

Theme Issue Article

Increased shear stress- and ristocetin-induced binding of von Willebrand factor to platelets in cord compared with adult plasma

Thomas Rehak^{1,2}, Gerhard Cvirn³, Siegfried Gallistl^{1,2}, Bettina Leschnik¹, Martin Köstenberger^{1,2}, Helga Katzer⁴, Volker Ribitsch⁴, Wolfgang Muntean^{1,2}

¹Ludwig Boltzmann Research Institute for Pediatric Hemostasis and Thrombosis, Graz, Austria

²Department of Pediatrics, Medical University of Graz, Graz, Austria

³Institute of Medical Chemistry and Pregl Laboratory, Medical University of Graz, Graz, Austria

⁴Institute of Chemistry, Colloid Science and Rheology, Karl Franzens University of Graz, Graz, Austria

Summary

Multiple indications do exist that the extensive neonatal platelet adhesion and aggregation, and the shorter closure time of neonatal compared with adult whole blood in the platelet function analyzer 100 are attributable to the physiological high plasma concentrations and high concentrations of unusually large von Willebrand factor (vWf) multimers in neonates. However, to date the direct experimental evidence is lacking. Therefore, we compared in the present study the ability of neonatal vWf to bind to platelets to that of adult vWf. Platelet-poor plasma of neonatal or adult origin, containing antibody-stained vWf, was incubated with neonatal or adult platelet suspension. Subsequently, vWf-platelet interaction was induced by exposing the mixture to shear stress by means of a cone/plate measuring system or by incubating the mixture with

ristocetin. Finally, samples were analyzed in a FACScan flow cytometer. Detected fluorescence intensities directly correlate with the amount of vWf attached to the platelet surface. We found that significantly higher amounts of neonatal vWf were attached to platelets in the presence of shear stress or ristocetin. This efficient neonatal vWf-platelet interaction is an effect intrinsic to the neonatal vWf, and not to the neonatal platelet: the amount of neonatal vWf attached to neonatal platelets was not different from the amount of neonatal vWf attached to adult platelets. Furthermore, decreasing the vWf content in cord plasma to adult level resulted in significantly suppressed vWf-platelet attachment in the presence of ristocetin, indicating that the high neonatal vWf level contributes to the efficient vWf-platelet binding in neonates.

Keywords

Neonatal and adult platelets, von Willebrand factor, flow cytometry, shear stress

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Introduction

There is an apparent contrast between an *in-vitro* hypofunctional state of neonatal platelets (1-6) and evidence of normal or even short *in-vivo* bleeding time of the newborn (7), the traditional *in-vivo* test for assessing primary hemostasis (8).

However, all the *in-vitro* studies revealing low activity of neonatal platelets were performed under static or flow shear-free conditions using either platelet aggregometry, biochemical assays of the release reactions, or FACS analysis. Shenkman et al. designed a study more compatible with the physiological milieu (9): citrated neonatal and adult whole bloods were sub-

Correspondence to:
Prof. Dr. Wolfgang Muntean
Ludwig Boltzmann Research Institute for Pediatric Hemostasis and Thrombosis at
the Department of Pediatrics
Medical University of Graz
Auenbruggerplatz 30
A-8036 Graz, Austria
Tel.: +43 316 385 2609, Fax: +43 316 385 2619
E-mail: wolfgang.muntean@meduni-graz.at

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jected to shear stress on subendothelial extracellular matrix (ECM)-coated wells in a cone and plate(let) analyzer. In contrast to the previous findings, neonatal platelets demonstrated more extensive adhesion and aggregation on EMC than adult platelets. The authors suggested that the extensive neonatal platelet deposition on EMC is mediated by plasma von Willebrand factor (vWf), which is known to be present in neonatal plasma at higher concentrations and in a more multimerized state than in adult plasma, and, therefore, is probably more active in neonates than in adults (10-13). Furthermore, studies utilizing the Platelet Function Analyzer (PFA 100, Dade Behring, Miami, FL, USA) also demonstrate a well-functioning primary hemostasis in neonates. The PFA 100 measures the "closure time (CT)", an *ex-vivo* surrogate of the *in-vivo* bleeding time (14). Detected shorter CTs in cord compared to adult blood were also attributed to vWf since addition of anti-vWf antibody prolonged the PFA 100 CT (15).

