Mechanisms of thrombocytopenia in platelet-type von Willebrand disease

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ABSTRACT

Platelet-type von Willebrand disease (PT-vWD) is an inherited platelet disorder characterized by thrombocytopenia with large platelets caused by gain-of-function variants in GP1BA leading to enhanced GPIba-von Willebrand factor (vWF) interaction. GPIba and vWF play a role in megakaryocytopoiesis, thus we aimed to investigate megakaryocyte differentiation and proplatelet-formation in platelet-type von Willebrand disease using megakaryocytes from a patient carrying the Met239Val variant and from mice carrying the Gly233Val variant. Platelet-type von Willebrand disease megakaryocytes bound vWF at an early differentiation stage and generated proplatelets with a decreased number of enlarged tips compared to control megakaryocytes. Moreover, they formed proplatelets upon contact with collagen, differently from normal megakaryocytes. Similarly, collagen triggered megakaryocytes showed defective activation of the RhoA-MLC2 axis, which prevents proplatelet formation, and increased phosphorylation of Lyn, which acts as a negative regulator of GPVI signaling, thus preventing ectopic proplatelet-formation on collagen. Consistently, human and murine bone marrow contained an increased number of extravascular platelets compared to controls. In addition, platelet survival of mutant mice was shortened compared to control mice, and the administration of desmopressin, raising circulating vWF, caused a marked drop in platelet count. Taken together, these results show for the first time that thrombocytopenia in platelet-type von Willebrand disease is due to the combination of different pathogenic mechanisms, i.e. the formation of a reduced number of platelets by megakaryocytes, the ectopic release of platelets in the bone marrow, and the increased clearance of platelet/vWF complexes.

Introduction

Platelet-type von Willebrand disease (PT-vWD) is an autosomal dominant inherited bleeding disorder caused by gain-of-function variants of GP1BA conferring enhanced affinity for von Willebrand factor (vWF) to platelet integrin GPIba.1 This disorder is characterized by a mild to moderate bleeding phenotype2 associated with fluctuating thrombocytopenia, which is conventionally explained by the formation of vWF-platelet complexes that are rapidly cleared from the circulation.1 Findings in support of this interpretation are the presence of circulating vWF-platelet complexes in a murine model of PT-vWD3 and the increased platelet clearance of mice with type 2B-vWD, a condition in which mutated vWF shows enhanced affinity for GPIba.4 No studies, however, have shown enhanced platelet clearance in either patients or mice with PT-vWD.

On the other hand, GPIba is an important regulator of megakaryocytopoiesis, as shown by defective proplatelet formation (PPF) by megakaryocytes incubated with GPIba-blocking antibodies5 and by megakaryocytes from Bernard-Soulier syndrome patients.6,7 Indeed, GPIba regulates the polarization of the megakaryocyte demarcation membrane system and transendothelial platelet biogenesis through intracellular signals that involve the small GTPases Cdc42/RhoA.8 von Willebrand factor also plays a role in megakaryocytopoiesis, by accelerating and boosting PPF and by increasing platelet production upon megakaryocyte expo-
sure to high shear rates. Megakaryocytes from type 2B-vWD patients form a reduced number of abnormally large proplatelets, explaining the macrothrombocytopenia in PT-vWD. Besides GPIbα, other megakaryocyte receptors for adhesive proteins play an important role in the regulation of PPF. In particular, the interaction of αβ3, and GPPIV with type I collagen inhibits PPF, in this way preventing ectopic platelet release in the bone marrow endostal niche. The interaction of megakaryocyte αβ3, with type I collagen activates the Rho-ROCK pathway which induces the phosphorylation of myosin light chain 2 (MLC2), thus inhibiting PPF, while GPPIV triggers inhibitory signaling mediated by Src family kinases (SKF), a family of kinases acting on an array of downstream effectors, including adaptor, enzyme, and cytoskeletal proteins, that collectively co-ordinate cytoskeletal remodeling.

