Thrombin Generation in Platelet-Poor Plasma Is Normal in Patients with Hereditary Mucocutaneous Haemorrhages

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Key Words
Thrombin generation · Mucocutaneous haemorrhages

Abstract
Mild hereditary bleeding disorders presenting with mucocutaneous haemorrhages are usually difficult to diagnose. We measured thrombin generation in platelet-poor plasma (TG-PPP) in 206 patients with a clinically unequivocal bleeding tendency: 45 with von Willebrand disease (vWD), 49 with platelet aggregation/secretion defects (PASD), 10 with a combination of both and 102 who did not fit the diagnostic criteria for any known haemostatic disorder. TG-PPP was not significantly different from controls in all patient groups, indicating that an abnormality in the plasmatic clotting system is unlikely to contribute to the bleeding in patients with type 1 vWD and PASD. In patients with undiagnosed mild hereditary bleeding disorders, there must be other mechanisms which explain the abnormal haemorrhagic tendency, most likely as yet unrecognized defects in platelet-vessel wall interaction. As a next step we plan to investigate thrombin generation in PRP.

Introduction
Patients presenting with non-thrombocytopenic mucocutaneous haemorrhages (MHC) of a hereditary nature are usually difficult to diagnose. It is widely accepted that most of these patients have a mild disorder of primary haemostasis, which involves the successive interactions of blood platelets with the vessel wall concluding the formation of the platelet plug. However, even after repeated testing, a large proportion of them have no demonstrable alterations in plasma von Willebrand factor (vWF) and ex vivo platelet function, which define the most frequent and best-known diseases of this system [type 1 von Willebrand disease (vWD) and platelet aggregation/secretion defects (PASD)]. In a preliminary retrospective study including 589 patients with this type of bleeding, we could not establish a definitive diagnosis in 56% of them after a first complete clinical and laboratory study \cite{1}. These observations indicate that the pathogenesis of the bleeding disorder in many of these patients is unknown. Furthermore, no diagnostic assays are currently available to explore endothelial or vascular haemostatic function.

A significant proportion of patients with these diseases characteristically exhibit a prolonged bleeding time (BT), commonly considered a hallmark of primary haemostasis...
Patients and Methods

Patients and Controls

This prospective study was approved by the Medical Ethics Committee of the School of Medicine, P. Catholic University of Chile. Patients and controls were recruited from different centres in Santiago, Chile, and all of them gave their informed consent to participate. They were always interviewed by the same physician using a standardized questionnaire and were subjected to the same study protocol. Each interview with the patients or their family lasted at least 15 min and was directed to judge objectively the relevance of the following symptoms: nosebleeds, easy bruising, gum bleeds, bleeding after tooth extraction, bleeding after any surgery (with special mention of 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 first or second grade relatives with established bleeding disorders or bleeding symptoms). All the patients recruited for this study (n = 206, women/men = 128/78, age = 14.4 ± 9.7 years, range 4–45 years) presented with MCH appearing early in childhood; 80 and 20% of them had a first and second grade family history of similar bleeding, respectively. Age- and sex-matched controls were recruited from the same centres by the same physicians for preoperative assessment for minor, elective surgery (i.e., hernia, phimosis). Patients or controls with concurrent drug intake, other concomitant diseases, acute or chronic infections of any type, platelet count below 130,000/μl, vWD variants, severe platelet function disorders (i.e., Glanzmann disease, Bernard-Soulier syndrome), all clinical forms of haemophilia A or B and other clotting factor deficiencies were excluded from the study. Volunteers were allowed to enter the study 1 week or 3 days after intake of aspirin or NSAIDs, respectively. Patients or controls with abnormalities in blood cell count with smear inspection, or in serum biochemical tests including transaminases (ALT/AST), creatinine, albumin and C-reactive protein were also excluded from the analysis.

Laboratory Tests

Routine haemostatic testing included PT, APTT, TT, clot lysis in saline and urea. Plasma fibrinogen was measured by the Clauss assay (Diagnostica Stago, Asnieres, France). The forearm BT was evaluated using a commercial device (SimpleT® IR and SimpleT® Pediatric, Organon Teknika, Durham, N.C., USA). 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 activities of factors VIII (FVIIIc), IX (FIXc) and XI (FXIc) were determined by one-stage, modified APTT assays using plasmas depleted in the respective factors (Dade-Behring, USA). Plasma vWF was measured by a sandwich-type ELISA, using a capture monoclonal antibody (vW1, kindly provided by Dr. Robert R. Montgomery, Milwaukee, Wisc., USA) and a peroxidase-conjugated rabbit antibody for detection (Dako, Carpinteria, Calif., USA). vWF:RiCof was determined by the slope of aggregation of formaldehyde-fixed platelets. vWF collagen binding assay (CBA) was performed as described [8]. Platelet aggregation and 125I-5-HT (serotonin) secretion in platelet-rich plasma (PRP: 2 × 10⁵ platelets/μl) were performed as previously described [9]. Low concentrations of ADP (4 μM), collagen (1 μg/ml) and epinephrine (8 μM), known to elicit primary and secondary waves of aggregation in most healthy subjects, were employed. Higher concentrations of ADP (8 μM), collagen (2 μg/ml) and sodium arachidonate (1 mM) were selected in order to elicit full aggregation. The extent of aggregation was assessed by measuring the maximum change in light transmission, which had been previously observed to closely correlate with the maximum rate of aggregation.

