intact platelets was assayed by flow cytometry [14]. Washed, fixed platelets (Bio/Data, Horsham, PA, USA) were incubated with recombinant VWF and varying doses of ristocetin (American Biochemical and Pharmaceutical, Marlton, NJ, USA) at room temperature for 1 hour. A labeled anti-VWF antibody (DAKO, Carpinteria, CA, USA) was then added, and the presence of VWF-platelet conjugates measured on a flow cytometer.

Platelet-binding assays were also performed using a recombinant GPIIb α construct and recombinant VWF or patient plasma bound to an ELISA plate via a monoclonal antibody to GPIIb α [16]. The GPIIb α construct contained two gain-of-function mutations, D235Y and M239V, to allow for spontaneous binding of VWF in the absence of ristocetin as well as a C65A mutation to prevent dimerization. Plasma samples or recombinant VWF samples were added, and presence of binding detected by monoclonal antibodies to VWF. No ristocetin was used in this assay.

Collagen binding assays

VWF collagen binding assays were performed using recombinant VWF or patient plasma [17]. Type III collagen binding was performed using Immulon IB plates (Thermo Scientific, Rochester, NY, USA) coated with 1 mcg/mL human placental collagen (Southern Biotech, Birmingham, AL, USA). Type I collagen binding was performed using Nunc Maxisorp plates (Nalge Nunc, Rochester, NY, USA) coated with 5 mcg/mL human placental collagen (Sigma, St. Louis, MO, USA). Type VI collagen binding was performed using kits imported from Technoclone (Austria) through Diapharma (West Chester, OH, USA).

Results

Four unrelated subjects were identified in the Zimmerman Program with type 2M VWD and novel mutations. All subjects had decreased VWF:RCO/VWF:Ag ratios (< 0.6) with normal multimer distribution, consistent with the diagnosis of type 2M VWD. Clinical laboratory

data are summarized in table 1. DNA sequencing demonstrated heterozygosity for a novel mutation in each proband. The four mutations, S1358N, S1387I, S1394F, and Q1402P, all localize to the VWF A1 domain. None of these mutations have previously been reported. No other *VWF* mutations were found for any of the four subjects under investigation.

Reported bleeding symptoms included easy bruising or minor cutaneous bleeding in 3 subjects, menorrhagia in 2 subjects, epistaxis in 2 subjects, and gingival bleeding in 1 subject. Three of the subjects were age 18 or younger at the time of study enrollment and had not yet experienced childbirth or major dental extractions, which may have led to an underestimation of the bleeding score. Affected family members had low VWF:RCo/VWF:Ag ratios, while all unaffected family members had normal VWF levels.

Normal expression, secretion, and multimerization of 2M VWD variants

When these mutations were expressed in recombinant VWF, no difference in expression levels was seen (data not shown). No intracellular retention of VWF was observed, consistent with the diagnosis of type 2M rather than type 2A VWD. As expected, multimer distribution was normal for all of the mutations studied (data not shown).

Defective platelet binding in 2M VWD variants

Since type 2M VWD is typically characterized by a defect in VWF-platelet interactions, we examined the ability of the mutant constructs to bind platelets by two methods; flow cytometry with intact platelets and ELISA with recombinant GPIb α . When ristocetin-induced binding to intact platelets was measured using flow cytometry, all four mutations displayed absent platelet binding (figure 1). Only the wild-type VWF construct gave measurable results, while platelet binding to all 4 type 2M mutants was undetectable. Results differed slightly with recombinant GPIb α as compared to intact platelets. When platelet binding was measured by ELISA using a gain-of-function GPIb α construct in the absence of ristocetin (figure 2), 2 mutations showed decreased binding (1358N and 1394F) while 2 mutations showed absent binding (1387I and 1402P). The ELISA assay was also performed using patient plasma samples from the type 2M VWD subjects. Results varied, perhaps due to the heterozygous nature of the mutations. The subjects with S1358N, S1394F, and Q1402P all had decreased binding to platelet GPIb α using the ELISA assay, while the subject with S1387I had a relatively preserved GPIb α binding compared to the antigen level. Interestingly, the 1387I mutation had absent binding using the homozygous recombinant VWF construct, suggesting that presence of some normal VWF was sufficient to compensate for the mutation in the ELISA assay.

Defective collagen binding in 2M VWD variants

Another function of VWF is to bind exposed subendothelial collagen. We therefore examined binding to types I, III, and VI collagen for the four type 2M mutations using recombinant VWF homozygous for the mutations of interest (figure 3). Two mutations, 1358N and 1394F, displayed normal binding to all 3 collagen types. One mutation, 1402P, had decreased binding to only type VI collagen. The 1387I mutation, on the other hand, had decreased binding to all the collagen types tested. Collagen binding was also tested using patient plasma from the type 2M VWD subjects, all of whom were heterozygous for the mutations under study. Results were similar, for the most part, to the recombinant results. The subject with S1387I had decreased binding to types I and VI collagen, although relatively preserved binding to type III collagen. The subject with S1358N had slightly decreased binding to type I collagen as well. As the mutations were all heterozygous, presence of some wild-type VWF may have compensated for the mutant VWF in the ELISA assay.

