Thrombosis Research 129 (2012) e18–e24

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Assessment of a Cohort of Primarily Pediatric Patients with a Presumptive Diagnosis of Type 1 von Willebrand Disease with a Novel High Shear Rate, Non-citrated Blood Flow Device

Eric F. Grabowski *, Marjorie A. Curran, Elizabeth M. Van Cott

Massachusetts General Hospital and Harvard Medical School, Boston, MA

ARTICLE INFO

Article history:
Received 4 August 2011
Received in revised form 2 December 2011
Accepted 6 December 2011
Available online 20 January 2012

Keywords:
von Willebrand disease (vWD)
shear rate
blood flow
digital videomicroscopy
ristocetin cofactor activity
bleeding score

ABSTRACT

Background: A precise approach to the diagnosis of von Willebrand disease (vWD) remains elusive. One important reason is that vWD is a blood flow-related disorder: a vW Factor-platelet GPIb binding defect exists in this condition under the high shear-rate (>1000 sec-1 in whole blood; >3000 sec-1 in PRP) conditions of physiologic blood flow which exist in the arterioles of mucous membranes, from which most bleeding in vWD occurs.
Methods: We therefore studied 28 patients (mean 18.9 yrs) with vWD, diagnosed according to the 2007 NHLBI clinical guidelines, and 26 healthy controls (mean 17.5 yrs). Blood was collected into a plastic tube containing 4 U/ml FC dalteparin, 1.75 μg/ml of the Tab (anti-CD41) monoclonal antibody directed against platelet GPIIb, and 1.0 μg/ml of an ALEXA 555-conjugated rabbit anti-mouse second antibody. Within 30–90 min, the blood was then withdrawn at 667 and 1330 sec^{-1} through a special flow chamber allowing for real-time epifluorescence digital videomicroscopy of platelets interacting with a microfibrillar collagen substrate. With MetaMorph software (Universal Imaging) we quantified the percent area (PA) covered by and total volume (TV) of adherent platelet aggregates within a 435 μm × 580 μm field of view.
Results: At 667 sec^{-1} after 1 min PA and TV were similar for patients and controls, but at 1330 sec^{-1} PA was 9.32 ± 4.21 (mean ± SD) for patients, a value lower ($p<0.001$) than the 12.8 ± 3.39 for controls. TV was $(1.43 \pm 0.91)x10^4$ for patients, a value also lower ($p<0.001$) than the $(2.22 \pm 0.77)x10^4$ for controls. PA or TV was below the 2.5th percentile for controls in 10 patients (36%) and both PA and TV were below the 2.5th percentile in eight.
Conclusions: The novel flow device found that PA and TV were significantly reduced under high shear stress in vWD patients compared to normal controls. However, there was some overlap between the vWD and the control group, suggesting that some vWD patients had normal platelet adhesion/aggregation under the conditions studied. Further study with a higher shear rate appears indicated.

Introduction

Von Willebrand disease (vWD) is one of the most common bleeding disorders worldwide, yet a precise approach to the diagnosis of this condition and prediction of its severity remains elusive. One reason for this is that the components of the standard vWD laboratory panel, which include levels of factor VIII clotting activity, ristocetin cofactor activity, and von Willebrand factor (vWF) antigen, are subject to acute changes as "acute phase reactants." Levels of these components can rise significantly above their true baselines with infection, injury, stress, pregnancy, medications such as oral contraceptive pills, and inflammatory disease states, making clear diagnosis problematic. A second reason is that the severity of bleeding manifestations often does not correspond to the level of vWF measured by the above parameters. A third reason is the view by many investigators [1–3] that vWD is a blood flow-related disorder: the defect in this condition is brought out under the high shear-rate conditions of physiologic blood flow which exist in the arterioles of mucous membranes, from which most bleeding occurs. We therefore believe that measurements of functional vWF should be carried out under flow conditions which simulate those in the arterioles of mucous membranes, and not under so-called static conditions used in most current laboratory tests.