The loss of physiological suppression of PPF by type I collagen, and consequently the ectopic release of platelets in the bone marrow, have been reported to cause thrombocytopenia in WAS mice, a model of Wiskott-Aldrich syndrome, and in patients with MYH9-RD, two inherited disorders characterized by reduced platelet number.

We studied proplatelet formation using human and murine PT-vWD megakaryocytes and show that vWF is bound to megakaryocyte surface GPIbα at early differentiation stages. We also show that megakaryocytes form a reduced number of large platelets compared to control megakaryocytes. Moreover, suppression of proplatelet formation by type I collagen is impaired and associates with abnormalities of the intracellular signaling pathways triggered by collagen, involved in the suppression of proplatelet formation. An enhanced number of free platelets were consistently observed in the bone marrow of PT-vWD mice. Increased clearance of platelet/vWF complexes is also evident, and it contributes to reduce the platelet count, especially in stress conditions in which circulating vWF levels are increased.

Methods

Throughout the manuscript the terms control and PT-vWD will refer to human megakaryocytes/platelets and the terms TgWT and TgG233V to murine megakaryocytes/platelets.

This study was approved by the ethics committee Comitato Etico Aziende Sanitarie (CEAS) of the Region of Umbria (approval number 2663/15).

Animals

The generation of mice expressing the human GPIbα transgene carrying the G233V variant in homozygous form (TgG233V) and of control mice expressing a wild-type human GPIbα transgene (TgWT) has been previously described. These animals express a human GPIbα transgene and no mouse GPIbα, and both TgG233V and TgWT have been consistently backcrossed with C57BL/6 mice.

Human and murine megakaryocyte cultures

To obtain human megakaryocytes, CD45+ or alternatively CD34+ cells were separated from peripheral blood of a PT-vWD patient carrying the Met239Val mutation (studied on 12 different occasions) and of 15 healthy controls. The obtained cells were then induced to differentiate into megakaryocytes in StemSpan serum free expansion medium (SFEM) supplemented with human recombinant stem cell factor (SCF) (25 ng/mL) and thrombopoietin (TPO) (10 ng/mL) for seven days and TPO alone for the following seven days, as previously described. All subjects gave their informed consent and all studies were carried out according to the principles of the Declaration of Helsinki. Murine megakaryocytes were cultured from bone marrow cells flushed from mouse femurs in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and recombinant murine TPO (10 ng/mL) for four days, as previously described. For details see the Online Supplementary Appendix.

Megakaryocyte spreading and proplatelet formation

Spreading and proplatelet formation in human and murine megakaryocytes were evaluated by immunofluorescence, as previously described. For details see the Online Supplementary Appendix.

von Willebrand factor binding to megakaryocytes

Exogenous vWF was not added to the medium in any of the experiments. vWF binding to human and murine megakaryocytes was assessed by confocal microscopy and by flow cytometry. For details see the Online Supplementary Appendix. vWF secretion by megakaryocytes was measured in cell culture supernatants at days 3, 7 and 14 of cell differentiation using an ELISA kit (Aserachrom VWF:Ag, Stago Italia, Milan, Italy).

Megakaryocyte intracellular signaling triggered by type I collagen

Megakaryocytes were plated for 16 hours in 12-well plates pre-coated with 25 μg/mL of type I collagen or 1% BSA and then lysed in HEPES-glycerol lysis buffer (HEPES 50 mM, 10% glycerol, 1% Triton X-100, MgCl2 1.5 mM, EGTA 1 mM, 1% protease inhibitors). RhoA activity (RhoA-GTP), the phosphorylation of MLCK2, of Src-family kinases (SKF) and of Lyn were assessed by Western blotting. For details see the Online Supplementary Appendix.

Megakaryocyte migration assay

Megakaryocyte migration assay was performed as described in transwell migration chambers (8 μm, Millipore) coated or not with 25 μg/mL type I collagen, and cells were counted by flow cytometry. Results were expressed as chemotaxis index (CI). For details see the Online Supplementary Appendix.

