Relevance of platelet desialylation and thrombocytopenia in type 2B von Willebrand disease: preclinical and clinical evidence

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Major Resources Table

Reagents

Name	Vendor or Source	Catalog #
Ricinus communis agglutinin I (RCA)	Vector Labs	FL-1081
Erythrina cristagalli (ECL)	Vector Labs	FL-1141
Biotinylated *Maackia amurensis* lectin II (MALII)	Vector Labs	B-1265
β-lactose	Sigma-Aldrich	L3750
α2-3,6,8 neuraminidase from *C. perfringens*	Sigma-Aldrich	N-3001
PE-streptavidin	Calbiochem	189737
PNGase F	New England Biolabs	P0704
O-sialoglycoprotein endopeptidase (OSGE)	Cedarlane	CLE100
DANA (N-Acetyl-2,3-dehydro-2-deoxyneuraminic Acid)	Santa Cruz	SC-215433A
Oseltamivir phosphate	Selleckchem	S2597

Antibodies

Target antigen	Vendor or Source	Catalog #
FITC-rat anti-mouse GPIbα mAb (clone XiaG5)	Emfret Analytics	M040-1
FITC-rat anti-mouse GPVI mAb (clone JAQ1)	Emfret Analytics	M011-1
FITC-rat anti-mouse integrin $\alpha_{IIb}\beta_3$ mAb (clone Leo.H4)	Emfret Analytics	M021-1
FITC-rat IgG negative control	Emfret Analytics	P190-1
FITC-rat anti-mouse CD41 mAb (clone MWReg30)	BD Pharmingen	553848
FITC-rat IgG negative control	BD Pharmingen	553924
Neu1 (clone H-300)	Santa Cruz	SC-32936
Purified rat anti-mouse GPIbα (clones XiaG5/XiaG7)	Emfret Analytics	M040-0/M042-0
Purified rat anti-mouse integrin αIIb (clone 386627)	R&D	MAB 4118
Purified rat anti-mouse integrin β3 (clone Luc.A5)	Emfret Analytics	M030-0
Purified rat anti-mouse GPVI (clone JAQ1)	Emfret Analytics	M011-0
Alex fluor 488 goat anti-rabbit secondary antibody	Invitrogen	A11008

Methods

Recombinant mVWF/p.V1316M. Baby hamster kidney cells were transfected with pNUT-mouse VWF and pNUT-mouse VWF/p.V1316M encoding mouse VWF and mouse VWF/p.V1316M, as previously described.[1] Serum-free-conditioned medium was collected, and VWF antigen levels were quantified, taking normal pooled human plasma as a reference (100%).

Desialylation by VWF. Citrated plasma from WT or 2B mice was mixed 9:1 (v/v) with citrated platelet-rich plasma from WT mice. Washed platelets (prepared as previously described)[2] were incubated in the presence of WT or 2B recombinant murine VWF

(p.V1316M). All samples were incubated for 1 hour at 37°C without stirring.

RCA pull-down. Washed platelet (500 µL at 3.10^8/mL) were lyzed (NaCl 0.15 M, HEPES 5 mM, NP40 1%, octylglucopyranoside 0.1%, SDS 0.1%, EDTA 1 mM, cantharidine 10 µM, 5 µg/ml leupeptin, 10 µg/ml aprotinin) and centrifuged 14 000*g* during 20 min. Biotinylated-RCA (5 µg/mL) was added to the supernatant and incubated during 1h at 4°C while rotating. Then, samples were incubated for 1h with streptavidin magnetic beads at room temperature while rotating. The beads were then washed and proteins were eluted with SDS sample buffer.

Western-blot. Samples were separated by 4-12% SDS-PAGE, and immunoblotted with anti-GPIbα antibodies (mix of clones XiaG5/XiaG7, 1/100), GPVI (1/100), αIIb (1/1000), β3 (1/200). Immunoreactive bands were visualized using Enhanced Chemiluminescence Detection Reagents (Pierce). Images of the chemiluminescent signal were captured using G:BOX Chemi XT16 Image Systems and quantified using Gene Tools version 4.0.0.0 (Syngene).

***In vivo* sialidase inhibitors.** The neuraminidase inhibitors N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA, 0.1 mg/g total body weight) and oseltamivir phosphate (0.025 mg/g total body weight) were respectively injected intraperitoneally and intravenously into 2B and WT mice. An injection of Hank's balanced salt solution was used as a control. After 6, 24, 48, 96, and 168 h (*i.e.* up to 7 days), the mice were bled, and the platelet count and RCA binding were measured.

***In vivo* desialylation**. WT mice received an intravenous injection of *C. perfringens* α2-3,6,8 neuraminidase. The platelet count and RCA binding were measured after 1 hour.

REFERENCES

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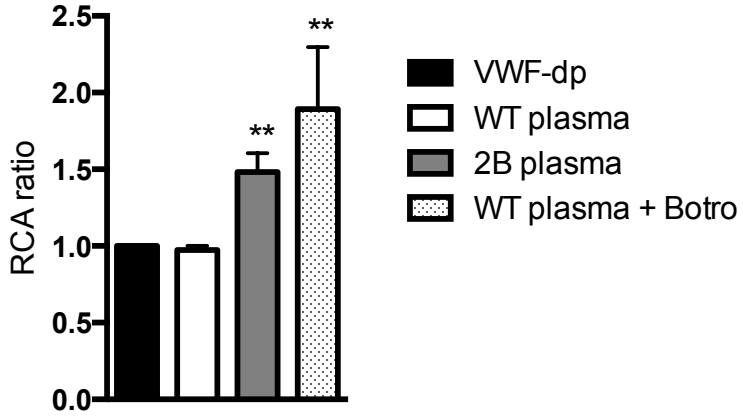
2. Adam F, Kauskot A, Nurden P, et al. Platelet JNK1 is involved in secretion and thrombus formation. *Blood*. 2010;115(20):4083-4092.

Supplemental data

Supplemental Figure 1: Histogram of RCA lectin binding on WT mouse platelets in the presence of VWF-deficient plasma (KO VWF), WT plasma, 2B plasma or WT plasma in the presence of botrocetin (5 µg/mL). The fold increase in each experiment was calculated with the VWF-deficient plasma as set as 1. (Mean ± SD, n=3, One-way Anova followed by Dunnett's test. **p<0.01.



Supplemental Figure 2: Histogram of MFI of MALII/streptavidin (left) and RCA (right) lectin binding on WT mouse platelets treated or not with neuraminidase (0.05 U/mL).