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High Shear Dependent von Willebrand Factor Self-assembly Fostered by Platelet Interaction and Controlled by ADAMTS13

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ABSTRACT

Introduction: The paradigm of activation induced platelet aggregation has recently been refuted under blood flow conditions with shear rates exceeding 20,000 s^{-1}. These lead to reversible rolling platelet aggregates, which were dependent on the presence of immobilized and soluble von Willebrand factor.
Material and Methods: In vitro experiments using direct fluorescence video-microscopy were performed in wall parallel and stagnation point flow chambers with shear rates raised from 20,000 to 50,000 s^{-1}. Washed blood cell suspension containing recombinant von Willebrand factor (rVWF) was perfused over rVWF or collagen coated surfaces.
Results: Here we show for the first time with the visualization of rVWF that not only colloid and polymer, i.e. platelets and VWF, form a composite, but that VWF itself is capable of entirely reversible self-assembly. On a collagen surface the platelet-VWF-conglomerates did not roll but VWF nets bound permanently to the collagen fibers and captured and immobilized platelets from the flow. Lowering the shear rate below the threshold of 20,000 s^{-1} no longer dissolved these deposits. Ultralarge multimer containing rVWF was most effective compared to normal sized rVWF. The presence of ADAMTS13 limited rolling aggregate and platelet-VWF-conglomerate formation to a time window of 7-8 minutes. Changing wall parallel flow to stagnation point flow halved the required shear rate threshold.
Conclusion: We conclude that flow dynamics can trigger reversible von Willebrand factor self-assembly and platelet-VWF-conglomerate accrual, which are regulated by ADAMTS13 to a time span needed by coagulation to stabilize it, e.g. in case of vessel injury.

Introduction

Platelet accrual at sites of vascular injury requires arrest of non-activated platelets from fast flowing blood, succeeded by their activation and stabilization as an aggregate and ultimately a thrombus. The initial step is thought to be single platelet adhesion to immobilized von Willebrand factor via their GPIbα receptor. However in the case of a vessel leak this platelet accumulation – one at a time – may not suffice for efficient stop of bleeding. In addition vessel leaks or stenosis may exhibit flow conditions with excessively elevated shear rates that appear to counteract cellular deposition. Ruggeri and coworkers have put forward a mechanism of rolling platelet aggregate formation independent of platelet activation and signaling. The process was entirely reversible if the shear rate fell below a critical threshold of 20,000 s^{-1} [1]. We recently applied it to blood-clotting-inspired reversible polymer-colloid composite assembly in flow [2]. There model simulations were used to show the flow-driven self-assembly between polymer and colloid, i.e. VWF and platelet. Interestingly, the propensity to form such composites is in contrast to our intuitive expectation, where laminar shear flow conditions may rather be expected to decrease association. Thus the clotting scenario in which flow controls the reversible assembly of complex composites was described to represent a completely new aggregation paradigm in blood flow that cannot be understood in terms of purely diffusion-limited and/or reaction-limited aggregation concepts [2]. The examined shear rates were up to 14,000 s^{-1} and the corresponding VWF GPIbα-bond breakup force was estimated to be in the order of ~ 10pN. In the present paper we have taken this a step further and examined the influence of two different flow conditions -wall parallel

Abbreviations: BSA, bovin serum albumin; EDTA, ethylenediaminetetraacetic acid; GPIbα, glycoprotein Ibα; PBS, phosphate buffered saline; PGE1, prostaglandin E1; PPACK, H-D-Phe-Pro-Arg-chloromethylketone trifluoracetate salt; rADAMTS13, a recombinant disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; rVWF, recombinant von Willebrand factor; UL, ultra large.

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and stagnation point flow- on the self-assembly of the VWF polymer in solution and on the surface while increasing the shear rates up to 50,000 s^{-1} and introducing the cleaving enzyme ADAMTS 13. In addition we examined the effect of a fibrillar collagen surface and the size distribution of the VWF multimers.

Material and Methods

All procedures involving human subjects were conducted in accordance with the Declaration of Helsinki and were approved by the Ethics Commission of the Ludwig-Maximilians-University Munich. Informed consent was obtained from all participating subjects.

Fluid Preparation for Flow Experiments

Blood sample collection and preparation was performed as previously described [1]. For perfusion studies, blood from healthy human volunteers was drawn from an antecubital vein and collected into S-Monovettes (Sarstedt AG&Co, Nümbrecht, Germany) containing the disodium salt of ethylenediamine tetraacetic acid (EDTA, 1.6 mg/ml blood; Sarstedt). H-D-Phe-Pro-Arg-chloromethylketone trifluoracetate salt (PPACK, final concentration 46 μM; Bachem Bioscience, King of Prussia, PA), prostaglandin (PG) E_1 (final concentration 14 μM; Sigma-Aldrich Chemie, Taufkirchen), and apyrase from potato (Grade 3, 1.3 ATPase U/ml blood; Sigma-Aldrich Chemie) were added to the blood when indicated to inhibit platelet activation and block integrin function.

For washed cell suspensions the blood was centrifuged at 1400 *g* for 13 min at room temperature (22 °C – 25 °C). The supernatant plasma was discarded and the platelets and leukocytes sedimented on top of the erythrocyte cushion were resuspended with an equivalent volume of divalent cation-free Hepes/Tyrode buffer (17 mM Hepes [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 130 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, and 2.8 mM dextrose), pH6.5, containing PPACK (46 μM), PG E_1 (14 μM), and a reduced amount of apyrase (0.65 ATPase U/ml). The gently mixed cell suspension was centrifuged twice with the supernatant fluid removed and replaced by a fresh aliquot of Hepes/Tyrode buffer, containing PG E_1 (14 μM) and a reduced amount of apyrase (0.325 ATPase U/ml), respectively. To adjust platelet count and hematocrit to physiological levels (180,000 - 390,000/μl and 38% - 43%, respectively), the cell fraction was resuspended in Hepes/Tyrode buffer, pH7.4, containing 50 mg/ml bovine serum albumin (BSA; Sigma-Aldrich Chemie).