Bone marrow histology

The VA collection of platelets was carried out in ten sections of human bone marrow from the PT-vWD patient, from three patients with immune thrombocytopenia (ITP), and from three controls and ten sections of murine bone marrow from femurs and tibiae of 3 TgWT and 3 TgG233V mice. For details see the Online Supplementary Appendix.

Measurement of platelet life span in mice

Mice were injected intravenously with 0.5 μg/g body weight of an anti-GPIX mAb (Emfit Analytics, Eibelstadt, Germany) conjugated with DyLight 488 (Life Technologies, Italia) and the percentage of residual fluorescent platelets was assessed for five days by flow cytometry as described. Blood was obtained from tail tips to cuts in tubes containing 4% sodium citrate. The same experiments were repeated by administering to mice desmopressin (DDAVP) (0.3 μg/kg) by subcutaneous injection immediately after intravenous injection of the DyLight 488-conju-
gated anti-GPIX mAb. For details see the Online Supplementary Appendix.

von Willebrand factor-bound circulating platelets

Blood smears were prepared from five different samples of EDTA-anticoagulated human or mouse blood from cardiac puncture and analyzed by fluorescence microscopy, as previously described.\textsuperscript{21,22} For details see the Online Supplementary Appendix.

Statistical analysis

Data are presented as means±Standard Error of Mean (SEM). The \textit{t}-test for unpaired data was used to analyze results. \( P<0.05 \) was considered statistically significant.

Results

Platelet-type von Willebrand disease patient

The characteristics of the PT-vWD patient carrying the Met239Val variant in this study have been reported previously.\textsuperscript{20} Platelet count was mildly and variably reduced (41-168x10\(^9\)/L) and platelet volume mildly increased (15.5 fl., normal: 8.0-12.0 fl.), with 94\% normal, 5\% large, and 1\% giant platelets (normal: 95-100\%; 0-4\% and 0-1\%, respectively).\textsuperscript{29} Similar to the platelet count, the percentage of reticulated platelets was highly heterogeneous, ranging from 5.7\% to 23.6\% (normal values 7-10\%). The lowest platelet counts (41 and 80x10\(^9\)/L) and the highest

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Figure 1. von Willebrand factor (vWF) binding to megakaryocytes. (A) vWF binding to human megakaryocytes at different days of culture as assessed by confocal microscopy. vWF binding was analyzed by confocal microscopy of megakaryocytes cytospun on glass coverslips. vWF is stained green (Alexa Fluor® 488 Goat Anti-Rabbit IgG), CD42b is stained red (Alexa Fluor® 568 Donkey Anti-mouse IgG), and nuclei are stained blue with Hoechst. Samples were mounted using the ProLong Antifade medium (Molecular Probes) and analyzed at room temperature using a TCS SP II confocal laser system equipped with a DM IRBE inverted microscope and a 40x oil NA objective (Leica, Bensheim, Germany). Representative merging images are shown. Surface localization of vWF was established by analyzing the intensity of the fluorescence signal along the x-axis on the optical section for each fluorochrome using Image J software. Scale bars=20 \( \mu \)m. P: PT-vWD megakaryocytes; C: control megakaryocytes. Representative images are of megakaryocytes from three controls and of three different preparations from the PT-vWD patient. (B) Binding of vWF (mean fluorescence intensity, MFI) to human resting control and PT-vWD megakaryocytes at days 7, 10 and 14 of differentiation, as assessed by flow cytometry. Data represent mean±SEM for five repeated measures (*\( P<0.05 \) vs. control). Raw FACS data are shown in Online Supplementary Figure S11A. (C) Binding of vWF to murine megakaryocytes from Tg\(^{WT}\) and Tg\(^{G233V}\) mice at day 4 of differentiation induced by increasing doses of ristocetin, as assessed by flow cytometry. Data represent mean±SEM for five repeated measures (*\( P<0.05 \) vs. 0). Raw FACS data are shown in Online Supplementary Figure S11B.
von Willebrand factor binding to megakaryocytes