Multiple indications do exist that high plasma concentrations and high concentrations of unusually large vWf multimers in neonates lead to efficient vWf-platelet interaction, resulting in extensive neonatal platelet adhesion and aggregation and in short PFA 100 CTs. However, to date the direct experimental evidence for this suggested high neonatal vWf-platelet interaction is lacking. Therefore, our study was performed to examine the supposed high ability of neonatal vWf to interact with platelets in the presence of a shear field or ristocetin in order to quantitate the amount of vWf attached to the platelet membrane.

Material and methods

Both reconstituted cord and adult plasmas were exposed to shear stress by means of a rotational viscometer or were incubated with increasing amounts of ristocetin, to induce conformational change in the vWf molecule that occurs *in-vivo* when circulating vWf is attached to collagen at the site of injury, enabling vWf to bind to the GPIb part of the GPIb-V-IX receptor on the platelet membrane. Subsequently, the amounts of vWf attached to the platelet surface were comparatively evaluated by means of a FACS analyzer using a primary anti-vWf antibody and a secondary fluorescein isothiocyanate (FITC) IgG antibody.

Reagents

FITC-conjugated mouse IgG1 control was from Becton Dickinson Biosciences, San Jose, CA, USA. FITC-conjugated monoclonal antibody anti-CD42b directed against GPIb was from Immunotech, Marseille, France. Monoclonal mouse anti-human vWf antibody M0616 (clone F8/86) and FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins were from DAKO A/S, Glostrup, Denmark. For washing procedures PBS containing 2% bovine serum albumin (Sigma, St. Louis, USA), in case of staining PBS containing 2% bovine

serum albumin and 0,1% NaN₃ was used. The fibrin polymerization inhibitor H-Gly-Pro-Arg-Pro-OH (GPRP; Pefabloc™ FG) was purchased from Pentapharm LDT (Basel, Switzerland).

Blood sampling

Cord blood was obtained immediately following delivery of 18 full term infants (39-42 weeks gestational age). Newborns with Apgar scores of 9 or less five minutes after delivery were excluded from the study. Blood (2.7 ml) was collected into pre-citrated S-Monovette™ pre-marked tubes from Sarstedt, Nümbrecht, Germany, containing 300µl 0.106 M citrate. The hematocrit of cord blood was slightly, but not significantly, elevated over adult values. Additionally, blood was taken from the antecubital vein of 16 healthy adults, not having ingested medications known to affect platelet function. Platelet-rich plasma (PRP) was prepared by centrifuging citrated whole cord and adult blood at room temperature for 10 min at 1300 rpm, respectively. Subsequently, PRP was centrifuged at room temperature for 10 min at 2300 rpm and the upper phase (platelet-free plasma, PPP) was removed, pooled and stored at -70°C until assayed. The remaining platelet pellet was resuspended in 2 ml phosphate buffered saline (PBS) containing 2% bovine serum albumin and centrifuged at room temperature for 10 min at 2000 rpm. Platelets were washed twice in this way. Platelet counts were determined on a Sysmex KX-21N Automated Hematology Analyzer (Sysmex, Illinois, USA) and platelet suspensions were adjusted to contain 50 000 cells/µl.

Measurements of vWf antigen

Plasma vWf:Ag was measured by an enzyme immunoassay using rabbit anti-human vWf A0082 (DAKO, Denmark) for capture and peroxidase-conjugated rabbit anti-human vWf P0226 (DAKO, Denmark) for detection. The concentration of vWf was calculated from a standard curve obtained with pooled, normal plasma.

Analysis of the multimeric structures of neonatal and adult vWf

The multimeric structure of vWf was evaluated by SDS-argarose gel electrophoresis on a low-resolution gel system (0.8% low-gelling-temperature argarose, 16).

Determination of the GPIb-IX complex expression on the platelet surface

Platelet suspension (50 000 cells/µl, from neonatal or adult origin) was incubated with fluorescent antibody directed against GPIb for 20 min at room temperature in the dark. Subsequently, samples were fixed in 2% paraformaldehyde solution and analyzed within 24 h in a FACScan™ flow cytometer (Becton-Dickinson, New Jersey, USA, 17).

