Cosegregation of von Willebrand Factor Gene Polymorphisms and Possible Germinal Mosaicism in Type IIB von Willebrand Disease

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Recent reports of the mutations resulting in von Willebrand disease (vWD) have indicated that some cases of type IIA vWD are caused by single nucleotide substitutions in the gene encoding von Willebrand factor (vWF). However, the molecular pathogenesis of type IIB vWD remains unresolved and, with the complex posttranslational processing required for fully functional vWF, the mutations responsible for this phenotype may occur at loci other than the vWF gene. This study has used six intragenic vWF polymorphisms to assess the linkage of type IIB vWD to this gene in three families (48 individuals). The results of these studies indicate that there is significant linkage between the vWF gene and the type IIB phenotype (logarithm of the odds ratio of 7.2 at $\theta = 0$), suggesting that the mutations responsible for this disorder frequently occur at this locus. Results from one of these families indicates that the disorder has been transmitted from an unaffected parent to two children who have inherited the same vWF gene as seven unaffected siblings. This finding is suggestive of the presence of germinal mosaicism for the mutation in the father.

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MATERIALS AND METHODS

Materials. Tris, sodium dodecyl sulphate (SDS), boric acid, sodium citrate, sodium chloride, magnesium chloride, phenol, butanol, chloroform, and polyacrylamide were obtained from BDH Inc (Toronto, Ontario, Canada). All reagents were analytical grade.

Ultrapure agarose, proteinase K, deoxyribonucleotide triphosphates (dNTPs), restriction endonucleases, and Taq polymerase were purchased from Gibco/BRL (Burlington, Ontario, Canada). Gene Screen Plus hybridization membrane was from Dupont Canada Inc (Pointe Claire, Quebec, Canada). $\alpha^32P-dCTP$ was obtained from ICN Radiochemicals (Montreal, Quebec, Canada).

Study population. Three three-generation families (48 individuals) with type IIB vWD formed the basis for the study. Informed consent was obtained from all participating subjects and the study protocol had been approved by an ad hoc human ethics committee from the Faculty of Medicine, Queen’s University, Kingston, Ontario, Canada.

vWF assays. Blood was collected into 3.8% (wt/vol) sodium...
citrate (9 vol blood to 1 vol anticoagulant). Platelet-poor plasma was prepared by centrifugation at 3,000 g for 15 minutes at 4°C.

Factor VIII:C was measured by a one-stage method.28 vWF antigen (vWF:Ag) was measured by Laurell rocket immunoelectrophoresis using a rabbit polyclonal antihuman vWF antibody.29 vWF ristocetin cofactor (vWF:RiCof) was measured according to the method of Weiss et al.30 All assays were standardized by the simultaneous testing of a commercial reference plasma (Assayed Reference Plasma, Helena Laboratories, Beaumont, TX). All tests were performed on a minimum of two samples from each individual collected on different occasions and the results reported as a mean of these studies. The molecular weight profile of vWF in plasma and platelet lysates was assessed by crossed immunoelectrophoresis31 and by multimeric assay in a 1.4% agarose gel in the presence of 0.1% SDS.32 Pseudo-vWD was excluded in each of the families by assessing the aggregation response of washed patient platelets to low-dose ristocetin (0.5 mg/mL) when mixed in normal platelet-poor plasma.33,34

vWF gene polymorphisms. vWF cDNA probes for this investigation were kindly provided by the following investigators: probe vWF 2280, Dr Hans Pannekoek, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam);

| Table 1. Coagulation Studies in the Three-type IIB vWD Families |
|-------------------|------------------|------------------|-----------------|
|                   | VI\(\text{II})\)C | vWF:Ag (U/mL)    | vWF:RiCof (U/mL) | vWF Multimers*   |
|                   | Plasma           | Platelets        | RIPA+           |
| Family 1          |                  |                  |                 |
| I.1               | 0.61             | 1.10             | 0.62            | II              |
| I.3               | 1.14             | 1.46             | 1.26            | N               |
| II.1              | 1.20             | 0.87             | 0.94            | N               |
| II.2              | 0.32             | 0.33             | 0.22            | II              |
| II.3              | 0.59             | 0.62             | 0.30            | II              |
| II.4              | 0.43             | 0.57             | 0.29            | II              |
| III.1             | 0.33             | 0.44             | 0.17            | II              |
| III.2             | 0.78             | 1.16             | 0.66            | N               |
| III.3             | 0.35             | 0.44             | 0.14            | II              |
| III.4             | 1.52             | 1.00             | 0.75            | N               |