One aim of the present study, therefore, was to show that platelet adhesion/aggregation for patients with vWD is reduced compared to that for normal controls at an arteriolar shear rate of 1330 sec-1, characteristic of mucous membranes, but not at a lower shear rate of 667 sec-1. We used a novel flow-based test system developed by one of the authors [4]. A second aim was to correlate quantitative findings to the Bleeding Score (BS) of Rodeghiero et al. [5], and to compare this correlation to that for the conventional vWD panel (e.g., ristocetin cofactor activity).

* Corresponding author at: Pediatric Hematology/Oncology, Yawkey 8B, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. Tel.: +1 617 726 2737; fax: +1 617 724 0702.
E-mail address: Grabowski.Eric@MGH.Harvard.edu (E.F. Grabowski).

0049-3848/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2011.12.016

Methods

As part of an IRB-approved study, we enrolled 36 patients with Type 1 vWD, diagnosed according to the 2007 NHLBI clinical guidelines [6] and 34 healthy controls without any overt bleeding disorder. Eight patients and two controls were excluded from the analysis because of substandard image quality or non-recorded images in an early phase of this study. Three other controls were excluded because of a bleeding disorder (two) or aspirin ingestion (one) which became known only after the subject had been enrolled and more personal history came to light. Three further controls were excluded because of probable aspirin ingestion, as they showed a decrease in platelet deposition with increasing shear rate indicative in our experience of unacknowledged aspirin [4] or other NSAID ingestion, or, less likely, an inherited platelet defect. This left 28 patients (21 females and 7 males) and 26 controls (13 females and 13 males), which constituted 81% of enrolled subjects. The greater number of females reflects the frequent referrals to our practice for menorrhagia. Nonetheless, there was no significant difference in platelet adhesion/aggregation between male and female patients (see Results). All eight patients for whom images were not available were included in the correlations of platelet adhesion/aggregation, lowest ristocetin cofactor activities, and bleeding score, for a total of 36 patients. Most patients (24) were in the pediatric age group (<18 years), but some younger adults (12) were included. Median patient age was 14 years (first and third quartiles, 6 and 29 years, respectively), while the median control age was 15.5 years (difference not significant by the Wilcoxin rank-sum test; first and third quartiles, 3 and 37.5 years).

Blood was collected into a plastic tube containing 4 U/ml FC low MW heparin (dalteparin), 1.75 μg/ml of the Tab (anti-CD41) monoclonal antibody directed against platelet GPIIb, 1.0 μg/ml of an ALEXA 555-conjugated rabbit anti-mouse second antibody, and PBS (9 parts blood:1 part PBS). The TAB monoclonal antibody was developed by Dr. Rodger McEver, is well-characterized, and has been shown not to affect platelet aggregation induced by ADP or thrombin [7] or 125-I-fibrinogen binding [8]. Blood was then transported to our laboratory and, within 30–90 min of blood collection, rewarmed to 37 °C in a water bath, and then immediately withdrawn at 670 and 1330 sec-1 through a special flow chamber [4] allowing for blood exposure to 150 μm glass cover slips (Fig. 1) to which had been preadsorbed microfibrillar collagen (equine tendon; Helena Laboratories, Beaumont, TX) from a 100 μg/ml solution over 15 min. The surface concentration of adsorbed collagen was considered for each cover slip to reach the same final value over several minutes, according to the Langmuir adsorption equilibrium [9]. Adsorption on the cover slips took place in a covered tissue culture plate with rectangular wells, the cover slips thereby never being allowed to dry prior to mounting in the chamber. Using epifluorescence digital videomicroscopy (Nikon Optiphot microscope, 20x objective; Photometrics CoolSnap HQ camera–4095 grey levels, Roper Scientific; Lumen 200 PRO light source, Prior), we imaged platelets interacting in real time with the collagen substrate at times 1, 2, 3 and 4 min at the lower shear rate, and at times 1 and 2 min at the higher shear rate. Platelet deposition after one minute at 1330 sec-1 was diminished with blood from a subset of patients as compared to blood from controls (Fig. 2A and B). Resolution was better than 1 μm, which permitted the identification of single platelets, the estimation of the mean platelet area in image pixels, and the estimation of the total platelet volume as the sum of the products, for all fluorescence intensities, of fluorescence intensity and the number of image pixels at that fluorescence intensity. In preliminary work, the average area for single platelets (from images for two patients and two controls) was 58.3 ± 20.7 (mean ± SD, $N = 69$) pixels, or about 10.2 square microns. This value is consistent with an adherent platelet diameter of 3.6 microns. We therefore defined as an image object any region with a fluorescence intensity above background whose area equaled or exceeded that for the 2.5th percentile for a single platelet, or 18 pixels.

