Supplementary data

Materials and Methods

Materials

Fetal Bovine Serum (FBS) from GIBCO Life Technologies, Carlsbad, CA. 100 µM cell screens were from EMD Millipore, Germany. Acid-Citrate-Dextrose (ACD) was from BD Biosciences, Franklin Lakes, NJ. Streptomyces hyaluronidase, Benzonase® and prostaglandin E1 were from Sigma Aldrich, St. Louis, MO. Thrombin Receptor Activating Peptide (TRAP-6) was from Tocris Bioscience, UK. Anion exchange spin columns were from Thermo Scientific, Waltham, MA. Hyaluronan ELISA-like assay was from Echelon Biosciences, Salt Lake City, UT. Select-HA™ was from Lifecore Biomedical, LLC, Chaska, MN. Vectasheild Mounting Medium with DAPI was from Vector Labs, Burlingame, CA.

Cell Isolation and Culture

Human intestinal mucosal smooth muscle cells (M-SMCs) were isolated from colon surgical specimens (obtained from the Department of Surgical Pathology at the Cleveland Clinic) and cultured as previously described. Briefly, 3-centimeter strips of the colon mucosal layer were incubated in Hanks’ BSS (HBSS) containing 0.15% dithiothreitol (w/v) for 30 min, washed in HBSS containing 1 mM EDTA for 3 h, and finally washed in HBSS for 2 h. The strips were cut into small pieces and then digested with 0.1 mg/mL collagenase and 0.1 mg/mL DNase for 18 h. The liberated cells were filtered through 100 µM cell screens, washed, suspended in DMEM/F12 media containing 10% FBS and cultured on tissue culture plastic incubated at 37°C in a 5%CO₂ humidified environment.

Platelet Isolation

Blood was collected by sterile venipuncture (without use of a tourniquet) directly into tubes containing Acid-Citrate-Dextrose (ACD) from consented healthy donors as approved by the Cleveland Clinic Institutional Review Board. The citrated blood was centrifuged for 20 min at 200g without breaking. The Platelet-Rich Plasma (PRP) layer was then collected, prostaglandin E1 was added to a final concentration of 35 ng/ml, and the PRP centrifuged for 20 min at 600g. The resulting platelet pellet was then washed twice with HBSS containing 35 ng/mL PGE1.

Immunofluorescence histochemical staining of M-SMCs

M-SMCs from the co-culture assay grown on coverslips were washed and then fixed in cold methanol for 5 min. Coverslips were incubated in blocking buffer (2% FBS in HBSS) for 1 h at RT, and then in blocking buffer containing 5 µg/mL biotinylated HA binding protein (EMD Millipore, Germany) and anti-CD42b (1:100) for 18 h at 4°C. Coverslips were then washed and incubated with blocking buffer containing detection reagents Alexa Fluor® 488 streptavidin (1:500) and Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000) (Life Technologies, Carlsbad, CA) for 45 min at RT. The stained coverslips were washed and mounted onto slides. Images were obtained using Leica TCS SP5 II Confocal or Leica upright microscope DM5500 B (Leica, Germany).
Platelet microparticle isolation

Platelet microparticles were isolated as previously described.² Briefly, freshly isolated platelets were incubated without or with 15 μM TRAP for 10 minutes at 37°C and centrifuged at 750g for 20 minutes (platelet fraction). The supernatant platelet releasate was placed on ice and centrifuged at 10,000g for 30 minutes at 4°C to obtain the microparticles fraction. Platelet fraction (750g pellet), microparticles’ fraction (104 pellet), and platelet releasate (104 supernatant) were analyzed by western blotting for the presence of HYAL2. Microparticle formation was also induced as described previously³. Washed mouse platelets were incubated with 1 U/mL thrombin and 50 μg/mL collagen at 37°C for 45 minutes. Following activation, platelets were removed by centrifugation at 1000g for 20 minutes, and supernatant was analyzed for HYAL2 by immunoblotting.

Immunohistochemical staining of platelets

Resting or TRAP-activated freshly isolated platelets were fixed in 3.5% paraformaldehyde for 30 min at RT. Fixed platelets were spun onto a poly-l-lysine coated 8-well chamber slide at 250g for 10 min. Where indicated, permeabilization of platelets was done by adding cold methanol for 5 min. Fixed Specimens were incubated with blocking buffer (2% FBS in HBSS) for 1 h. Non-permeabilized platelets were incubated with antibodies against HYAL2 and P-selectin. Permeabilized specimens were incubated with an antibody against HYAL2 and an antibody against one of the following: CD42b, LAMP2, PDI, KDEL, LAMP-1, NEU1, P-selectin, vWF, and fibrinogen. A permeabilized specimen was incubated with antibodies against P-selectin and vWF as a positive control for co-localization. After washing, specimens were incubated with the appropriate Alexa Fluor® secondary antibody for 45 min at RT, washed again, and Vectasheild Mounting Medium with DAPI was added. Images were obtained using either Leica TCS SP5 II Confocal/Multi-Photon high speed upright microscope or Leica upright microscope DM5500 B (Leica, Germany). Pearson’s correlation coefficients were obtained by analyzing individual images of the Z-stack using Image-Pro Plus software (Rockville, MD). See supplementary table 1 for a list of antibodies and concentrations used.