von Willebrand factor bound to the surface of human PT-vWD megakaryocytes, co-localizing with GPIbα, was detected by confocal microscopy (Figure 1A) and flow cytometry (Figure 1B) on days 7, 10 and 14 of cell differentiation, while control megakaryocytes did not show bound vWF at any of the differentiation times. vWF did not co-localize with GPIbα intracellularly (Online Supplementary Figure S2). vWF was secreted from megakaryocytes starting from day 7 until day 14 of differentiation (Online Supplementary Figure S3A).

von Willebrand factor was not detected on the surface of megakaryocytes from TgG233V mice (Online Supplementary Figure S3B), in line with previous observations.19 It cannot be excluded that vWF is bound to murine mutant megakaryocytes in vivo but that the quantity is too low to be detected by immunofluorescence, or that vWF bound to the megakaryocyte surface is lost during murine megakaryocyte isolation, culture and analysis. However, megakaryocytes from TgG233V mice bound vWF after stimulation with a lower dose of ristocetin compared to megakaryocytes from control mice, confirming increased affinity for vWF (Figure 1C).

MegaKaryocyte differentiation, spreading, proplatelet formation and migration

The percentage of peripheral-blood derived CD45+/CD34+ cells differentiating in megakaryocytes was comparable in PT-vWD and controls (PT-vWD 37.3±7.2%, controls 41.2±9.3%), with a slightly but significantly lower percentage of PT-vWD megakaryocytes reaching stage IV of differentiation (Figure 2A). Transmission electron microscopy of PT-vWD megakaryocytes did not show ultrastructural abnormalities (Online Supplementary Figure S4).

The fraction of megakaryocytes spreading on immobilized fibrinogen, vWF or type I collagen was similar in PT-vWD and controls (Online Supplementary Figure S5A and B). The fraction of megakaryocytes generating proplatelets, either in suspension or on fibrinogen- or VWF-coated coverslips, was also comparable in PT-vWD and controls.

Figure 2. Proplatelet formation from human megakaryocytes. (A) Megakaryocyte percentage and maturation. The percentage of CD41+ cells at day 14 of culture was measured by flow cytometry. Maturation of megakaryocytes was determined by fluorescence microscopy based on ploidy, cell diameter, and CD41 expression. For each sample, at least 100 megakaryocytes were evaluated. Data represent means±Standard Deviation (SD) for five controls and for the platelet-von Willebrand disease (PT-vWD) patient on five different occasions; *P<0.05 vs. control. (B) Percentage of megakaryocytes-extending proplatelets in suspension, or onto glass coverslips coated with fibrinogen (FBG), vWF or type I collagen (Coll I). Data represent means±Standard Error of Mean (SEM) of five controls and of five different preparations from the PT-vWD patient (*P<0.05 vs. control). (C) Representative images of control and PT-vWD megakaryocytes plated on type I collagen. Scale bars=20 μm. β-tubulin is stained green (Alexa Fluor® 488 Goat Anti-Rabbit IgG; Molecular Probes, Life Technologies, Milan, Italy), polymerized actin is stained red (rhodamine-phalloidine; Molecular Probes), and nuclei are stained blue with Hoechst. Specimens were mounted with the ProLong Antifade medium (Molecular Probes), analyzed at room temperature with a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Inc., Oberkochen, Germany) using a 63x/1.4 Plan-Apochromat oil-immersion objective and images acquired using the AxioVision software (Carl Zeiss Inc.). All polynucleated cells extending protrusions with terminal tips were defined as proplatelet-forming megakaryocytes while those displaying a flattened shape with actin organized into focal adhesion points and fibers as spreading megakaryocytes. Scale bars=20 μm. (D) Number of proplatelet tips generated by megakaryocytes. Individual data, means and 95% Confidence Interval (95%CI) are shown (*P<0.05 vs. control). Measures were carried out on megakaryocytes from five controls and five different preparations from the PT-vWD patient. (E) Diameter of proplatelet tips generated by megakaryocytes. Individual data, means and 95%CI are shown (*P<0.05 vs. control). Measures were carried out on megakaryocytes from five controls and five different preparations from the PT-vWD patient. (F) Percentage of control megakaryocytes-extending proplatelets of type I collagen (Coll I) under resting conditions or after incubation with 1.5 mg/mL of ristocetin. Data represent means±SEM of five different experiments (ASTe<0.05 vs. resting). (G) Migration of megakaryocytes through transwell filters uncoated or coated with type I collagen in response to SDF-1α (100 ng/mL). Chemotaxis index (CI) expresses the number of cells that have passed through the filter in response to SDF-1α divided by the number of cells passed in the absence of SDF-1α (n=4; *P<0.05 vs. control). Measures were carried out on megakaryocytes from four controls and four different preparations from the PT-vWD patient.
PT-vWD megakaryocytes extended very long and branched proplatelets on fibrinogen and vWF, probably due to the boosting role of bound vWF on proplatelet formation.\textsuperscript{9-11} However, the number of proplatelet tips was reduced and tip diameter was larger in PT-vWD megakaryocytes. In particular, we identified a subset of cells producing a number of tips below the 95% Confidence Interval (95%CI) of controls, and which had tips of a diameter above the 95%CI of controls (Figure 2C and D). Moreover, a significantly higher number of PT-vWD megakaryocytes extended proplatelets when plated on type I collagen, compared to control megakaryocytes (Figure 2B and E). Comparable results were obtained using mouse Tg\textsuperscript{Camly} megakaryocytes (Online Supplementary Figure S6).