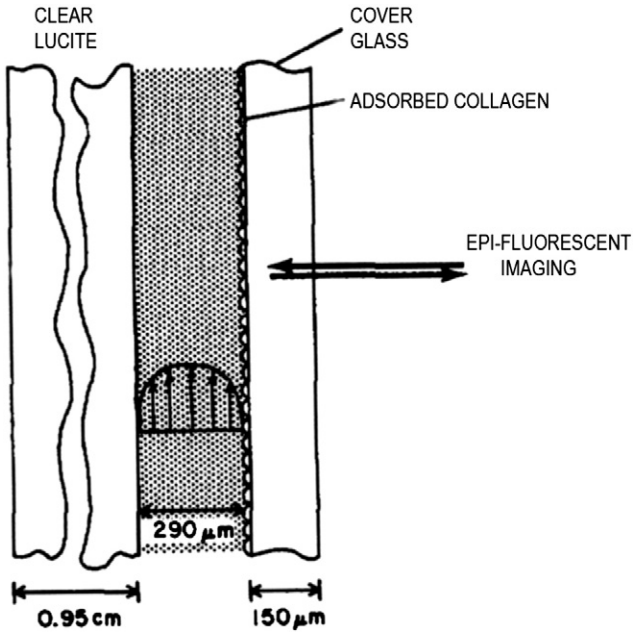

Fig. 1. Schema of flow path in blood flow chamber. Lengths of arrows represent local magnitude of flow velocity.

Blood was collected into a low MW heparin insofar as platelet function is more physiologic owing to the presence of normal levels (mM) levels of ionized calcium. Collection of blood into sodium citrate, in contrast, leads to artifacts in platelet adhesion/aggregation (see Discussion). In order to confirm this in the present flow system, we compared platelet adhesion/aggregation with blood from the same donor collected into 0.23% sodium citrate vs the same concentration of low MW heparin as above. In two separate high shear rate experiments, platelet aggregates formed in citrate were significantly smaller in both surface attachment area and volume as compared aggregates formed in low MW heparin (Fig. 3A and B).

We quantified platelet deposition at both shear rates using MetaMorph software (Universal Imaging) in terms of 1) the percent area (PA) covered by adherent single platelets and platelet aggregates (field of view 435 μm × 580 μm, or 0.2523 mm2), 2) the platelet thrombus volume (TV), as platelets per mm2 for the field of view, and 3) the number of objects (NO), whether single platelets or platelet aggregates, in the field of view. Measurements of TV in units of fluorescence intensity-pixels were converted to platelets per mm2 by dividing by the mean volume for a single platelet in units of fluorescence intensity-pixels. The platelet mean volume proved to be $25{,}500 \pm 2{,}390$ (mean ± SE) fluorescence intensity-pixels. In this step we assumed that local fluorescence intensity is proportional to the number of platelets in the vertical or z-direction at a particular x-y location. This assumption is reasonable for aggregate thicknesses (estimated to be in the range of 5 to 30 μm) small compared to the flow chamber channel height (290 μm), a condition best assured at the earliest time point (1 min) of the study.

Clinical BS [5], ristocetin cofactor activity (performed on an AggRam platelet aggregometer, Helena Laboratories, Beaumont, TX) and vWF antigen (Liatest vWF reagent, Diagnostica Stago, Asnieres, France; performed on an MDAII analyzer, TCoag, Parsippany, NJ) as measured by the Massachusetts General Hospital Special Coagulation Laboratory, were also recorded. When multiple values of the ristocetin cofactor activity or vWF antigen were available, the lowest and/or a replicate value was used to make a diagnosis of mild vWD and thereby define who was a patient. However, for correlation analyses involving flow device parameters, lowest and same-day ristocetin cofactor activities were both used. Same-day ristocetin cofactor activity was defined as ristocetin cofactor activity measured on the same