Platelet membrane glycoprotein polymorphisms do not influence the clinical expressivity of von Willebrand disease type I

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Summary

Von Willebrand disease (VWD) is characterized by a significant variation in bleeding symptoms among patients with similar laboratory profiles and equivalent plasma levels of von Willebrand factor (VWF) activities. Considering the recent suggestion that platelet membrane glycoprotein polymorphisms (PltGPs) may play a role as modulators of thromboembolic or haemorrhagic diseases, we investigated the role of different PltGPs and GPVI content in the clinical expression of patients with VWD type I. The diagnosis of VWD (n = 76) was based on laboratory findings (VWF:Ag, VWF:RCo, VWF:CB, FVIII:C, and multimer analysis), family and personal history of bleeding. All patients were interviewed using a standardized questionnaire, and classified into two categories: bleeders (unequivocal bleeding tendency, n = 53) and non bleeders (absence of bleeding symptoms, n = 23). PltGPs, HPA-1, 2 and 5 and C807T of GPIa were determined by fluorophore-labelled hybridization probes on a LightCycler™. GPVI content was measured by western blotting.

VWF:Ag, VWF:RCo, VWF:CB and FVIII:C levels were not significantly different between symptomatic and asymptomatic patients. There were no differences in the genotype distribution and allele frequencies between bleeders and non bleeders for the platelet alloantigen systems HPA-1, 2, 5 and the GPIa C807T polymorphism. The levels of platelet GPVI were similar in symptomatic and asymptomatic VWD patients (109.6 ± 58.4 vs 114.1 ± 52.5, respectively; p: 0.77). These results show that PltGPs HPA-1, 2 and 5 or the C807T dimorphism of GPIa do not influence the clinical expressivity of VWD type I. The wide variation in GPVI content was not associated with the severity of bleeding in the patients. Other genetic factors that may contribute to the variable expressivity of VWD type I should be investigated.

Keywords

don Willebrand disease, platelet glycoproteins, polymorphisms, bleeding diathesis

Introduction

Platelet membrane glycoproteins play a pivotal role in thrombus formation on areas of damaged vessel wall in a process involving several steps. Endothelial damage and/or plaque rupture leads to collagen exposure, von Willebrand factor (VWF) immobilization and the adhesion of circulating platelets to the subendothelial extracellular matrix (ECM). Under conditions of high shear rate the initial tethering of platelets occurs through the interaction of glycoprotein Ib/IX (GPIb/IX) with the A1 domain of VWF molecule. Stable adhesion to ECM requires additional binding of collagen to glycoprotein Ia/IIa (integrin...


α₂β₃) and glycoprotein VI. Platelet-collagen interactions, as well as platelet tethering result in platelet activation, characterized by shape change, release of granule contents, activation of glycoprotein Ib/IIa (GPIb/IIa, integrin α₂β₃) and generation of platelet membrane procoagulant activity.

Most of the genes encoding for platelet membrane glycoproteins contain polymorphisms, which may potentially affect the receptor function of these glycoproteins. In fact, several of these polymorphisms have been associated with increased platelet adhesiveness and aggregation, and some studies have shown that they constitute risk factors for arterial thrombosis (1).

The common variation of GPIIIa, HPA-1, is characterized by a T to C transition at nucleotide 1565, which results in a Pro to Leu substitution at amino acid 33. The Pro³ allele (HPA-1b) has been associated with enhanced adhesive phenotype (2-4), increased thrombin generation (5) and a modest although controversial increased risk for arterial thrombosis (6). Two polymorphisms of GPIb/IX, the variable number tandem repeat and the HPA-2 system, have been associated with increased risk of ischemic cerebrovascular disease (7) and ischemic stroke (8); however, the studies on acute coronary events, have reported controversial results (9, 10). The two major platelet collagen receptors are GPIa/IIa and GPVI. Two silent polymorphisms at nucleotide 807 (T or C) and 873 (A or G) of the GPIa gene correlate with platelet GPIa/IIa density Platelets expressing the 807T allele show increased surface expression of GPIa/IIa and enhanced binding to immobilized collagen under shear stress (11). The 807T allele has been shown to be associated with increased risk of myocardial infarction and stroke, especially in young patients (12, 13), findings not confirmed by other studies (14-16).