In order to exclude the possibility that defects of the col-

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**Figure 3. Collagen-triggered signaling in human megakaryocytes.** (A) RhoA activation (RhoA-GTP) of megakaryocytes in suspension (-) or after 16 hours (h) of adhesion to type I collagen (+). Densitometric analysis was performed using the Image J software. Quantification of RhoA-GTP is relative to total RhoA expression and is expressed in arbitrary units (AU) (n=5; #Significantly different from control on type I collagen; \( P < 0.05 \)). (B) MLC-2 phosphorylation (pMLC-2) of megakaryocytes in suspension (-) or after 16 h of adhesion to type I collagen (+). Densitometric analysis was performed using Image J software. Quantification of pMLC-2 is relative to total MLC-2 expression and is expressed in arbitrary units (AU) (n=5; #Significantly decreased vs. control on type I collagen; \( P < 0.05 \)). (C) SFK phosphorylation (p-Src Tyr416) in megakaryocytes in suspension (-) or after 16 h of adhesion to type I collagen (+). Densitometric analysis was performed using Image J software. Quantification of p-SFK is relative to total SFK expression and is expressed in arbitrary units (AU) (n=5; *Significantly increased vs. control in suspension; \( P < 0.05 \)). (D) Lyn phosphorylation (p-Lyn) in megakaryocytes in suspension (-) or after 16 h of adhesion to type I collagen (+). Lyn was immunoprecipitated and western blotting was carried out using the anti-SFK antibody. Densitometric analysis was performed using Image J software. Quantification of P-Lyn is relative to total Lyn expression and is expressed in arbitrary units (AU) (n=5; *Significantly different from resting control; \( P < 0.05 \)). Blots are representative of megakaryocytes from five controls and five different preparations from the platelet-type von Willebrand disease patient.
lagen receptors were responsible for this peculiar phenotype, we sequenced the GP6, ITGA2, and ITGB1 genes in our PT-vWD patient. However, we did not find any rare pathogenic variant or any common genetic variant associated with decreased response to collagen. In addition, we assessed the expression of αβ, by flow cytometry, which was also normal (Online Supplementary Table S1).

