FOCUS ON HEMATOLOGY

Low Platelet $\alpha_2\beta_1$ Levels in Type I von Willebrand Disease Correlate With Impaired Platelet Function in a High Shear Stress System

By Jorge Di Paola, Augusto B. Federici, P.M. Mannucci, Maria T. Canciani, Marcie Kritzik, Thomas J. Kunicki, and Diane Nugent

Platelet adhesion to collagen-coated surfaces in whole blood under flow conditions is mediated by both von Willebrand factor (vWF)-dependent recruitment of the platelet glycoprotein Ib-IX receptor complex and collagen interaction with the integrin $\alpha_{2}\beta_{1}$. In type 1 von Willebrand disease (vWD), platelet adhesive functions are impaired due to the decrease in vWF levels in plasma and platelets. There are at least three alleles of the human $\alpha_2$ gene, distinguishable by a cluster of silent or noncoding sequence differences within a segment of the gene. Two alleles, associated with low receptor density, can be distinguished by nucleotide 807C, while the third allele associated with high receptor density, expresses nucleotide 807T. Gene frequencies of these alleles in a normal population ($n = 167$) are 0.58 for 807C and 0.42 for 807T. We measured the frequencies of these alleles in symptomatic patients with five types of vWD (type 1, $n = 78$; type 2A, $n = 25$; type 2B, $n = 14$; type 2M, $n = 10$; and type 3, $n = 20$). Compared with the normal group, no significant difference in allele frequencies was observed among individuals with types 2A, 2B, 2M, or 3 vWD. However, the frequency of the 807C allele, associated with low collagen receptor density, among type 1 vWD patients ($807C = .71$; $807T = .29$) was significantly higher than that of the normal population ($P = .007$). Also, in patients with vWD type 1 and borderline to normal ristocetin-cofactor (vWF:RCo) activity, collagen receptor density correlates inversely with closure time in a high shear stress system (platelet function analyzer [PFA-100]). We propose that low platelet $\alpha_2\beta_1$ density results in less efficient primary platelet adhesion and may result in increased tendency to bleed, as evidenced by the high frequency of this polymorphism in patients with type 1 vWD compared with normal individuals. In addition, this may account for the variability between patients with similar levels of vWF antigen, but strikingly different bleeding histories.

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Platelet adhesion to collagen in damaged vascular subendothelium is of pivotal importance to both the initiation of platelet thrombus formation and procoagulant activity. This first step in thrombus formation is mediated by both von Willebrand factor (vWF)-dependent recruitment of the glycoprotein Ib-IX receptor complex and collagen interaction with the integrin $\alpha_{2}\beta_{1}$. The integrin, $\alpha_{2}\beta_{1}$, mediates platelet adhesion to both fibrillar (types I, III, and V) or nonfibrillar (types IV, VI, VII, and VIII) collagens. While the levels of the integrins $\alpha_{2}\beta_{1}$ (fibronectin receptor) and $\alpha_{2}\beta_{3}$ (fibrinogen receptor) can vary from one individual to the next, this difference never exceeds a fraction of the mean population level. However, $\alpha_{2}\beta_{1}$ levels can vary up to 10-fold in the general population and this correlates with differences in adhesiveness to type I or type III collagens. This variation in $\alpha_{2}\beta_{1}$ receptor density is associated with differential inheritance of three alleles of the human $\alpha_2$ gene that can be distinguished by multiple sequence differences in selected introns and silent sequence differences in a segment of the coding region.10 Because there is a correlation between platelet adhesion to collagen and $\alpha_{2}\beta_{1}$ receptor levels, differences in $\alpha_{2}\beta_{1}$ receptor levels may influence the risk for thrombosis or bleeding in vivo. While the risk is apparently negligible among healthy individuals, it can become an important factor in individuals who are otherwise predisposed toward thrombosis or bleeding by an unrelated genetic or acquired condition, such as von Willebrand disease (vWD).

Among inherited bleeding disorders, vWD is certainly the most common, with a reported prevalence ranging from 0.8% to 1.3%. The pathogenesis of vWD is based on quantitative and/or qualitative abnormalities of vWF, a large, multimeric protein, encoded by a gene in chromosome 12, which circulates in the blood plasma, but is also stored in granules of endothelial cells and platelets. The most common form of vWD, known as type 1, accounts for more than 70% of patients and is caused by a quantitative deficiency of vWF. Type 1 is very heterogeneous and several subtypes have been described according to platelet vWF content. Moreover, in type 1 vWD, platelet vWF cooperates with plasma vWF to determine the degree of abnormalities in platelet-vessel wall interaction.

