PLATELET ADHESION TO SURFACE CAPTURED DISULFIDE-INTACT A1 AND RCAM A1 UNDER SHEAR FLOW

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INTRODUCTION

Type 2A Von Willebrand disease (VWD) is clinically identified by a loss of high and intermediate Von Willebrand factor (VWF) multimers resulting in a loss of platelet-dependent function. The majority of mutations that cause type 2A disease occur in the A2 domain and are grouped by their effects on cellular retention of VWF or the susceptibility of VWF to proteolysis by ADAMTS13. However, a few mutations also classified as 2A occur in the A1 domain. The most puzzling are those that abolish the single disulfide bond in the A1 domain, which is required to maintain the native structure of the domain. These mutations could cause severe problems in hemostasis that lead to microvascular thrombosis because of the enhanced affinity of this intermediate conformation for platelet GPIba.

This customer case study provides a functional comparison of the two thermodynamic conformational states using native disulfide-intact A1 and reduced and carboxyamidated (RCAM) A1 as a model of the intermediate state. The conformational dependence of platelet adhesion to the native and molten globule states of the A1 domain is quantitatively compared using real-time high-speed video microscopy analysis of platelet translocation dynamics under shear flow in a parallel plate microfluidic flow chamber.

MATERIALS AND METHODS

The interaction of disulfide-intact A1 and of RCAM A1 with platelets was studied by immobilizing the proteins via the 6xHis-Tag on Cellix biochips in which the internal channel surface was coated with a Cu2+ chelating chemistry. To date, existing parallel plate flow assays for platelet adhesion studies with respect to VWF have been conducted by binding of proteins to the surface of microchannels using covalent bonding of proteins to pre-actived carboxyl, hydroxyl or carbonyl groups on the surface of glass or plastic. In contrast to this, Dr. Matt Auton's group devised a more robust and efficient method for binding of VWF A1 and RCAM A1 to a Cu2+ chelated PEG surface via the 6xHis-Tag. This results in a highly reproducible and specific protein coating for recruitment of platelets under shear flow.

Cellix manufactures Vena8 GCS biochips with microchannels of dimensions 800μm x 80μm (WxH). These plastic biochips are bonded to glass slides with a Cu2+ chelated PEG surface provided by Microsurfaces Inc. to which the A1 and RCAM A1 in TBS were captured by the 6xHis-Tag at a total concentration of 5 mM.
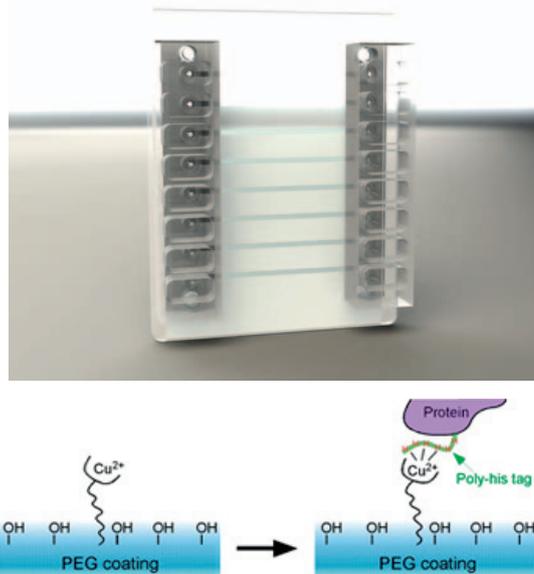

Figure 1: Vena8GCS biochip and Cu2+ coating for specific binding of vWf via poly-histidine tag.

Citrated whole blood was obtained from healthy volunteer donors and a 100 μL sample was perfused using Cellix's Mirus Evo Nanopump controlled by VenaFluxAssay software. This perfusion at 800 s-1 was followed by TBS buffer to wash out red blood cells. The shear was increased or decreased in a stepwise manner to measure the dynamics of platelet adhesion at high and low shear. The flow was equilibrated for 50s at each shear rate and 1 min movies were recorded at 24 fps in phase contrast at 200X magnification with 2 X 2 pixel binning on a Zeiss Axiocam Mrm camera (6.45 μm/px) attached to a Zeiss Axio Observer D1 inverted microscope. Tracking analysis was performed using Media Cybernetics ImagePro Premier. The distance traveled in the direction of flow was calculated. Trajectories were retained for statistical analysis if the platelet was present for >1 s and traveled a total distance greater than 1 μm. Distance traveled in pixels (px) was converted to (μm) using calibration 1 px = 0.0645 μm. Pause times were determined by the amount of time (seconds) a platelet velocity was 0 ± 0.23 μm/s. Data are reported as mean velocities and pause times attained by individual platelets averaged over all platelets analyzed.
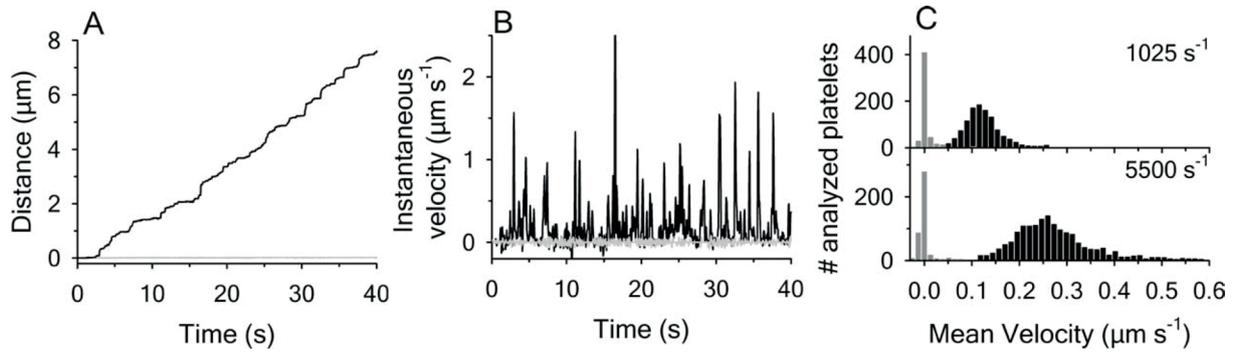
Figure 2: Comparison of platelet translocations on disulfide-intact A1 with RCAM A1. (A) Traveled distance; (B) Instantaneous velocities at 1025 s-1; (C) Histograms of the mean velocity at shear rates of 1025 and 5500 s-1. In (A), (B) and (C) platelets interacting with disulfide-intact A1 (black line/bar), RCAM A1 (grey line/bar)