VWf-staining

PPP (from neonatal or adult origin, diluted 1:10 in PBS containing 0.1% NaN_3) was incubated at a concentration of 4.6 $\mu\text{g}/\text{ml}$ for 30 min with mouse-anti-human-vWf antibody (DAKO) in the dark, and, subsequently, in the same way with a FITC-conjugated rabbit-anti-mouse antibody (DAKO).

Attachment of vWf to platelets

To 40 μl of neonatal or adult platelet suspension, 50 μl PPP (from neonatal or adult origin) containing the antibody-stained vWf were added (reconstituted plasma, 3). Subsequently, vWf-platelet interaction was induced by exposing the mixture to shear stress by means of a cone/plate measuring system (9) or by incubating the mixture for 10 min at room temperature with increasing amounts of ristocetin (0.3, 0.4, and 0.7 mg/ml final concentrations).

Subjection of shear stress to platelets

Both neonatal and adult platelets were exposed to shear stress by incubating the washed platelets with PPP and subsequent application of 500 μl of the mixture to the cone/plate measuring system UDS 200 from Paar Physica, Graz, Austria in the presence of 1 mg/mL GPRP. Platelets were subjected to shear at rates of 500, 750, and 1000 s^{-1} , respectively, at 37°C for 5 min using a rotating metal cone (3.75 cm radius, 2° angle).

Flow cytometric analysis

Following attachment of vWf to platelets, samples were fixed in 2% paraformaldehyde solution and analyzed within 24 h in a FACScan™ flow cytometer (Becton-Dickinson). Detected fluorescence intensities directly correlate with the amount of fluorescent antibody, and, thus, with the amount of vWf attached to the platelet surface (18). Fluorescent isotype-matched antibodies were used as negative controls. GeoMean of fluorescence intensity was taken for further statistical analysis.

Statistical analysis

Results obtained in cord and adult plasma were compared by means of Mann-Whitney U-test. The effects of increasing amounts of ristocetin on fluorescence intensities were analyzed using paired t-test. The significance level of p-values was set at 5%. Calculations were performed using WINSTAT 3.1 (Kalmia Co. Inc., USA).

Results

Multimeric structures of neonatal and adult vWf

Pooled cord blood contained significantly higher levels of vWf (vWf:Ag = 160%) than adult blood (vWf:Ag = 109%). Cord plasma contained ultralarge vWf multimers, not present in adult plasma.

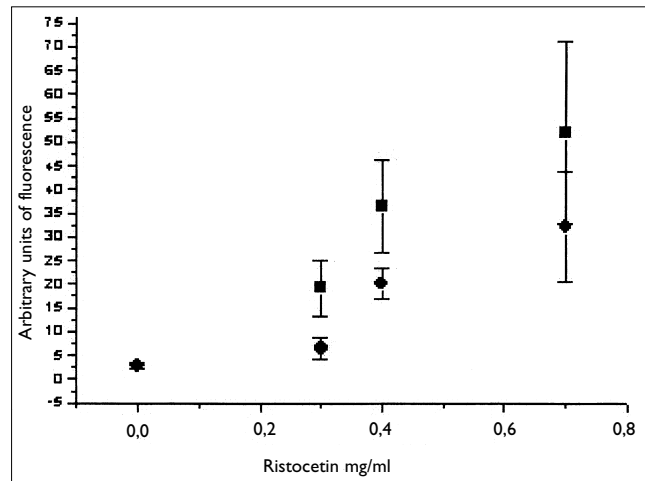


Figure 1: Effect of ristocetin on the amount of vWf attached to the platelet surface in reconstituted cord (■) and adult plasma (●), respectively, both containing stained vWf. The amount of vWf attached to the platelet surface corresponds with fluorescence intensities detected by means of FACS analysis.