Discussion

These results show that the novel 2M VWD mutations display a variable range of functional defects. Ristocetin-induced platelet binding to intact platelets was diminished for all the mutations under investigation. However, binding to isolated GPIb α in the absence of ristocetin was not as profoundly affected for the 1358N and 1394F constructs. It is possible that static assays of VWF function reflect a different aspect of VWF activity. It is also possible that these mutations also affect VWF-ristocetin interactions, and do not represent a significant functional defect. However, none of these mutations affect the known ristocetin-binding domains of VWF, unlike the previously reported 1467S and 1472H mutants which affect ristocetin binding but do not alter platelet binding [14,15]. In addition, the subjects reported here all had bleeding symptoms associated with their diagnosis of type 2M VWD, unlike the subjects with 1467S and 1472H.

Collagen binding results also varied, with no defect observed for 1358N and 1394F, and a defect only in type VI collagen for 1402P as previously reported [20]. The 1387I mutation had decreased binding to all collagen types tested. Several reports have also demonstrated exclusive defects in collagen binding, all with mutations located in the A3 domain [21–23]. Type I and III collagen bind VWF via both the A1 and A3 domains [10] while type VI collagen binds VWF exclusively through the A1 domain [11]. Subtle conformational changes induced by some of the type 2M point mutations may affect collagen binding; alternately, the mutations may affect the binding site directly.

These results, however, did not necessarily correlate with the amount of bleeding as measured by the bleeding score. While correlation with bleeding symptoms is difficult given the limited number of affected subjects, the lowest bleeding score was seen in the subject with 1394F. This construct had normal collagen binding and only showed a slight decrease in platelet GPIb binding for the ristocetin-independent assay. On the other hand, the highest bleeding score was seen in the subject with 1358N, which also showed no defect in collagen binding.

Previous studies have demonstrated variable bleeding in type 2M VWD patients. It is tempting to theorize that bleeding may correlate with the severity of the functional defect. Most type 2M patients have significant bleeding, with menorrhagia and epistaxis frequently reported [12]. However, decreased bleeding symptoms have been seen in type 2M as compared with type 2A VWD [24]. Symptoms may vary across affected family members, as has been reported for several other type 2M VWD mutations [25]. Other modifier genes may be involved given the variability seen across families.

Federici and colleagues reported that only 14% of type 2M patients in their study met criteria for response after a desmopressin trial [13]. Therefore, many clinicians opt to treat type 2M VWD with VWF-containing concentrates, particularly for surgical procedures. Desmopressin may be used in some patients for minor bleeding, but does not address the functional problem present in 2M VWD patients. Alternative non-specific treatments may be useful, such as anti-fibrinolytics for mucosal bleeding and estrogen therapy for menorrhagia. Future developments, including recombinant VWF, may ultimately allow for more specific treatment of the underlying defect.

Conclusion

Type 2M VWD represents a spectrum of functional defects, including altered platelet binding, altered collagen binding, or both. Treatment of affected patients may ultimately require targeted analysis of the underlying mutation for optimal efficacy.

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Figure 1. Absent ristocetin-induced binding to intact platelets for recombinant type 2M VWF
 Binding to intact platelets in the presence of ristocetin was measured by flow cytometry for the type 2M mutants and compared to wild-type (WT) recombinant VWF. Mean fluorescence is shown on the y axis and ristocetin concentration (in mg/mL) on the x axis for a representative experiment.

Figure 2. Defective binding to recombinant GPIIb/IIIa for recombinant type 2M VWF
Platelet binding was measured by ELISA for the type 2M mutants and compared to wild-type (WT) recombinant VWF. This graph shows the ratio of platelet GPIIb/IIIa binding to antigen (VWF:Ag) using a gain-of-function GPIIb/IIIa in the absence of ristocetin. Data represent mean of 3 separate transfections and error bars denote 1 standard deviation.

Figure 3. Collagen binding for recombinant type 2M VWF

Collagen binding was measured for the type 2M mutants and compared to wild-type (WT) recombinant VWF. This graph shows the collagen binding (VWF:CB) to antigen (VWF:Ag) ratio using type I collagen (black bars), type III collagen (gray bars), and type VI collagen (white bars). Data represent mean of 3 separate transfections and error bars denote 1 standard deviation.

Table 1

VWF laboratory findings for type 2M VWD subjects.

	Subject 1	Subject 2	Subject 3	Subject 4
Mutation	S1358N	S1387I	S1394F	Q1402P
Sex	Female	Female	Female	Female
Bleeding score	11	5	2	3
FVIII (IU/dL)	80	42	90	48
VWF:Ag (IU/dL)	59	25	88	31
VWF:RCo (IU/dL)	32	11	37	14
VWF:RCo/VWF:Ag ratio	0.54	0.44	0.42	0.45
Multimer distribution	Normal	Normal	Normal	Normal
VWFpp (IU/dL)	64	64	81	71
VWF platelet ELISA (U/dL)	14	32	25	5
VWF:CB (III) (IU/dL)	57	27	111	41
VWF:CB (I) (IU/dL)	27	11	130	52
VWF:CB (VI) (U/dL)	53	13	100	28

FVIII = factor VIII activity, VWF:Ag = VWF antigen, VWF:RCo = VWF ristocetin cofactor activity, VWFpp = VWF propeptide, VWF:CB (III) = VWF collagen binding with type III collagen, VWF:CB (I) = VWF collagen binding with type I collagen, VWF:CB (VI) = VWF collagen binding with type VI collagen.