Family 2

I.1 | 1.53 | 1.09 | 1.24 | N |
II.1 | 0.56 | 0.84 | 0.90 | N |
II.2 | 0.52 | 1.61 | 0.99 | N |
II.3 | 1.10 | 1.52 | 2.92 | N |
II.4 | 0.49 | 0.78 | 0.77 | N |
II.5 | 0.53 | 1.25 | 0.75 | N |
II.6 | 0.44 | 0.43 | 0.20 | II |
II.7 | 1.11 | 1.60 | 1.20 | N |
II.8 | 0.65 | 0.84 | 0.60 | N |
II.10 | 0.60 | 2.20 | 1.48 | N |
II.11 | 0.89 | 0.63 | 0.59 | N |
II.12 | 0.62 | 0.60 | 0.54 | N |
II.13 | 0.31 | 0.27 | 0.11 | II |
II.14 | 0.39 | 0.68 | 0.14 | II |
II.15 | 0.68 | 0.62 | 0.22 | II |
II.16 | 0.82 | 1.38 | 1.28 | N |
II.17 | 0.97 | 0.58 | 0.67 | N |
II.18 | 0.67 | 0.62 | 0.71 | N |
II.19 | 0.98 | 0.76 | 0.80 | N |
III.1 | 0.33 | 0.25 | 0.11 | II |
III.2 | 0.88 | 0.72 | 1.06 | N |
III.3 | 0.94 | 0.70 | 0.96 | N |

Family 3

I.1 | 0.78 | 0.76 | 0.80 | N |
I.2 | 0.64 | 1.31 | 0.81 | II |
II.2 | 0.25 | 0.26 | 0.11 | II |
II.3 | 0.90 | 1.25 | 0.96 | N |
II.4 | 0.57 | 0.99 | 0.64 | N |
II.5 | 0.49 | 0.66 | 0.30 | II |
II.6 | 0.26 | 0.75 | 0.37 | II |
II.7 | 0.25 | 0.26 | 0.11 | II |

Abbreviations: RIPA, ristocetin-induced platelet aggregation; ND, not done.

*II, Loss of highest molecular weight multimers; N, Normal multimer pattern.
‡+, Aggregation to 0.5 mg/mL ristocetin concentration; –, no aggregation below 1 mg/mL ristocetin concentration.
In contrast to the other members of this family, due to geographical constraints, these two individuals were tested on only one occasion. Their plasma samples arrived in our laboratory thawed; thus, the values for VIII:C may be artifically low.
Probe pVWE6, Dr David Ginsburg, Howard Hughes Medical Institute, Ann Arbor, MI; and vWF cDNA clone pDL34, Dr Dennis Lynch, Dana-Farber Cancer Institute, Boston, MA.

The probe inserts used in this study were as follows: an 1,100-bp Pst I fragment from probe pvWF 2280, a 2,000-bp EcoRI–Sac I fragment of pVWE6, a 1,900-bp Sac I fragment of the vWF cDNA clone, and a 4,500 bp Kpn I fragment from the cDNA clone.

The vWF polymorphisms tested were a Bgl II RFLP (polymorphism information content [PIC] 0.43), a Taq I RFLP (PIC 0.49), a second Taq I RFLP (PIC 0.09), a Sac I RFLP (PIC 0.47), a
BamHI RFLP (PIC 0.27), and a 4-bp repeat vWF VNTR polymorphism in intron 40 of the vWF gene.  

Southern hybridization studies. High molecular weight leukocyte DNA was obtained from individuals by standard techniques. Five micrograms DNA were digested to completion with the appropriate restriction endonucleases according to the manufacturer’s instructions (GIBCO/BRL). The digested DNA was electrophoresed on a 0.8% to 1.0% agarose gel at 50 V for 20 hours and capillary blotted to Gene Screen Plus nylon membrane. Prehybridization was performed at 65°C overnight in 1.0 M NaCl, 1% SDS, and 10% dextran sulphate. The membrane was hybridized with an α-32P-dCTP-labeled vWF probe overnight at 65°C and subsequently washed to a stringency of 0.1 M sodium chloride/ sodium citrate (SSC; 0.15 mol/l NaCl and 0.015 mol/l sodium citrate) and 0.1% SDS at 65°C for 30 minutes. Finally, the membrane was exposed to Kodak XAR-5 x-ray film (Rochester, NY) with an intensifying screen at −70°C for 1 to 7 days.

