The role of neuraminidase 1 and 2 in glycoprotein Ibα-mediated integrin αIIbβ3 activation

Dianne E. van der Wal, April M. Davis, Melanie Mach, Denese C. Marks

1Australian Red Cross Lifeblood (formerly known as Blood Service) and 2Sydney Medical School, University of Sydney, Sydney, NSW, Australia

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Correspondence: DIANNE E. VAN DER WAL - divanderwal@redcrossblood.org.au
Supplementary Information

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Running head: The role of neuraminidases in platelet function

Dianne E. van der Wal¹, April Davis¹, Melanie Mach¹, Denese C. Marks¹,²

1) Research and Development, Australian Red Cross Blood Service, Sydney, Australia
2) Sydney Medical School, The University of Sydney, NSW, Australia

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Reagents and sources

We used the following products (with sources): collagen, ristocetin and arachidonic acid (AA, Helena Laboratories, Beaumont, TX, U.S.A.), human α-thrombin, fibrinogen (plasminogen, VWF-, fibronectin-depleted (Enzyme research Laboratories, South Bend, IN, U.S.A.), mono-sialo-dihexosylganglioside (GM3, Avanti Polar Lipids, Alabaster, Alabama, U.S.A.), VWF FXIII-free (Abcam, Melbourne, VIC, Australia), prostaglandin I2 (PGI2, Cayman Chemical, Ann Arbor, MI, U.S.A.), collagen (NEU-activity assay, Chrono-log corporation, Havertown, PA, U.S.A.), O-sialo-glycoprotein Endopeptidase (OSGE, Cedarlane, ON, Canada). ADP, 2’-(4-Methylumbelliferyl)-α-D-N-acetyleneuraminic acid sodium salt hydrate (MUNANA), N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), N-acetyl-D-glucosamine (GlcNAc), Arg-Gly-Asp-Ser (RGDS) peptide, Triton-X-100, Native human D-Dimer protein and bovine serum albumin (BSA), apyrase, BAPTA-AM-AM, indomethacin, all sourced from Sigma/Merck (Darmstadt, Germany). Peptide-N-Glycosidase F (PNGase F, PNGase, New England Biolabs, San Francisco, CA, U.S.A) recombinant neuraminidase (Clostridium perfringens, Roche, Sydney, NSW, Australia), laminin (mouse, Life Technologies, Carlsbad, CA, U.S.A.) and fixation buffer (4% paraformaldehyde, BioLegend, San Diego, CA, U.S.A.), IbiTreat microchamber µ-slide VI 0.4 (µ-slide VI0.4, IBIDI, Martinsried, Germany) and ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Antibodies were obtained (with sources): anti-factor V (Haematologic Technologies Inc., Essex Junction, U.S.A.), anti-lysosomal-associated membrane protein-1 (LAMP-1), anti-fibrinogen and anti-VWF (Abcam, Cambridge, MA, U.S.A.), anti-NEU1, anti-NEU2, anti-β-Galactosidase (Santa Cruz Biotechnology, Dallas, TX, U.S.A.), anti-NEU1, anti-mitochondria (Abcam), membrane stain CellBrite 640 (Biotium, Fremont, CA, U.S.A.). Anti-P-selectin antibody (microscopy) and secondary antibodies anti-mouse (Alexa (A)488-conjugated), anti-rabbit (A647) and anti-goat (A647), all sourced from Thermo Fisher, anti-P-selectin-PE (CD62P) and PAC-1-FITC (BD Biosciences, San Diego, CA, U.S.A). Glycan binding lectins Ricinus communis agglutinin-1 (RCA-1), wheat germ agglutinin (WGA) and Wheat Germ Agglutinin (WGA), Erythrina cristagalli (ECL), Peanut agglutinin (PNA), Maackia amurensis lectin-1 (MAL-1), Sambucus nigra (SNA) were conjugated to fluorescein and all sourced from Vector Laboratories (Burlingame, CA, U.S.A.).
Methods

**Platelet incubations**

As large volumes were required, apheresis platelets (Day 1 post-collection and 100% in autologous plasma) were used and diluted to 200x10⁹/mL in Tyrode’s. Platelets were perfused 5 times (continuous flow) through a microchannel, flow rate of 10,000s⁻¹ (59.5mL/min using equation: \( \gamma \text{ (s}^{-1}) = 176.1 \varphi \times \text{flow rate (mL/min)} \) and \( \varphi \) = viscosity (1.047). Prior to perfusion, platelets were stimulated \( \pm \)risto (0.5mg/mL, 5min 37°C).