Flow Cytometry

Freshly isolated resting or TRAP-activated (25μM for 1 min at RT) platelets were fixed in 1% paraformaldehyde for 2 h at RT. Fixed platelets were washed then incubated for 1 h at RT in flow cytometry buffer (0.1% BSA, 0.05% sodium azide in PBS) with anti-HYAL2 (Thermo) (1:100), Allophycoerythrin (APC)-conjugated anti-P-selectin (Ebioscience) (1:1000), and anti-CD16/CD32 (to block the Fc receptors, 1:100). Platelets were washed then incubated with Alexa Fluor® 568 Goat Anti-Rabbit IgG for 30 min at RT. 30,000 events/sample were acquired on a BD Bioscience LSR II (BD Biosciences) flow cytometer. Data was analyzed using FlowJo (Tree Star, Ashaland, OR).

Subcellular fractionation of platelets using differential centrifugation

Freshly isolated platelets were sonicated and subcellular fractionation was achieved as previously described.⁴,⁵ Briefly, platelet lysates were centrifuged at 1,000g to remove unlysed platelets, and then at 19,000g to obtain pellets that contain granules and lysosomes. Supernatants were ultracentrifuged at
100,000g to obtain pellets containing membranes and supernatants containing cytosol. Membranes were further fractionated into dense tubular system (DTS) and plasma membranes by layering on 40% sucrose and ultracentrifuging at 100,000g. Fractions containing equal total proteins were analyzed by immunoblotting for the presence of HYAL2 and CD42b.

**Immuno-electron microscopy**

Freshly isolated resting or TRAP-activated platelets were fixed with 3.5% paraformaldehyde and 0.09% gluteraldehyde for 30 min at RT and then centrifuged. Platelet pellets were washed with PBS, dehydrated with ethanol (30%-100%), embedded with LR White medium, and polymerized at 50°C for 48 h. Ultrathin sections cut with a diamond knife (87 nm) were mounted on formvar-coated nickel grids. Grids were rehydrated with PBS, blocked with 1% BSA for 30 min, and incubated with rabbit anti-HYAL2 antibody (Abcam) (1:50) for 18 h. Grids were washed and incubated with 10 nm gold-conjugated goat anti-rabbit IgG (Redding, CA) (1:10) for 1 h. Grids were washed again, fixed with 1% gluteraldehyde, and washed with distilled water. Grids were then stained with uranyl acetate and lead citrate, dried and examined with a FEI Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (FEI Company, Hillsboro, OR) at 60 kV.

**Statistical analysis**

All statistics were performed using the Student t test. Data is presented as mean ± standard error unless otherwise mentioned. Patient data was presented as scatter plot showing mean ± standard error and as box-and-whiskers plot showing median and 10-90 percentiles. P-values below 0.05 were considered statistically significant.

**References**


Supplementary Table 1: List of antibodies used for the immunohistochemical detection of platelet organelles.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species</th>
<th>Company</th>
<th>Concentration used</th>
<th>Secondary antibody used*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYAL2</td>
<td>Rabbit polyclonal</td>
<td>Thermo Scientific (Waltham, MA)</td>
<td>1:50</td>
<td>Alexa Fluor® 488 Donkey Anti-Rabbit IgG (1:1000)</td>
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<tr>
<td>CD42b</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000)</td>
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<tr>
<td>LAMP1</td>
<td>Mouse monoclonal</td>
<td>Ebioscience (San Diego, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
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<tr>
<td>LAMP2</td>
<td>Mouse monoclonal</td>
<td>Ebioscience (San Diego, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
<tr>
<td>PDI</td>
<td>Mouse monoclonal</td>
<td>Abcam (Cambridge, England)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
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<td>KDEL</td>
<td>Mouse monoclonal</td>
<td>Abcam (Cambridge, England)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
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<td>vWF</td>
<td>Goat polyclonal</td>
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<td>Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000)</td>
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<tr>
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<td>Rabbit polyclonal</td>
<td>Abcam (Cambridge, England)</td>
<td>1:100</td>
<td>Alexa Fluor® 488 Donkey Anti-Rabbit IgG (1:1000)</td>
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<td>P-selectin</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
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<td>Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000)</td>
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<tr>
<td>Fibrinogen</td>
<td>Mouse monoclonal</td>
<td>Cedarlane Labs (Burlington, ON)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
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<tr>
<td>NEU1</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
</tbody>
</table>

* All secondary antibodies were purchased from Life Technologies (Carlsbad, CA).