Aside from the variation in vWF levels from one patient to the other, it is difficult to explain the significant variation in bleeding tendencies observed between patients with similar laboratory profiles and similar levels of vWF. Thus, genetic and nongenetic factors may augment or attenuate the likelihood of bleeding symptoms in these patients. We postulate that variation in the integrin $\alpha_{2}\beta_{1}$ density on the platelet surface might be a major factor that influences bleeding in patients with type 1 vWD. To test this hypothesis, we determined the $\alpha_2$ genotype in 148 patients with vWD by using restriction digest analysis of genomic DNA. To show correlation between collagen receptor density and platelet function, we also performed platelet adhesion and aggregation studies in the platelet function analyzer (PFA-100, Behring, Dade, FL), a high shear...
Patients. We studied 148 symptomatic patients diagnosed with one of five types of vWD as follows: type 1, n = 78; type 2A, n = 25; type 2B, n = 14; type 2M, n = 11; and type 3, n = 20. The diagnosis of vWD was based on laboratory findings (plasma vWF levels, ristocetin cofactor activity [vWF:RCo], factor VIII coagulant activity, and multimer analysis), family and personal history of bleeding, and a prolonged bleeding time. We also studied 99 age-sex–matched Italians and 68 non-Italian whites without evidence of bleeding disorders and used them as controls. Patient blood samples were collected at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milano, Italy and shipped to Children’s Hospital of Orange County (CHOC), Orange, CA. An additional 15 patients samples were harvested and processed at CHOC. Informed consent was obtained in all cases following institutional guidelines.

Isolation and amplification of patient genomic DNA. Genomic DNA was isolated from peripheral blood mononuclear cells by using the method of Miller et al. The desired segment of the \( \alpha_2 \) gene (581 kb) was amplified by polymerase chain reaction (PCR) using a Perkin Elmer Cetus DNA Thermal Cycler (Norwalk, CT) and Taq polymerase (Pharmacia Biotech, Piscataway, NJ). The primers used in the PCR reaction were: (1) 5'GATTTAATTTTGGGGAAGTTGG 3' and (2) 5'CA TAGGTTTTTGGGGAACAGGTGG 3'. The PCR cycling conditions were: 94°C × 10 minutes, one cycle, followed by 94°C × 1 minute, anneal 1 minute, 72°C × 1 minute, where the annealing was 69°C for two cycles, 67°C for two cycles, and 65° for 35 cycles. The PCR product is a 581-bp segment located within the intron separating two exons that encode the allelic polymorphisms at bp 807 and 873. Genomic DNA cloned from individuals homozygous for 807C or 807T served as positive controls.

Detection and typing of 807T and 807C polymorphisms using Bgl II restriction site. Digestion of the PCR product was performed with the restriction enzyme Bgl II (Pharmacia Biotech). Briefly, for each 40-µL reaction, 15 µL of the PCR product, 4 µL of 10x restriction buffer (One-Phor-All buffer Plus, Pharmacia Biotech), and 1 µL of Bgl II enzyme (× U/µL) were used. The PCR product was digested at 37°C for at least 2 hours and analyzed on a 1.5% agarose gel in 1x tris acetate EDTA (TAE) buffer. The gel was stained with ethidium bromide and visualized under ultraviolet (UV) light.

Platelet function studies. Platelet adhesion and aggregation was evaluated in 32 patients with vWD type 1 in the PFA-100, as previously described by Fressinaud et al. The PFA–100 assay was performed with two different types of cartridges (collagen/epinephrine and collagen/adrenaline diphasphate [ADP]), and values shown represent the mean of duplicate measurements. The statistical analysis of the results was performed using \( t \)-test.

RESULTS

We have previously shown that there are at least three alleles of the human \( \alpha_2 \) gene distinguishable by a cluster of silent and/or noncoding sequence differences within a 4-kb segment of the gene (Fig 1). A naturally occurring restriction site for the enzyme, Bgl II, was found in intron G of the 807T allele.
Thus, PCR products amplified from the 807T allele and spanning the Bgl II site can be digested with Bgl II to generate two fragments of 343 and 238 bp, while product amplified from the 807C allele remains undigested. Bgl II digestion of the 581 bp PCR product amplified from genomic DNA of an individual heterozygous for these alleles yields three fragments (581 bp, 343 bp, 238 bp) (Fig 2). The gene frequencies of the 807C and 807T alleles in the control group (n = 167) were .58 and .42, respectively.

Genomic DNA from 148 individuals diagnosed with vWD was analyzed, and these results were compared with the control group as seen in Table 1. By χ² analysis, it was determined that the frequency of 807C allele associated with low collagen receptor density in type 1 vWD patients is higher than that of the normal population (P = .007), the frequencies of these alleles being .71 for 807C and .29 for 807T. In the type 2M patients, the P value was significant at P = .050, albeit not as strong an association as that seen with type 1 vWD patients. On the other hand, there is not a significant difference in allele frequencies among patients with vWD type 2A (P = .246), 2B (P = .353), or 3 (P = .351), compared with the normal group.

Platelet adhesion was analyzed in 32 patients with vWD type 1 in the PFA-100 and results are shown in Fig 3. At critically low vWF:RCo levels (<30 U/dL), the deficiency of vWF overrides the allele effect on closure time in the PFA-100. When vWF:RCo levels reach values between 40 to 80 U/dL, as seen in many type 1 patients, the effect of the allele on platelet adhesion to collagen is evident. As seen in Fig 3, patients with similar or identical vWF:RCo levels in the 40 to 80 U/dL range, the closure time is significantly longer in those patients who are homozygous for the 807C allele (open circles). By t-test, the differences in closure times between the patients homozygous for the 807C allele and the patients homozygous for the 807T are statistically significant, whether all the subjects with vWF:RCo greater than 40 (P = .018) or just the ones between 40 and 80 (P = .003) are included.