Polymerase chain reaction analysis of intron 40 VNTR polymorphism. Priming oligonucleotides for the polymorphic vWF VNTR in intron 40 of the vWF gene were synthesized using the phosphoramidite method on an Applied Biosystems (Foster City, CA) 380A DNA synthesizer. The two oligonucleotide primer sequences were 5'-AGCTATATATCTATTTATCAT-3' and 5'-AGATACATATATATCTATTTATCAT-3'. One microgram genomic DNA was used in the amplification reactions with 10 μL Cetus (Montreal, Quebec, Canada) PCR buffer (500 mmol/l KCl, 100 mmol/l Tris–HCl, pH 8.3, and 20 mmol/l MgCl2, 0.1% gelatin), 8 μL 2.5 mmol/l dNTPs, 25 pmol each primer, 1 μL BSA (3 mg/ml), 2.5 U Taq polymerase (GIBCO/BRL), and nanopure water to a final volume of 100 μL. Amplification was performed by PCR using an automated Perkin Elmer Cetus (Montreal, Quebec, Canada) DNA Thermal Cycler. Samples were amplified using a cycle of 1 minute at 94°C to denature, 2 minutes at 50°C to anneal primers, and 1.5 minutes at 72°C to extend. The procedure was repeated for 30 cycles. The resulting product was electrophoresed on a 12% polyacrylamide gel using a Tall Mighty Small electrophoresis unit (Hoefer Scientific, San Francisco, CA). Gels were stained with ethidium bromide and visualized under ultraviolet light. Endonuclease-digested pGEM plasmid was used for molecular weight estimation and three previously genotyped samples (demonstrating six different alleles) were included in each electrophoresis run.

RESULTS

Coagulation studies. The phenotypic coagulation profiles for the members of these families are shown in Table 1. Variably normal or low values for Factor VIII:C, vWF:Ag, and vWF:Ricof were demonstrated in all affected individuals and intermittent mild thrombocytopenia (50 to 100 × 10⁹/L) was documented in most of these subjects. Hyperresponsiveness of affected subjects’ platelets for ristocetin-induced aggregation (ristocetin concentration 0.5 mg/ml) was evident in all patients tested. All patients demonstrated a type II vWD profile on analysis of plasma vWF multimers and/or crossed immunoelectrophoresis. Platelet lysate vWF multimers were normal in all the affected individuals tested. These results are all consistent with a diagnosis of type IIB vWD. Washed patient platelets did not aggregate in response to low-dose ristocetin when mixed with normal plasma, thus excluding the possibility of platelet-type vWD in these families.

RLFPl studies. The five RFLPs and the vWF VNTR analyzed in this study are shown in Fig 1. The five conventional RFLPs are all biallelic systems, whereas the VNTR was found to have eight alleles comprising 6 to 14 repeats of the four-nucleotide repeat sequence (VNTR 6–14). All six polymorphic sites are situated in the 3’ half of the vWF gene.

The results of the family studies with these polymorphisms are shown in Fig 2. It can be seen that the type IIB phenotype cosegregates consistently with the vWF RFLP haplotype 1/2/1/1 in family 1. In families 2 and 3 only the VNTR polymorphism was informative.

In family 2, the VNTR allele 8 segregates with the vWD phenotype in the third generation. In the second generation, two affected subjects and seven unaffected individuals have inherited the VNTR 8 allele from the unaffected father (I.2). This raises the possibility of several potential phenomena, including gonadal mosaicism in the father, variable penetrance of the mutation, or the involvement of a locus other than the vWF gene in this kindred. In family 3, the analysis of individual III.1 indicates cosegregation of the IIB phenotype with the VNTR 12 allele.

Although incomplete penetrance and variable expression of the phenotype is well described in type I vWD, there is no evidence of such phenomena in type II disease, where the biochemical defect is a distinct qualitative entity. Therefore, we propose that the findings in family 2 are best explained by the presence of gonadal mosaicism in the first-generation male, and that if this assumption is correct, we can conclude that these families showed cosegregation of the type IIB vWD phenotype to the gene for vWF with a combined logarithm of the odds ratio (LOD score) of 7.2 (0 = 0.0), indicating greater than 10 million to 1 odds in favor of linkage. Even if the results from family 2 are considered to be the consequence of several recombination events in the second generation, the maximum LOD score (2.25 at 0 = 0.14) is still more in favor of linkage to either the vWF gene itself or an adjacent locus, rather than a distant site on the genome. These results provide further independent support for the hypothesis concerning the existence of germline mosaicism in the first-generation male.

Fig 1. (Cont’d). (E) Four of the eight VNTR alleles: VNTR 12–14 and VNTR 10 seen in three heterozygous individuals.

VNTR 14

VNTR 10

Table 1. Phenotypic coagulation profiles for the members of these families are shown.
Fig 2. vWF gene polymorphism data on the three type IIB families studied. In each family only the polymorphic markers for which key individuals were informative are shown. In family 1 (A), the IIB phenotype cosegregates consistently with the polymorphic haplotype 1/2/1/2 from the first-generation affected male (I.1). In family 2 (B), the two affected second-generation males (II.5 and II.12) both inherited the VNTR 8 allele from the unaffected father and the same allele was transmitted to all three of the affected third-generation individuals. In family 3 (C), the only informative data involve the transmission of the VNTR 12 allele to the affected female (III.1).