Glycoprotein VI is the major signalling receptor for collagen on platelets (17). Deficiency of GPVI is associated with bleeding and a reduction in the response to collagen. A three to five-fold variation in GPVI content has been demonstrated which was in direct correlation with prothrombinase activity (18). Polymorphic variations at the GPVI locus have been recently demonstrated (19, 20) associated with platelet function differences (20) and increased risk of myocardial infarction (19).

Although it has been suggested that platelet membrane glycoprotein polymorphisms may contribute to an increased risk of bleeding or thrombosis, most of the attention has been focused to the prothrombotic phenotypes. However, it may well be that under certain circumstances, genetic variations affecting the structure or level of platelet receptors could play a role in haemorrhagic states. Very few studies have addressed the impact of these polymorphisms in bleeding disorders. Di Paola et al demonstrated that bleeding manifestations were exacerbated in von Willebrand disease (VWD) associated with lower levels of platelet surface GPIa/IIa (21) and Ghosh et al found that Glanzmann’s thrombasthenia patients homozygous for HPA-1b allele had a milder course of the disease (22).

Von Willebrand disease (VWD) is the most common inherited bleeding disorder in humans with a reported prevalence in the general population ranging from 0.8% to 1.3% (23, 24). It is a heterogeneous disease due to quantitative or qualitative abnormalities of VWF. Most of the cases correspond to a partial quantitative deficiency of VWF (type 1 VWD) characterized by autosomal dominant inheritance and generally mild to moderate bleeding. One of the main features of type 1 VWD is its extremely variable penetrance and expressivity with a significant variation in bleeding tendencies among affected individuals (25, 26). From a clinical standpoint it is difficult to understand the wide variation in bleeding symptoms among patients with similar levels of VWF. Thus it is highly possible that other genetic factors may alter the severity of bleeding manifestations in patients with VWD type 1.

Considering the critical role of platelets in the arrest of bleeding by initiating the formation of the haemostatic plug and the exposure of procoagulant activity, we undertook this study to know if different polymorphisms of platelet membrane glycoproteins influence bleeding in patients with VWD type 1.

**Methods**

**Patients and controls**

We studied 76 patients diagnosed with VWD type 1 as part of a prospective study of patients with mucocutaneous hemorrhages. Patients and controls were recruited from different centers in Santiago, Chile, and all of them gave their informed consent to participate. To evaluate the bleeding symptoms they were always interviewed by the same physician using a standardized questionnaire and were subjected to the same study protocol. Each interview with the patient or family lasted at least 15 minutes and was directed to judge objectively the bleeding diathesis. The questionnaire evaluates the presence and severity (scored from 1+ to 4+) of the following symptoms: nosebleeds, easy bruising, gumbleeds, bleeding after tooth extraction, bleeding after any surgery (with special mention to tonsillectomy/adenoidectomy), menorrhagia and bleeding at or after delivery, prolonged bleeding from small wounds, superficial haematomas, muscle and joint bleeding, blood in urinary, digestive or respiratory tracts, and history of fist or second grade relatives with established bleeding disorders or bleeding symptoms. Normal controls were referred by the same physicians, and were recruited among patients undergoing preoperative haemostatic assessment for minor, elective surgery (i.e., hernia, phimosis) or were volunteers from an elementary school. After careful evaluation of the bleeding history, patients were assigned to three categories (1 to 3), unequivocally bleeders, undeterminate history of genuine bleeding diathesis and patients who were definitively non bleeders (asymptomatics). For the purpose of the analysis and association with glycoprotein polymorphisms, we compared bleeders (n= 53) with non
bleeders (n=23). Patients in whom the history was not clear with respect to haemorrhagic symptoms were not considered for the analysis. Patients or controls with concurrent drug intake, other concomitant diseases, acute or chronic infections of any type, platelet count below 130.000/µL or VWD variants were excluded from the study. This prospective study was approved by the Medical Ethics Committee of the School of Medicine, P. Catholic University of Chile.