vWD was diagnosed in the patient population when two or more of the following tests were abnormally low: vWF:Ag, RiCof, CBA or...
FVIIIc. Moreover, a laboratory study in which an abnormal prolongation of the BT was associated with low values of one or more of the vWF components (vWF:Ag, RiCof, CBA) was also classified as vWD. Normal values for the vWF/FVIII complex, adjusted for age, sex and ABO type, had previously been established in our laboratory in a school age population (n = 503) [10] and in blood donors (n = 822). PASD was diagnosed when the platelet aggregation or 14C-5-HT secretion was normal with arachidonate and abnormal with the following agonists or their combinations: ADP + epinephrine + collagen, ADP + epinephrine, ADP + collagen, epinephrine + collagen, collagen alone in low and high concentration, or ADP alone in low and high concentration. A defective aggregation/secretion with arachidonate, associated with defects with all the other agonists, was initially considered as evidence of an aspirin, NSAIDs or even some unknown food effect. Patients with this pattern of defects were included only if they had a previous platelet function test, or if a repeated study confirmed these findings. For ADP, collagen and arachidonate, the presence of a reversible platelet aggregation or a suboptimal maximum change in light transmittance determined during 5 min after adding the agonist was considered as evidence of an abnormality. Platelet aggregation with epinephrine was considered defective if only a primary wave of aggregation was observed. For 14C-5-HT secretion, the reaction was stopped at 5 min with saline solution containing EDTA and formaldehyde, and the radioactivity in supernatant PPP was counted. Normal values for each parameter of platelet aggregation and secretion were established in our laboratory with data of 43 healthy individuals.

Thrombin Generation in PPP
TG-PPP was determined by monitoring the continuous production of thrombin measuring the fluorescent split product of the substrate Z-Gly-Gly-Arg AMC (Bachem, Torrance, Calif., USA) in a Fluoroskan Ascent microtiter plate fluorescence reader (Labsys-A

Results
We found that among 206 patients with a clinically unequivocal bleeding tendency, 102 did not fit the diagnostic criteria for any known haemostatic disorder. The remaining 104 were classified as vWD type 1 (n = 45), PASD (n = 49), or a combination of both (n = 10). Patients with vWD, irrespective of their sex, age and blood ABO type, had mean ± SD percentages of vWF:Ag, RiCof, CBA and FVIIIc of 34.6 ± 15.3, 31.1 ± 13.6, 29.8 ± 15.4 and 53.4 ± 26.5%, respectively. Forty patients with PASD presented both aggregation and secretion defects according to the diagnostic criteria described in Patients and Methods, whereas 9/49 had only a defect in platelet 14C-5-HT secretion. The age, sex and number of patients with abnormal BT in each diagnostic category are shown in table 1. In the group of patients with unknown disease, the PT, APTT, clot lysis in saline and urea and concentration of fibrinogen and FVIIIc, FIXc and FXIc were within the normal range (>50%). TG-PPP in controls and patients are shown in figure 2. The area under the thrombin generation curve for each patient and control was divided by the area obtained with the pool of normal plasmas in the same run, and expressed as a ratio. The ratios for controls and vWD type 1, PASD, vWD + PASD and patients with unknown disorders were, respectively, 0.86 ± 0.19, 0.84 ± 0.27, 0.86 ± 0.21, 0.97 ± 0.25 and 0.92 ± 0.28. No significant differences of mean values were observed between the control group and patients in each diagnostic category (ANOVA...
Fig. 2. Endogenous thrombin generation in controls and patients with MCH classified according to diagnostic categories. The ordinate expresses the ratio of the patient (or control) to pool of normal plasmas processed in the same run. A group of 11 patients with clotting factor deficiencies is included, who had a significant decrease (p < 0.0001) in TG-PPP ratio when compared with controls and patients with MCH. No significant differences were detected between controls and patients as well as among the different patient subgroups.

with multiple comparisons of Dunnet). In the group of patients with vWD, FVIIIc was significantly correlated with TG-PPP (r = 0.46, p < 0.0001). Similarly, the mean thrombin generation in patients with an unknown cause of bleeding was not significantly different than that of patients with vWD, PASD or both. Figure 2 also shows that patients with deficiencies of clotting FVIIIc, FIXc and FXIc have a distinct decrease in thrombin generation, which is significantly lower than in controls (p < 0.001) and patients of all groups.

The ratios of TG-PPP presented in figure 2 show values slightly lower than 1, both in controls and in all the patient categories. This can be explained with the fact that the pool of normal plasma used as a standard in each run was obtained from healthy volunteers whose mean age was 37 years, which is significantly older than the mean age of controls and each of the patient groups; it is known that the thrombin generation increases with aging.