DISCUSSION

We investigated the genetic linkage of type IIB vWD with the gene for vWF in three families. Using six intragenic polymorphic markers, we have demonstrated that type IIB vWD is linked to the gene for vWF with a significant LOD score of 7.2, assuming that our hypothesis concerning the presence of germline mosaicism in family 2 is correct. Probing with cDNA probes from both ends of the vWF gene has indicated that large deletions, insertions, or rearrangements are unlikely as the cause for vWD in these families. In one of these families we identified a pattern of disease inheritance that, if the disease is linked to the gene for vWF, is consistent with gonadal mosaicism.

vWD is characterized by marked variability in its clinical manifestations and laboratory phenotype, all of which suggests that there is likely an underlying mutational heterogeneity. The type II forms of vWD are all characterized by the absence from plasma of the hemostatically important large molecular weight multimers. Patients with type IIB vWD synthesize a form of vWF that has an increased affinity for the platelet glycoprotein Ib receptor; thus, there is some evidence to suggest that the molecular abnormality in this disorder may be situated in the glycoprotein Ib binding domain of the mature vWF subunit between amino acids 449 and 728 corresponding to the A1 domain.

Phenotypic studies in both types IIA and IIB vWD have demonstrated significant heterogeneity both in clinical expression of the disorder and in tests of the physical properties of the mutant vWF protein. In light of the complex posttranslational processing of this molecule, there has been speculation as to whether the underlying genetic defect in these conditions is consistently in the vWF gene itself or at an alternative genetic locus involved in these modifying events. In types I and III vWD, where a similar diversity of disease is evident, this issue has been addressed by the observation of vWF gene deletions in some type III patients and the cosegregation of vWF RFLPs with types I and III vWD in a small number of families. Similarly, a study using two vWF RFLPs in one large type IIA vWD kindred demonstrated complete linkage of this phenotype with the vWF gene locus (LOD score 5.88). Recently two
adjacent point mutations in the vWF gene sequence corresponding to the A1 protein domain have been reported in a type IIB vWD patient. There also have been point mutations reported in the vWF gene in type IIA vWD and a deletion in an undefined variant form of type II vWD. Thus, the few studies to date indicate that these various forms of vWD are caused by mutations in the gene encoding vWF and not at loci involved in the posttranslational modification of the protein. Nevertheless, in the one type IIB study reported, several possibilities must be considered before the molecular changes identified can be claimed to be the causative mutation. Expression studies of this mutant protein have not been carried out and the issue is complicated by the high rate of polymorphism that appears to exist at this locus, as well as the fact that this region of the gene is duplicated on chromosome 22 as a partial pseudogene sequence. The phenomenon of genetic locus heterogeneity also requires consideration, as has recently been demonstrated in another autosomal-dominant trait (polycystic kidney disease), where the disorder in a specific ethnic population appears to be caused by a mutation at a “variant” locus.

The results of the VNTR polymorphic studies in family 2 raise several additional issues that may further complicate the interpretation of RFLP linkage analysis in vWD. The transmission of the same vWF gene to two affected and seven unaffected children from an unaffected father requires an explanation. Although variable penetrance of vWD has been documented previously in type I disease, there is no evidence to suggest either incomplete penetrance or variable expression of intermediate forms of type II vWD. All members of this kindred have been interviewed and tested for their vWF phenotypic status on three separate occasions and in none of these instances was there clinical or laboratory evidence to suggest vWD in the father and his seven unaffected children. This is in sharp contrast to both the clinical symptomatology and consistently abnormal phenotypic studies in the five affected subjects. The two other explanations that should be considered in this family are that the disorder is segregating with a mutation at a genetic locus other than the vWF gene or that the vWF gene mutation occurred during development of the germ cells in the father, resulting in both normal and mutant vWF genes marked by the VNTR 8 allele. The ratio of unaffected to affected subjects in the second generation of this family supports the existence of the latter phenomenon, that of gonadal mosaicism, and we are currently pursuing more direct methods that would enable confirmation of this hypothesis.

This investigation has shown cosegregation of the type IIB phenotype with several vWF RFLPs in three large families. Despite the fact that the statistical test of linkage between this phenotype and the vWF gene is highly significant, the possibility that some forms of this disorder may be the consequence of mutations at other loci must be kept in mind until more families have been studied. Studies pursuing the mutational basis of type IIB vWD are in progress based on this linkage data and the known abnormality of interaction between type IIB vWF and the platelet glycoprotein Ib.

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