Effect of ristocetin on the ability of neonatal and adult vWf, respectively, to interact with platelets

The amounts of both neonatal and adult vWf (in terms of fluorescence intensities) being attached to the platelet surface due to addition of ristocetin dose-dependently increased with increasing amounts of ristocetin, whereby, significantly higher amounts of neonatal vWf were attached to platelets compared with adult vWf (Fig. 1). In order to investigate whether the more efficient vWf-platelet interaction in neonates is an effect intrinsic to the neonatal vWf and not to the neonatal platelet, mixing experiments were performed: the ability of neonatal vWf to interact with both neonatal or adult platelets was compared with the ability of adult vWf to bind to both neonatal or adult platelets. The amount of neonatal vWf attached to neonatal platelets was not different from the amount of neonatal vWf attached to adult platelets. The same was true for adult vWf attached to adult or neonatal platelets (data not shown).

Effect of shear stress on the ability of neonatal and adult vWf, respectively, to interact with platelets

Corresponding to the results obtained after ristocetin addition, neonatal vWf displayed significant higher ability to interact with platelets under shear stress compared to adult vWf. As observed in the presence of ristocetin, there was no difference whether adult or neonatal platelets were used (Fig. 2).

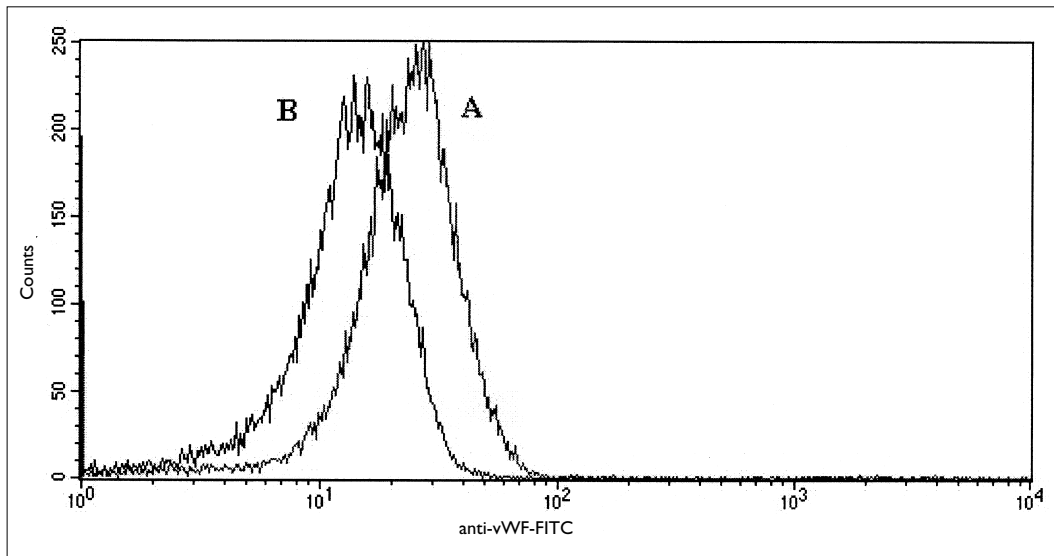


Figure 2: Effect of shear stress (750 s⁻¹) on the amount of vWf attached to platelets in reconstituted cord (A) and adult plasma (B), respectively, both containing stained vWf. Fluorescence intensity curve, evaluated on a FACS analyzer, in reconstituted cord plasma was shifted to the right compared with adult plasma (15 +/- 2 vs. 25 +/- 3, GeoMean) indicating higher affinity of neonatal vWf to the platelet membrane.

Expression of GPIb on neonatal and adult platelets

In order to demonstrate that expected high neonatal vWf-platelet interaction is not due to a possible higher number platelet receptors, the glycoprotein (GP) Ib (the platelet membrane receptor responsible for vWf-platelet interaction in platelet adherence) content on platelets of neonatal origin was compared to that on adult platelets. The platelet surface expression of the GPIb-V-IX complex was not significantly different between neonates and adults (data not shown).

Neonatal vWf plasma concentration effects amount of neonatal vWf being attached to platelet surface in the presence of ristocetin but not under shear stress

In order to investigate whether the higher plasma concentration of neonatal vWf is responsible for the more efficient vWf-platelet binding in neonatal plasma, vWf attachment to platelets was evaluated in reconstituted cord and adult plasma in the presence of ristocetin or shear stress and compared to that in cord plasma diluted to contain vWf at adult concentration.