<table>
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<tr>
<th>Table 1. Diagnostic categories, age, sex and prolonged BT in patients with familiar, non-thrombocytopenic MCH and controls</th>
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<tr>
<td><strong>Patient number</strong></td>
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<tr>
<td>vWD type 1</td>
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<tr>
<td>PASD</td>
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<tr>
<td>vWD + PASD</td>
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<td>Unknown disease</td>
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<td>Controls</td>
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Figures in parentheses represent percentage.
Discussion

We measured TG-PPP in patients with non-thrombocytopenic MCH of a hereditary nature, excluding patients with clotting factor deficiencies (i.e., mild haemophilias), and compared the results with those of healthy, age- and sex-matched volunteers. Forty percent of the patients of our study presented an abnormally prolonged BT, which, associated with clinical bleeding, is characteristic of defects of primary haemostasis. No significant differences in the type and severity of bleeding were observed in patients with type 1 vWD and/or with mild forms of platelet dysfunction and those without a known diagnosis. In all these groups of patients, TG-PPP was not significantly different from that in controls, irrespective of the etiologic diagnosis. Moreover, we found that PPPs of patients with type 1 vWD and/or with mild forms of platelet dysfunction produce similar amounts of thrombin to those of patients with identical symptoms (MCH appearing early in life with a positive family bleeding history), but without an identifiable cause for them. From this point of view, one major conclusion of our study is that in patients with all these types of disorders of primary haemostasis no abnormality in thrombin production in PPP is involved in the pathogenesis of their haemorrhages.

Considering the existing difficulties and uncertainties in the diagnosis of diseases of primary haemostasis [13, 14], and the relative lack of specificity of individual symptoms [15, 16], as well as that of some laboratory tests [13], we took great care in including patients with unequivocal bleeding symptoms, and controls with an absence of pathological bleeding and normal laboratory tests. Accordingly, our study populations did not include either patients with asymptomatic diseases of primary haemostasis or healthy controls with abnormal haemostatic tests. In this setting, the findings are particularly supportive of our conclusion that an abnormal TG-PPP does not contribute to the presentation of MCH in these patients. On the other hand, it is known that patients with mild haemophilia, who may have a decreased TG-PPP, as well as carriers of haemophilias A and B [17] may present with mild bleeding diathesis, undistinguishable from the MCH of the patients of our study. Taken together, these observations confirm that the same clinical presentation (i.e., non-thrombocytopenic MCH) may originate from different mechanisms.

In patients with type 1 vWD, the main abnormality which could affect the TG-PPP is the low FVIIIc. In patients with this diagnosis, the TG-PPP was normal, and this can be explained by their relatively high levels of FVIIIc (53.4 ± 26.5%). As expected, in this subgroup of patients FVIIIc levels were significantly correlated with TG-PPP. These observations indirectly confirm previous ones indicating that thrombin production in PPP is compromised when FVIIIc levels fall below 25% [18].

It is also known that patients with severe hereditary or drug-induced platelet functional disorders have an abnormally low thrombin production in PRP [4–6]. The exposure of anionic phospholipids in activated platelets is necessary for the assembling of the clotting factor complexes for thrombin generation. In our study, we added exogenous phospholipids for the reaction, excluding the patient platelets from the assay. Thus, it is probable that in patients with mild platelet defects the thrombin generation in PRP could be abnormally low if the activation process of the patient platelets does not result in a sufficiently active procoagulant surface. This is currently being tested in our laboratory.

Thrombin generation in patients with disorders of the clotting system is dependent on the concentration of coagulation proteins, and an endogenous thrombin potential less than 20% of normal is associated with severe bleeding [19]. But, it has also been demonstrated that thrombin generation is highly dependent on the relative concentration of procoagulant proteins and natural inhibitors [7]. Thus, it was conceivable that the concurrence of low normal concentration of one or more clotting factor (unable to affect routine clotting times), with a high concentration of natural anticoagulants (i.e., ATIII), resulted in decreased TG-PPP. Our current results do not have an imbalance of this type which could affect the TG-PPP in patients with MCH of unknown origin, who made up almost 50% of our study population. These results clearly dismiss a possible recommendation to determine this balance in the assessment of patients with this type of bleeding disorder.

The above findings suggest that other factors, independent of TG-PPP, are responsible for the bleeding in this group of patients. We are currently able to detect defects of vWF and platelet function in the platelet-vessel wall interaction, but we do not know how some factors of endothelial origin participate in this interaction at the site of haemorrhages, nor do we have laboratory tests to quantify their contribution. For example, the endothelial lining, locally or systemically, may produce excessive NO or PGI₂, inhibitors of platelet activation, aggregation and secretion, and which prolong the BT in vivo [20–22]. However, the effects of these antiplatelet agents ex vivo are undetectable by the current methods of assessing platelet function, due to their rapid inactivation. We can
also speculate about the existence of a yet unrecognized, mild platelet disorder affecting the formation of coagulation complexes on the platelet membrane and decreasing the generation of thrombin, but not detected by ex vivo aggregation and secretion tests. This possibility is being examined by determining thrombin formation in the presence of patient platelets.

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References