**NEU activity**

NEU activity was measured in plasma, in presence of 75µM sodium acetate (pH 4.5), 0.1% Triton X-100, 0.5mM 2′-(4-Methylumbelliferyl)-α-D-N-acetyllneuraminic acid sodium salt hydrate (MUNANA) in 96-wells plates after 10min (\( \lambda_{ex}=365\text{nm} \), emission \( \lambda_{em}=450\text{nm} \)). NEU activity was measured following Triton-X-100 permeabilisation (0.1%).

**Intracellular localisation of NEU1 and NEU2**

Platelets were stained with the following antibodies (final concentrations/dilutions) anti-LAMP-1 (1 µg/mL), anti-mitochondria (1.25 µg/mL), anti-factor V (FV, 10 µg/mL), anti-NEU1 (20 µg/mL), anti-P-selectin (1/50), CellBrite 640 (1/50) and anti-NEU2 (1/200, all O/N, 4°C).
Legends Supplementary Tables and Figures.

Suppl. Figure S1: Lectin binding and NEU expression following various treatments.
A) Washed platelets were pre-incubated +/- OSGE (80 μg/mL) or PNGase (10,000 U/mL+1/10 reaction buffer, 60 min, 37°C) and stimulated (VWF (10 μg/mL) + ristocetin (1.2 mg/mL), VWF/risto) and binding to fluorescein-conjugated MAL-1, ECL, PNA, SNA and RCA-1 lectins was measured by flow cytometry (n=3). Data +/- SEM. B) Washed platelets were stimulated with VWF/risto with or without recNEU (0.2 U/mL). Fluorescein-conjugated lectins MAL-1, ECL, PNA, SNA and RCA-1 (5 μg/mL) were added and samples were measured. Data was normalised and is shown as % of unstimulated control. *p<0.05 significant against unstim, (n=3, 1-way ANOVA). C) Washed platelets were stimulated with ristocetin only (1.2 mg/mL risto) or VWF/risto prior to measurement of binding to fluorescein-conjugated lectins by flow cytometry (n=3). D) Washed platelets were stimulated with α-thrombin (0.1 U/mL) and binding to fluorescein-conjugated lectins was measured by FACS (n=4).

Suppl. Figure S2: Granule and lysosome release following VWF-mediated GPIbα activation.
Washed platelets were stimulated with VWF/risto (Table 1), with or without recNEU (0.2 U/mL), calcium (1 mM), fibrinogen (500 μg/mL) and OSGE (80 μg/mL) and A) LAMP-1 (n=3), B) P-selectin (n=4) and C) PAC-1 binding (n=3) was measured. Results are shown as mean fluorescent intensities (MFI) values. Data+/- SEM. *p<0.05 significant against unstim; † = significant against control (1-way ANOVA).

Suppl. Figure S3: DANA inhibits desialylation.
PRP was incubated with/without NEU inhibitor DANA (1 mM, 15 min, 37°C) prior to addition of recNEU (0.2 U/mL, 30 min, 37°C) and fluorescein-conjugated RCA-1 lectin was added. Results are shown as MFI values +/- SEM. *p<0.05 significant against control, (n=3, paired t-test).

Suppl. Figure S4: Intracellular location of NEU2.
Unstimulated and stimulated (VWF/risto) non-permeablisised washed platelets were stained for NEU1 (red) and intracellular proteins (green): β-Galactosidase (β-Gal), LAMP-1, factor V (FV), mitochondria (Mt), visualised by fluorescence microscopy. Mitochondrial protein is not retained on the platelet membrane.
RCA-1 binding (MFI)

- recNEU
- recNEU+DANA

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