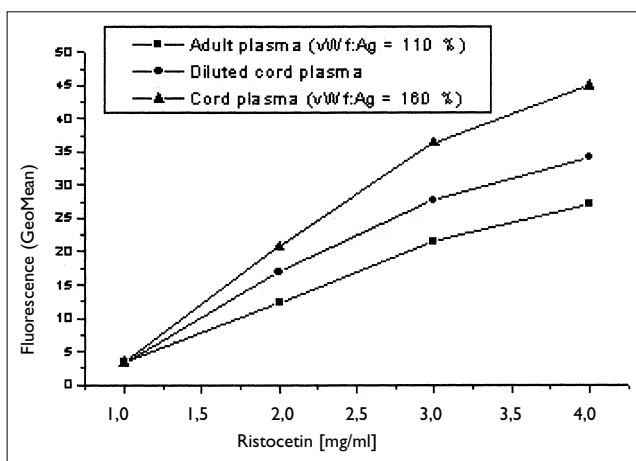


Figure 3: Effect of ristocetin on the amount of vWf attached to the platelet surface in reconstituted cord plasma containing vWf at neonatal level (vWf:Ag = 160%, ▲), cord plasma diluted to adult vWf-concentration (vWf:Ag = 110%, ●), and in reconstituted adult plasma (vWf:Ag = 110%, ■).

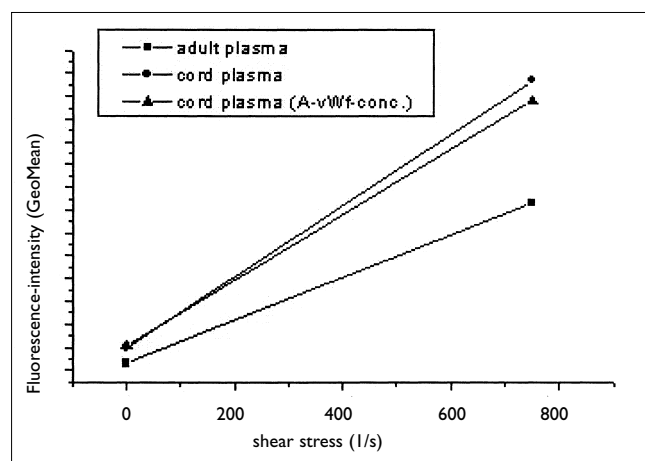


Figure 4: Effect of shear stress on the amount of vWf attached to the platelet surface in reconstituted cord plasma containing vWf at neonatal level (vWf:Ag = 160%, ●), cord plasma diluted to adult vWf-concentration (vWf:Ag = 110%, ▲), and in reconstituted adult plasma (vWf:Ag = 110%, ■).

Decreasing the vWf content in cord plasma to adult level resulted in significantly suppressed vWf-platelet attachment in the presence of ristocetin compared to that in cord plasma containing vWf at neonatal level, but was significantly more pronounced compared to binding of adult vWf to platelets (Fig. 3). In contrast, decreasing the vWf content in cord plasma to adult level did not result in a significant suppression of vWf-platelet interaction in the presence of shear stress compared to that in cord plasma containing vWf at neonatal level (Fig. 4).

Discussion

Impaired neonatal platelet aggregation in response to ADP, epinephrine, thrombin, and collagen has been previously described (19-21). Additionally, whole-blood flow cytometric analysis has confirmed that neonatal platelets are less reactive than adult platelets to physiological agonists (1). Defective thromboxane synthesis and serotonin release has also been shown (22, 23). Moreover, serotonin and ADP are present in dense granules at concentrations less than 50% of adult values (24), suggesting a storage pool deficiency in newborns.