To assess whether the constitutive binding of vWF to GPIba was directly responsible for the increased PPF on type I collagen, we incubated control human megakaryocytes with ristocetin (1.5 mg/mL) to induce vWF-GPIba binding, and evaluated their PPF on type I collagen. Control megakaryocytes incubated with ristocetin showed increased PPF on type I collagen, similar to PT-vWD megakaryocytes (Figure 2F), suggesting that the binding of vWF triggers proplatelet formation on collagen.

A significantly higher percentage of human PT-vWD megakaryocytes migrated through transwells coated with type I collagen in response to SDF1-α. In contrast, migration through uncoated transwells, compared to control human megakaryocytes (Figure 2G) or through transwells coated with vWF was normal (data not shown), confirming an altered interaction of PT-vWD megakaryocytes with type I collagen and not a generalized abnormality of migration.

Megakaryocyte intracellular signaling triggered by type I collagen

RhoA activation (RhoA-GTP formation) triggered by adhesion to collagen was lower in PT-vWD compared to control megakaryocytes (Figure 3A). Consistently, collagen-induced MLC2 phosphorylation, which depends on RhoA activation, was also impaired in PT-vWD megakaryocytes (Figure 3B). On the contrary, SFK phosphorylation was significantly higher in resting PT-vWD than in control megakaryocytes but, differently from control megakaryocytes, it did not increase further after adhesion to collagen (Figure 3C). Lyn showed the same activation pattern as SFK (Figure 3D). Comparable results were obtained using mouse TgWT and TgG233V megakaryocytes.
Interestingly, control megakaryocytes cultured in suspension showed phosphorylation of Lyn when incubated with ristocetin, thus confirming that the binding of vWF to GPIbα triggers Lyn phosphorylation (Online Supplementary Figure S8A). Finally, Cofilin, a protein that is phosphorylated by activated RhoA, was less phosphorylated upon adhesion to collagen in PT-vWD than in control megakaryocytes (Online Supplementary Figure S8B).

Platelets in bone marrow
Immunohistochemistry showed an increased number of platelets in the bone marrow of our PT-vWD patient compared to healthy controls (Figure 4A). In order to exclude the possibility that the increased number of platelets was simply due to the enhanced number of megakaryocytes in bone marrow (Online Supplementary Figure S1), we counted platelets in bone marrow from patients with ITP who also show increased megakaryocytes in bone marrow, but here the number of platelets was comparable to controls (Online Supplementary Figure S9A). Similarly, bone marrow from TgG233V mice showed an increased number of platelets compared to bone marrow from TgWT mice (Figure 4B). The ratio between bone marrow platelets and circulating platelets, as assessed by flow cytometry, was also significantly increased in TgG233V mice (Online Supplementary Figure S9B).

Platelet life span and platelet-bound vWF
The number of circulating platelets was 572.3±32.6x10^9/L in TgWT mice and 180.1±47.2x10^9/L in TgG233V mice. TgG233V mice showed a reduced platelet half-life (26 hours) compared to TgWT mice (47 h). A significantly lower percentage of DyLight 488-stained platelets was observed already 24 h after fluorescent anti-GPIX mAb injection, and the difference became even more evident 48 h after, when residual DyLight 488-stained platelets in blood were 12% in TgG233V and 46% in TgWT mice (Figure 4C). Moreover, 71.5±12.2% and 83.2±14.6% of circulating human and murine PT-vWD platelets showed surface-bound vWF, respectively, while no vWF could be detected on the platelet surface in controls. vWF bridged-platelet aggregates were also seen in PT-vWD, and not observed in controls (Figure 4D).

