Microparticle phenotypes are associated with driver mutations and distinct thrombotic risks in essential thrombocythemia

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doi:10.3324/haematol.2016.144279

Supplemental materials and methods

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Platelet-free plasma preparation

Blood was collected in the morning. Venipuncture of the antecubital vein was performed with a 21-Gauge needle following the application of light tourniquet. Blood was collected into 3.2% (0.109M) BD Vacutainer 2.7 mL citrate blood collection tubes. The first 3mL of blood were discarded. Samples were centrifuged at room temperature, 2500g for 15 min. Plasma was collected into a plastic tube, leaving 1 cm of plasma above the buffy layer. The plasma was then centrifuged a second time at 2500*g* for 15 min at room temperature. Then, platelet-free plasma (PFP) was collected, leaving approximatively 100µL at the bottom of the tube, homogenized, aliquoted and stored at -80°C until testing.

Labelling procedure for flow cytometry

Microparticles were enumerated and characterized in frozen-thawed PFP samples (maximum storage of 2 months at 80°C) with flow cytometry (Cytomics FC500, Beckman Coulter, Miami, USA). Thirty µL of freshly thawed PFP sample were incubated for 30 min at room temperature in the dark with 1 µL FITC-annexin V and 5 µL of fluorescent-labelled CD-specific monoclonal antibodies or 5 µL isotype-matched control monoclonal antibodies (all from Beckman Coulter, Miami, USA). Phenotyping was performed by colabelling with annexin V and the following lineage-specific antibodies: CD235a (red cell-MP, RMP; KC16), CD14 (monocyte-MP, MoMP; RMO52), CD11b (granulocyte-MP, GMP; Bear1), CD144 (endothelial-MP, EMP; TEA1/31), CD41 (thereafter referred to as PMP: platelet-derived MP; P2). PMPs were further characterized as coexpressing CD62P (P-selectin^{+} PMP; CLB-Thromb/16) or not. Incubation was followed by dilution with the Ca^{2+}-binding buffer (500 µL) provided by the manufacturer (annexinV kit, Beckman Coulter).

MPs enumeration was performed in duplicate among a gate delineated with 0.5-0.9 µm beads (Megamix® beads, BioCytex, Stago, Marseille, France). The cut off was adjusted on the basis of the results of matched isotype controls. Absolute values were determined using counting beads (Flow-Count®, Beckman Coulter). In addition, normal pooled platelet-free plasma from 20 healthy donors was included as inter run control. MPs counts were per µL.

Experimental conditions for determination of procoagulant activity of MPs

1-Phospholipid-dependent procoagulant activity of MPs

Phospholipid-dependent procoagulant activity of MPs was determined using the Zymuphen™ MP-activity assay (Hyphen BioMed). MPs were captured with immobilized annexin V, thereafter incubated with calcium, Xa-Va and prothombin. Under these experimental conditions, thrombin generation depends on the procoagulant phospholipid surface provided by MPs. MP-associated procoagulant activities were quantified as phosphatidylserine equivalents (nM).

2- Thrombin generation

Thrombin generation was investigated with Calibrated Automated Thrombography (CAT). PFP was supplemented with corn trypsin inhibitor (40 µg/mL, final concentration, Hematologic Technologies Inc., Essex Junction, USA) to limit activation of the contact phase. Results of CAT are represented as the ratio of the peaks of thrombin after initiation by 1 pM of TF without/with added phospholipids 4µM, to take into account the variable thrombin potential of plasmas (all reagents from Diagnostica Stago). Thrombin generation in the absence of added phospholipids depends on endogenous procoagulant surfaces, i.e. MPs. We used the maximal concentration (peak) of thrombin as a sensitive and reliable parameter of procoagulant surfaces.