A recombinant VWF drug candidate (rVWF) currently in clinical development [3–5] which contains ultralarge VWF multimers (/+ UL) and a preparation of rVWF derived thereof by chromatographic depletion of ultralarge multimers (/-UL) were used (Fig. 1E; friendly gift of Baxter Innovations GmbH,Vienna, Austria). Both preparations were described in detail by Turecek et al. [5] VWF in normal plasma concentration (rVWF, final concentration 1 VWF:Ag U/ml Hepes/Tyrode buffer)in physiological salt buffer, neutral pH, and low non-ionic detergent was added to the final cell suspension. For visualization the experiments were performed with a previously fluorescence-labeled rVWF (final concentration 1 VWF:Ag U/ml Hepes/Tyrode buffer). To clarify the effect of the VWF cleaving enzyme during flow conditions a recombinant ADAMTS13 solution (rADAMTS13, final concentration 1 U/ml Hepes/Tyrode buffer; friendly gift of Baxter Innovations GmbH, Vienna, Austria) in physiological salt buffer, neutral pH, and low non-ionic detergent was added to the flowing blood cell solution. Before the perfusion experiment solutions of magnesium chloride ($MgCl_2$, final concentration 0.3 mM) and calcium chloride ($CaCl_2$, final concentration 0.6 mM) were added to the fluid. Control experiments were performed with whole blood, i.e. no washing, and EDTA, PPACK, PG E_1, apyrase added.

Fluorescence-Labeling of the rVWF

The fluorescence protein labeling of the rVWF was performed with the Alexa Fluor® 488 Labeling Kit (Life Technologies GmbH, Darmstadt). One milliliter protein solution plus 100 μl of a 1 M sodium bicarbonate solution were incubated for two hours at room temperature with the Alexa Fluor® 488 dye while constantly stirred in the dark. The free dye was removed from the protein conjugates by extensive dialysis at 4 °C with Slider-A-Lyzer® Mini Dialysis Units (Thermo Fisher Scientific Inc., Rockford, IL) and phosphate buffered saline (PBS) containing sodium azide (13 mg/l; Sigma-Aldrich). For four days the dialysis buffer was refreshed thrice a day before the protein conjugates were stored in opaque tubes at 4 °C. During the labeling process half the active protein was lost without a change in the multimer distribution (Fig. 1E). The in the experiments used rVWF concentration was adjusted respectively.

Fluorescence-Labeling of Platelets

In order to fluorescence-label platelets the buffy coat was transferred into another reaction tube after the last blood washing centrifugation step. Per milliliter cell suspension 1.5 μl calcein red-orange AM solution (10 μg calcein/μl DMSO; Life Technologies GmbH) were added. After one hour incubation in the dark the buffy coat containing labeled platelets was resuspended in the red blood cell solution and processed as described above.

Preparation of Perfused Surfaces and In Vitro Perfusion Experiments

Multimeric rVWF with and without ultralarge multimers (coating concentration 1 VWF:Ag U/ml Hepes/Tyrode buffer; friendly gift from Baxter Innovations, Vienna) [6] or fibrillar collagen type I (coating concentration 100 μg collagen fibrils/ml buffer; Horm-Chemie; Nycomed, Munich) [7] were coated as substrates onto glass coverslips that were assembled into a parallel plate rectangular flow chamber (height: 127 μm, width: 2.5 mm, length: 25 mm). Coating procedures were performed for one hour at room temperature in a wet chamber. Perfusion experiments were conducted at 37 °C. For real-time visualization of platelet-VWF-interactions under high shear rates the flow chamber was mounted on the stage of an upright microscope (Axioskop 2 plus, Carl Zeiss, Jena). With a REGLO Digital MS-2/6 peristaltic pump (IDEX Health & Science GmbH, Glattbrugg) an artificial blood circulation was established, whereby the chamber was perfused at the desired flow rate (wall shear rates ranging from 10 000 s^{-1} up to 50 000 s^{-1}). Platelets and aggregates were observed in bright field illumination (100 W halogen lamp) and the labeled rVWF multimers and nets were observed by epifluorescence microscopy with a HBO-50 AC mercury lamp and a FITC filter block.

A second flow device with a radial stagnation point flow chamber was used to examine the dependence of changed hemodynamics on VWF-platelet interaction [8,9]. Glass coverslips coated as described above were placed in the flow chamber where the surface was perfused by a perpendicular incoming flow (inlet diameter: 650 μm, chamber height: 480 μm) that symmetrically radiated apart. In the outward regions of the chamber the fluid was collected in a ring channel and pumped off through the outlet. Washed blood cell suspensions containing rVWF/+ UL with labeled platelets was likewise perfused over an rVWF/+ UL or collagen type I coated surface.

Image Acquisition, Analysis and Statistics

All experiments were recorded live on double sided DVD-RAM/R with a Panasonic LQ-MD800 DVD video recorder at an acquisition rate of 25 frames per second. The images were acquired with a 2/3"-1-CCD-camera (AVT BC-71; Horn Imaging, Aalen) and an optical 0.33x – 1.6x zoom. The objective used was a Plan-Neofluar 40x/1.30 NA oil (Carl Zeiss, Ulm). Image analysis was performed offline with the Metamorph software package 6.1 (Molecular Devices). Along the middle axis of an aggregate