These data suggest that vWF/platelet complexes circulating in blood in PT-vWD patients are rapidly cleared from the circulation, thus reducing platelet lifespan. Indeed, the administration of DDAVP increased plasma vWF both in TgWT and TgG233V mice (+97±21% and +93±12%, respectively), but it caused a drastic reduction of platelet count only in TgG233V mice (Figure 5A). The percentage of circulating DyLight 488-stained platelets was reduced by 30% in TgG233V mice 60 min after DDAVP injection while it did not vary significantly in TgWT mice, confirming that vWF-bound platelets in PT-vWD are rapidly cleared from the circulation (Figure 5B). PT-vWD platelets did not show increased exposure of phosphatidylserine under resting conditions (% of Annexin V-positive platelets: PT-VWD, 4.1±0.6%, controls, 4.6±2.7%). From the comparative quantitative evaluation of our studies, it emerges that ectopic proplatelet formation is the mechanism that contributes the most to thrombocytopenia in PT-vWD (Online Supplementary Table S2).
Discussion

The formation of proplatelets by megakaryocytes is tightly regulated by the interaction with bone marrow matrix proteins. In fact, several molecules, such as type I-collagen, fibrinogen, fibronectin or vWF, support platelet biogenesis while type I-collagen inhibits it, thus preventing the premature release of platelets in the bone marrow.\(^{31}\) Defective PPF as a cause of inherited thrombocytopenias has been reported in Bernard-Soulier syndrome\(^ {67,68}\) and in type 2B-vWD,\(^ {69,70}\) but so far no studies have assessed PPF in PT-vWD.

Here we show that vWF binds to PT-vWD megakaryocytes at early stages of differentiation, causing a dysregulation of downstream signaling. This disturbs the suppression of PPF by type-I collagen, leading to the ectopic release of platelets in the bone marrow. Moreover, we show that megakaryocytes form a reduced number of large platelets, and that circulating PT-vWD platelets have vWF bound to their surface. We also observed that this leads to their clearance, especially when circulating levels of vWF increase. These mechanisms contribute to generate the thrombocytopenia typical of this disorder.

Differently from control megakaryocytes, which bind vWF only during PPF, PT-vWD megakaryocytes bind vWF at very early stages of differentiation. We show that, in vitro, differentiating megakaryocytes synthesize vWF and secrete it in the supernatant, with subsequent binding to mutant GPIbα in PT-vWD. It is conceivable that this happens in vivo in the bone marrow, too.

Platelet-type von Willebrand disease megakaryocytes developed proplatelets with a reduced number of tips and slightly enlarged with respect to controls, in line with the mildly increased diameter of circulating platelets seen in patients.\(^ {31}\) PPF requires a finely regulated cytoskeletal remodeling,\(^ {2,20}\) and, given that GPIbα interacts with several cytoskeletal proteins, it is likely that mutated GPIbα leads to cytoskeletal perturbation with the formation of enlarged proplatelets.

Platelet-type von Willebrand disease megakaryocytes developed proplatelets on type I collagen, differently from control megakaryocytes. This abnormal behavior was reproduced with control megakaryocytes when vWF-GPIbα binding was induced by ristocetin.

Platelet-type von Willebrand disease megakaryocytes also migrated through type I collagen-coated transwells more than controls, further showing a deranged interaction with collagen. A recent study showed normal response to type I collagen of platelets form Tg\(^ {233}\) mice.\(^ {32}\) However, in our experimental conditions, collagen was used as a substrate for adhesion of megakaryocytes and not in suspension as inducer of platelet aggregation, and this may account for the difference.

We did not detect defects of the collagen receptors, rare or common genetic variants of GPβ, ITGA2 and ITGB1 by flow cytometry or by sequencing. Therefore, we investigated the signaling triggered by the interaction of megakaryocyte αβ, with collagen.\(^ {11}\) We found decreased RhoA activation (RhoA-GTP) and MLC2 phosphorylation, showing impaired αβ-mediated signaling. Interestingly, a dysregulation of the RhoA pathway has been recently shown in type 2B-vWD, a condition associated with enhanced vWF-GPIbα interaction.\(^ {12}\) We also assessed SFK phosphorylation, given that GPV1-dependent SFK signaling inhibits PPF,\(^ {13}\) and found it to be increased in resting PT-vWD megakaryocyte.
References