### Table 1. Frequencies of the 807 Alleles in Patients With vWD Type 1, 2A, 2B, 2M, and 3 Compared With the Normal Controls

<table>
<thead>
<tr>
<th>VWD Patients and Controls</th>
<th>No. of Patients (n)</th>
<th>807 CC (low receptor density)</th>
<th>807 TT (high receptor density)</th>
<th>807C/807T (intermediate receptor density)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWD type 1</td>
<td>78</td>
<td>43 (55)</td>
<td>11 (14)</td>
<td>24 (31)</td>
<td>.007</td>
</tr>
<tr>
<td>vWD type 2A</td>
<td>25</td>
<td>14 (56)</td>
<td>1 (4)</td>
<td>13 (52)</td>
<td>.246</td>
</tr>
<tr>
<td>vWD type 2B</td>
<td>14</td>
<td>2 (14)</td>
<td>3 (22)</td>
<td>9 (64)</td>
<td>.353</td>
</tr>
<tr>
<td>vWD type 2M</td>
<td>10</td>
<td>6 (60)</td>
<td>3 (30)</td>
<td>1 (10)</td>
<td>.050</td>
</tr>
<tr>
<td>vWD type 3</td>
<td>20</td>
<td>9 (45)</td>
<td>1 (5)</td>
<td>10 (50)</td>
<td>.351</td>
</tr>
<tr>
<td>Controls</td>
<td>167</td>
<td>57 (34)</td>
<td>31 (19)</td>
<td>79 (47)</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Multiple advances in the molecular characterization, diagnosis, and treatment of vWD have occurred over the last few years. However, it is unclear why certain vWD type 1 patients develop more severe bleeding symptoms than other patients with equivalent vWF levels.21,22 Differences in severity of bleeding can even be detected in individuals with equivalent antigen level in the same immediate family. Our findings suggest that the level of α2β1 on platelets may represent one of the additional factors that determines severity of bleeding in this particular disease.

It is possible that genetic factors directly related to the vWF molecule and its mode of expression could also influence variability of clinical presentation. One such factor is multiple heterozygosity for mutations in the vWF gene.23 Furthermore, there is an association of O blood type with vWF levels in the lower range of normal.24 In addition, D’Alessio et al25 have emphasized the important contribution of platelet vWF to platelet adhesion to collagen in vWD type 1.

Other genetic factors, unrelated to the vWF gene, could also be involved. Our results are consistent with the hypothesis that a low level of the α2β1 receptor may result in delayed primary

![Allele Effect on Adhesion to Collagen](image-url)
platelet adhesion in patients with vWD type 1. Studies have shown that the primary mechanism of platelet attachment to the subendothelium is dependent on GPIb-IX–vWF interactions. A secondary mechanism involving αβ1 binding to the subendothelial collagen also plays an important role.10,26-28 The importance of αβ1 as a primary receptor that mediates platelet adhesion to collagen under flow conditions is underscored by the report of a secondary mechanism involving subendothelium dependent on GPIb-IX–vWF interactions. A study has shown that the primary mechanism of platelet attachment to collagen in patients with vWD type 1. Studies have suggested that the 807C homozygous state may account for the association of the low receptor phenotype in type 1 vWD bleeding. This increased prevalence of the 807C allele is associated with low αβ1 density. As the patients with more bleeding are more likely to be diagnosed, this increased association of the low receptor phenotype in type 1 vWD suggests that the 807C homozygous state may account for the variability in bleeding in vWD. In the PFA-100 test, the αβ1 receptor density in platelet adhesion in vWD type 1 patients who have a vWF:Rco activity in the 40 to 80 U/dL range. In patients with vWD type 1 whose vWF:Rco activity is less than 30 U/dL, the collagen receptor density does not seem to play a major role in closure time due to the fact that these patients’ platelet adhesion is extremely impaired. This may explain why the group of patients with type 3 vWD did not show a significant difference of allele frequencies when compared with the general population.

In most of the cases with type 2 and 3 vWD, a genetic defect has been correlated with moderate to severe bleeding symptoms, while no clear definition of the molecular basis of such defects is available for mild-moderate type 1 vWD. Therefore, additional information about the nature of bleeding in the latter group of patients will be very useful. We are currently performing expanded studies in families with vWD type 1 to study of 34 patients with various subtypes of type 1 vWD bleeding. A study of 34 patients with various subtypes of type 1 vWD bleeding. Br J Haematol 86:327, 1994

ACKNOWLEDGMENT

The authors would like to thank Peggy Nakagawa, MS and Diana Rozenshtein, BS for their excellent technical assistance and Dr Shirley Williams for her help and suggestions.

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Low Platelet $\alpha_\text{IIb}\beta_3$ Levels in Type I von Willebrand Disease Correlate With Impaired Platelet Function in a High Shear Stress System

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