**Laboratory tests**

Routine haemostatic testing included PT, APTT, TT, clot lysis in saline and urea. Plasma fibrinogen was measured by Clauss assay (Diagnostica Stago, Asnières, France). The forearm BT was performed using a commercial device (Simplate® II and Simplate® Pediatric, Organon Teknika Corp., Durham, NC). The upper normal limit of the method in our laboratory was set at 9.5 min for individuals >7 years old (n = 45) and at 6.5 min for those younger than 7 years old (n = 35). Coagulant activity of factor VIII (FVIII:C) was determined by one-stage, modified APTT assay using FVIII depleted plasma (Dade-Behring Inc., USA). Plasma von Willebrand factor was measured by sandwich-type ELISA, using a capture monoclonal antibody (vW1, kindly provided by Dr. Robert R. Montgomery, Milwaukee, Wisconsin) and a peroxidase-conjugated rabbit antibody for detection (Dako Corp., California). Plasma fibrinogen was measured by Clauss assay (Pharmacia). The developed gel was optically scanned and the content of platelet GPVI was determined in 66 patients (45 bleeders and 21 non bleeders) by a semiquantitative blot assay as described by Furihata et al (18) but using a polyclonal antibody anti-GPVI instead of convulxin. Briefly, platelet proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% slab gel under nonreduced conditions and transferred to a polyvinylidene difluoride membrane. The membrane was blocked, washed and incubated with rabbit polyclonal anti-human GPVI antibody (a kind gift of Kenneth J. Clemetson, Switzerland). After washing, the membrane was incubated for 1 hour with a 1:10 000 dilution of streptavidin conjugated to horseradish peroxidase, washed and detected by enhanced chemiluminescence reagents (ECL, Amersham Pharmacia). The developed gel was optically scanned and the bands corresponding to GPVI were quantified using Image J software (NIH) and expressed as a value relative to a pool of 6 normal platelet lysates.

**Genotyping for platelet glycoprotein polymorphisms**

Genomic DNA was isolated from 5 ml of whole blood using the Wizard Genomic DNA Purification Kit (Promega corporation, Madison, WI) according to the manufacturer’s protocol. Genotyping was performed by polymerase chain reaction (PCR) followed by melting curve analysis with specific fluorescent probes in a LightCycler™ System (Roche). For detection of the HPA-1a/b genotype, the sense and antisense primers for PCR were: 5' –TGCTCAAATGTACGGGTGTTAAC- 3' and 5' –CTGGGACAGTTATCCTTTGAG- 3', respectively (GenBank locus M20311). Following PCR, melting curve analysis was done using a LightCycler Red 640-labeled sensor probe (5'-CTGCTCCTCGGGCTCAAC- 3') and anchor probe, a 29 nm oligonucleotide, labelled at the 3' end with fluoroein (5'–GACTTCTCTTTGGGCTCTCTTACAGG- 3'). For the detection of HPA-2a/b dimorphism, the sense and antisense primers were 5'–GGGCTGGTGTCAGCTGACAA- 3' and 5' –CAGCGGGAGCTGCTCAA- 3', respectively (GenBank locus J02940). The nucleotide sequences of anchor probe and mutation probe for melting curve analysis were 5'–CTGGAGAAGCTAGTCTGGCTAACAACAC- 3' and 5'–CTCTGTAGCAGCCACACCCAA- 3', respectively. To investigate HPA-5a/b polymorphism the following primers were used: 5'–GCTTCTTTGATAGGTCGCCAACT- 3' (sense) and 5'–GGGGATCTCCCTAAAAATGTG- 3' (antisense) (GenBank locus X17033). The nucleotide sequence of the anchor probe and sensor probe were 5'–ACAGGTAGCTTCCTTCCTCCTTTTCT- 3' and 5'–ATTATTATTATTATTATTACCTTTTGTAAG- 3', respectively. Analysis of platelet glycoprotein Ia C807T genotype was performed as described by Morita et al (14). The sequences were 5'–AATGTATGTAGGACACATCC- 3' and 5'–TCTAATTTTTTTTTCCACCTTG- 3' for the sense and antisense primers respectively. The anchor and sensor probes sequences were 5'–GCTGTGTTGAGCTGCCACCATCTG- 3' and 5'–CTGGCATATTTGGCTCAGGAT- 3', respectively.