RESULTS

We have calculated platelet mean pause times and mean translocation velocities from the X–Y trajectories of moving platelets for each shear rate overall analyzing 1000 platelet trajectories [Fig. 3]. Mean platelet pause times increased from approximately 0.75 s at a shear rate of 530 s-1 to ~0.9 s at 1025 s-1 and then decreased again upon further increase of the shear rate. The mean platelet translocation velocities mirrored the pause times and were minimal at a shear rate of 1025 s-1 and increased at lower and higher shear rates. It was also observed that platelets began to detach from surface immobilized disulfide-intact A1 at shear rates ≤ 300 s-1.

In contrast to disulfide-intact A1, the interactions between surface-immobilized RCAM A1 and platelet GPIbα resulted in captured platelets that did not translocate across the surface at all applied shear rates. In this case, we did compare the distance traveled and instantaneous velocities. Figure 2(A) illustrates the traveled distance for a single platelet translocating on disulfide-intact A1 with a platelet on RCAM A1 over a 40 s time frame. The traveled distance was converted into instantaneous velocities in Figure 2(B). While the interaction of a platelet with A1 caused many translocation events yielding nonzero velocities, the velocity of a platelet translocating on RCAM A1 was statistically zero. This is also shown by the histograms given in Figure 2(C), which report velocity distributions of all analyzed platelet translocations at shear rates of 1025 and 5500 s-1 and even at higher shear rates up to 9000 s-1. However, the total number of platelets interacting with RCAM A1 was less, by approximately half, than with disulfide-intact A1 indicating that the on-rate for bond formation is reduced for RCAM A1 relative to disulfide-intact A1.

CONCLUSION

We show that RCAM A1 domain arrests platelets resulting in net zero translocation velocities. This is in stark contrast to disulfide-intact A1 where platelets are
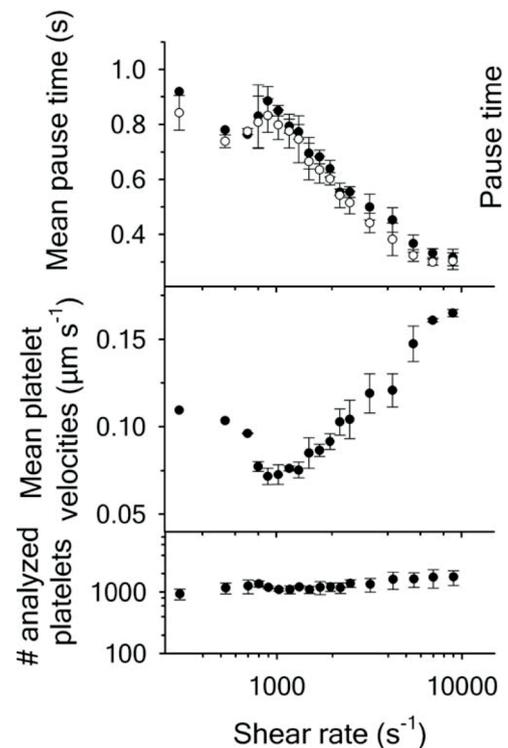

Figure 3: Platelet translocation on disulfide-intact A1 as a function of shear rate.

observed to stick and roll on the surface captured domain. Pause times determined for platelets translocating on disulfide-intact A1 represent the average length of time a platelet is immobile. This time has a complex dependence on the shear rate.

In our experiment, the mean pause times for disulfide-intact A1 are longer than in previously reported flow chamber measurements. We attribute these differences to the method of immobilization in which our protein is captured by the 6xHis-tag rather than by nonspecific interactions between the A1 domain and glass or plastic surfaces. This method of surface capture by specific chelation chemistry preserves the structure of the domain and allows one to distinguish the effects of different A1 domain conformations on platelet adhesion.

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