These results do not correlate with the short bleeding time of the newborn (7), indicating efficient primary hemostasis. A possible explanation might be that these *in-vitro* experiments were performed in stirred suspensions in the absence of both reactive surface and shear stress, which are present in the physiological milieu. As a consequence, vWf does not play a role in platelet adhesion and aggregation in these experiments. However, vWf, a glycoprotein synthesized in endothelial cells and megacaryocytes, has been extensively shown to play an essential role in platelet adhesion in the physiological milieu. Soluble vWf is attached to the exposed subendothelial matrix at the site of injury, resulting in a conformational change in the vWf A1 domain (25), facilitating the binding of vWf to the platelet surface. Additional interaction of vWf with the platelet membrane receptor GPIIb-IIIa results in subsequent platelet activation and aggregation (26-28).

The adhesive protein vWf exists in human plasma as a series of heterogeneous homo-multimers ranging in size from about 450 kDa to more than 10 000 kDa (29). However, only the largest multimers have been shown to be hemostatically active (30).

This leads to a possible explanation for the short bleeding times in neonates despite decreased *in-vitro* activity of neonatal compared to adult platelets. Neonatal plasma contains higher amounts particularly of the hemostatically active large multimers of vWf as shown by several authors (10) and reproduced in our study. As a result, increased neonatal platelet deposition on the subendothelium under flow conditions has been suggested to be related with the high content of large vWf multimers in neonatal plasma (9). Furthermore, the presence of high multimeric vWf in neonatal plasma has been shown to cause shorter

“closure times“ in the PFA 100 compared to adult plasma (15, 31). However, in these studies increased attachment of neonatal vWf to platelets in comparison to adult vWf has not directly been shown. Our study addresses this fact and provides evidence for high neonatal vWf-platelet interaction by means of FACS analysis. Enhanced amounts of vWf were attached to platelets (independent of origin) suspended in cord compared to adult plasma in a shear field or in the presence of ristocetin. Increased attachment of neonatal vWf to platelets was not due to the higher vWf:Ag concentration in cord plasma. Significantly more vWf was attached to platelets in cord plasma, even when the physiological high concentration of vWf (vWf:Ag, 160%) was decreased to adult level (109%). This effect was more pronounced in the presence of shear stress than in the presence of ristocetin, confirming the pivotal role of high multimeric neonatal vWf since it has been shown that mainly the high multimeric vWf molecules are hemostatically active in shear fields. Furthermore, no difference was detected between neonatal and adult platelets in their ability to bind vWf: similar amounts of neonatal and adult vWf bound to neonatal versus adult platelets. This is in good agreement with the findings of Michelson et al. (3) who have shown same concentrations of the vWf receptor GPIb on the platelet membranes of neonates and adults. This finding was reproduced in our study.

Two critical issues have to be stated. First, the amount of vWf attached to platelets was determined in a FACS analyzer by means of fluorescence antibodies. Measured fluorescence intensities are, however, not directly reporting on the number of surface-bound vWf molecules but on the amount of fluorescence antibodies attached to the surface-bound vWf. Thus, a difficulty arises from this fact. It can not be retraced whether these antibodies are attached to few, high multimeric surface-bound vWf molecules or to a greater number of surface-bound vWf at a lower degree of multimerization. Thus, the term “amount of vWf attached to the platelet surface” is related to the mass, i.e. the amount of vWf-subunits present on the platelet membrane, which is proportional to the amount of attached antibody and, hence, to the detected fluorescence signal, and not to the number of vWf molecules attached to the platelet surface.

Second, our experiments were performed in cord and not in neonatal plasma. The multimer pattern of vWf in cord blood might not exactly match that in neonatal blood. ADAMTS13, the metalloprotease that cleaves vWf in a shear-dependent manner (32), might not be as effective in the umbilical circulation due to lower levels of shear stress as compared to neonates, resulting in higher amounts of ultralarge vWf in cord than in neonatal blood (13). On the other hand, lower levels of vWf-cleaving protease activity in neonatal compared to cord blood has been reported (33). These two contrary effects obviously result in a similar content of high molecular weight multimers in cord and neonatal (heel-stick) blood, as detected by Weinstein et al (10).

In conclusion, although our laboratory experiments do not allow definite conclusions for physiological conditions, our data give support to the notion that the presence of ultralarge multimers of vWf in neonates results in efficient vWf-platelet inter-

action and might help to explain short bleeding times in the newborn infant and short closure times of neonatal blood in the PFA 100.

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