**Platelet GPVI content**

The content of platelet GPVI was determined in 66 patients (45 bleeders and 21 non bleeders) by a semiquantitative blot assay as described by Furihata et al (18) but using a polyclonal antibody anti-GPVI instead of convulxin. Briefly, platelet proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% slab gel under nonreduced conditions and transferred to a polyvinylidene difluoride membrane. The membrane was blocked, washed and incubated with rabbit polyclonal anti-human GPVI antibody (a kind gift of Kenneth J. Clemetson, Switzerland). After washing, the membrane was incubated for 1 hour with a 1:10 000 dilution of streptavidin conjugated to horseradish peroxidase, washed and detected by enhanced chemiluminescence reagents (ECL, Amersham Pharmacia). The developed gel was optically scanned and the bands corresponding to GPVI were quantified using Image J software (NIH) and expressed as a value relative to a pool of 6 normal platelet lysates.
Statistical analysis

The statistical analysis was performed on a PC by using the software package GraphPad Instat (GraphPad Software Inc.). Differences between patients and controls were assessed by unpaired Student’s t-test or Mann-Whitney test for data with normal or non-normal distribution, respectively. Genotype distribution and allele frequencies were compared by cross-tables using the $\chi^2$ test. Significance level was established at a value of $p < 0.05$.

Results

Among 250 patients and 150 controls enrolled in a prospective study on mucocutaneous haemorrhages, 76 fulfilled the diagnostic criteria for von Willebrand disease type 1 with an appraisable bleeding history. After standardized application of a comprehensive questionnaire on bleeding symptoms, the patients were classified into two categories: a) bleeders (n=53), in whom the assessment of the clinical history, with especial consideration to the presence and severity of symptoms, was unequivocal of a significant bleeding tendency and, b) non bleeders (n=23), in whom abnormal bleeding was absent. Non bleeder VWD patients were found mainly among relatives of index cases and subjects referred for the control group. Demographic information and laboratory findings of the 76 patients and controls included in the study are shown in Table 1. The group of bleeders was slightly older than that of non bleeders ($16.8 \pm 11.1$ vs $10.4 \pm 5.77$ years, respectively; $p=0.01$) and had a predominance of women ($74 \%$ vs $43 \%$; $p=0.018$). No difference between the 2 groups was found in ABO blood group distribution, as well as in plasma levels of VWF:Ag, RCo, CB and F VIII:C. Among the bleeders $45 \%$ had prolonged bleeding time, compared with $21 \%$ of the non bleeder patients ($p=0.046$).

The frequencies of HPA-1, HPA-2, HPA-5 and the GPIa C807T polymorphism genotypes were not significantly different among bleeders, non bleeders and healthy controls (Table 2). Similarly, the allele frequencies of HPA-1, HPA-2, HPA-5 and C807T in the different groups did not differ significantly (data not shown). No allele effect was found for all the systems studied in the PFA-100 system (data not shown).

Platelet GPVI content determined by western blotting confirmed the wide variation among normal individuals. The levels of platelet GPVI among VWD patients showed the same degree of heterogeneity but no significant differences were observed in the relative GPVI values between bleeders and non bleeders ($109.6 \pm 58.4$ vs $114.1 \pm 52.5$, respectively; $p=0.76$) (Fig. 1).

Discussion

Von Willebrand disease is a complex and heterogeneous inherited bleeding disorder. In the last few years a series of important
advances have contributed to a better understanding of the disease, especially its molecular characterization. However, it remains intriguing why VWD type 1 patients with similar levels of VWF present significant variations regarding the frequency and severity of bleeding symptoms. In this study we confirmed such a variation in a group of well characterized VWD type 1 patients. Using a standardized set of questions we classified the patients into those with unequivocally clinical bleeding and those who were essentially asymptomatic. The group of bleeders was slightly older than that of non bleeders and contained a larger proportion of females, probably due to the high frequency of menorrhagia in women with VWD type 1. Moreover, the proportion of individuals with prolonged bleeding time was higher among bleeders, likely reflecting the abnormality in platelet-vessel wall interaction. This observation supports the notion that plasma VWF levels are not the only determinants of the bleeding diathesis in VWD type 1. Despite their notorious clinical differences, the various activities of the VWF:FVIIIc complex were similar in bleeders and non bleeders, suggesting that other non genetic and genetic factors influence the likelihood of haemorrhages in these patients. In this context, it has been suggested that variations in platelet function could exacerbate or attenuate the clinical expression of VWD type 1 (21, 26). As some platelet glycoprotein polymorphisms have been linked to variations in receptor function and to increased risk for arterial thrombosis, we tested if the presence of these polymorphisms modulated the bleeding manifestations in young patients with VWD type 1.

The platelet alloantigen system HPA-1 has been extensively studied in relationship to arterial thrombosis. With respect to bleeding disorders, it has been described that homoizogosity for the b allele was associated with milder clinical course in patients with Glanzmann thrombasthenia (22), but this effect has not been detected in patients with primary intracerebral haemorrhage (29). Our study showed that the genotype distribution and allele frequencies were similar in bleeder and non bleeder VWD patients and in control individuals. The same lack of association was found when HPA-2 and 5 platelet alloantigen systems were evaluated; it should be pointed out that these 2 systems have not been investigated previously in bleeding disorders and have shown a very weak association with arterial thrombosis (1).

The C807T polymorphism of GPIa is significantly correlated with platelet receptor density and variations in platelet deposition on immobilized collagen under shear stress (30). Several studies have shown the association between the inheritance of 807T allele and an increased risk of early-onset arterial thrombosis (12, 13). With regard to bleeding disorders, Di Paola et al found an increased prevalence of the C allele (low platelet αβ1 density) in type 1 VWD patients, suggesting that this polymorphism contributed to disease severity. They also showed a correlation between closure time in a high shear stress system (platelet function analyzer, PFA-100) and αβ1 density in patients with borderline normal ristocetin cofactor (21). We found no significant differences in genotype distribution and allele frequency of C807T polymorphism between bleeder and non bleeder VWD type 1 patients. Moreover, in contrast with the findings of Di Paola the frequencies of genotypes and alleles were also similar between patients and controls (Table 2). This discrepancy may be the result of the difference in disease severity, since in the present study almost all of the patients had a ristocetin cofactor less than 50% which was not the case in the study of Di Paola. In fact, they showed that at very low VWF:RCo levels the allele effect disappears when tested in the PFA-100 system, an observation that was confirmed in the group of 52 patients studied by us with this test.

Platelet glycoprotein VI plays an essential role in the initiation of platelet attachment at sites of vascular injury and it has been proposed as a major determinant of arterial platelet plug formation (31). The platelet GPVI content is highly variable and recently described polymorphic variants may explain this heterogeneity (20). GPVI content may represent a genetic risk factor predisposing to haemorrhagic or thrombotic disorders, since it correlates directly with platelet prothrombinase activity, and alleles associated with reduced expression of GPVI exhibit a reduced response to collagen (18). Moreover, it has recently been demonstrated that allelic variants of GPVI gene correlate with functional differences and total and membrane-expressed GPVI (20). Accordingly, we hypothesized that an increased expression of platelet GPVI would moderate the bleeding symptoms in VWD type 1 patients. When this study was performed, the only way to determine platelet GPVI content was...
the semiquantitative blot assay. Using this technique, we confirmed the great variation in platelet GPVI content; however, the levels found in bleeders and non bleeders were identical. One limitation of this assay could be its relative low sensitivity, considering that small differences in platelet GPVI receptor density may significantly alter the responsiveness to collagen (32). For this reason, these findings should be confirmed with determination of surface density of the receptor and GPVI allele genotypes.

In summary, our results did not provide evidence for a significant role of different platelet glycoprotein polymorphisms in modulating the clinical expression of type 1 von Willebrand disease and other genetic and non genetic factors must be sought to explain its variability.

References


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