Supplementary Material to Guglielmini, Appoloni, et al. "Matrix metalloproteinase-2 enhances platelet deposition on collagen under flow conditions" (Thromb Haemost 2016; 115.2)

Suppl. Methods

High shear stress-induced platelet activation: effect of ADP

Citrated whole blood was preincubated for 2 min with ADP 6µM (Mascia Brunelli, Italy), prior to the filtration test.

TIMP-2 measurement

Blood was collected prior and at various intervals after the beginning of filtration, centrifuged at 3000xg for 20 minutes, and the supernatant plasma was then immediately frozen and stored at -80°C for later TIMP-2 concentration determination.

TIMP-2 measurements were performed using a commercial ELISA kit (TIMP-2: RayBiotech, Norcross, GA, USA) as previously described (Suppl. Ref 1).

β-thromboglobulin (β-TG) assay.

Blood was collected prior and at various intervals after the beginning of filtration, centrifuged at 3000xg for 20 minutes, and the supernatant plasma was then immediately frozen and stored at -80°C for later β-TG measurement.

β-TG measurement were performed using a commercial ELISA kit (Asserachrom β-TG: Diagnostica Stago, France), as previously described (17).

Platelet adhesion to immobilized collagen or active MMP-2 under static conditions.

Washed platelets were obtained with the Mustard method, as described (Suppl. Ref. 2).

200µl of washed platelets were then layered over a collagen (100µg/ml)- or MMP-2 (100µg/ml)-coated coverslip and let adhere for 60 minutes. Coverslip were then washed 3 times with PBS and fixed with PFA/PBS 4% for 20 minutes. PFA was then removed by washing with PBS and deposited platelets were permeabilized with Triton X100 0.1% in PBS for 20 minutes. Coverslips were then washed and blocked with BSA/PBS 1% 1h.
FITC-Phalloidin Staining.

Actin fibers where stained with FITC-Phalloidin (Sigma-Aldrich) diluted 1:250 in BSA/PBS 1%, for 30 minutes, and then washed 3 times with PBS and mounted with Mowiol (Suppl. Ref 3).

Adhesion was evaluated with a Carl Zeiss Axio Observer. A1 fluorescence microscope, using a 100X/1.4 Plan-Apochromat oil-immersion objective at 200x final magnification except for panel D (65X Plan-Apochromat oil-immersion objectives).

Pretreatment with active MMP-2 of a collagen-coated surface and subsequent platelet adhesion.

Active MMP-2 (50ng/ml), Inhibitor II (10µM), the combination of the two, or their vehicles were perfused at 580µl/min (i.e. the flow speed needed to obtain a shear rate of 3000 sec\(^{-1}\) with whole blood) for 5 min at 37°C over a collagen coated coverslip and, after washing the surface with 1% BSA/PBS, citrated whole blood was perfused at 3000 sec\(^{-1}\) for 5 min at 37°C. At the end of perfusion, coverslips were treated as described in the Methods section.

Suppl. References


Suppl. Figure 1: Modifications of TIMP-2, MMP-2/TIMP-2 ratio and β-TG levels induced by high-shear stress.

A) **TIMP-2 concentration before and after high shear-stress-induced platelet activation (O’Brien filtration test).** TIMP-2 concentration before and at various intervals after shear-induced platelet activation in plasma prepared from citrated human blood. Data are expressed as means±S.D. (n= 6).

B) **MMP-2/TIMP-2 ratio.** MMP-2/TIMP-2 ratio before and at various intervals after shear-induced platelet activation in plasma prepared from citrated human blood. Data are expressed means±S.D. (n= 6). *p<0.05 vs baseline

C) **β-TG concentration.** β-Thromboglobulin concentration before and at various intervals after shear-induced platelet activation in plasma prepared from citrated human blood. Data are expressed as means±S.D. (n= 6). * p< 0.05 vs baseline; ** p<0.01 vs baseline
Suppl. Figure 2: MMP-2 do not increase surface coverage on a collagen-coated surface.

Platelet interaction with immobilized collagen (Type I collagen from human placenta) under flow conditions was studied by confocal microscopy using a modification of the Hele-Shaw flow chamber. Human platelets were labeled in whole blood by the fluorescent dye mepacrine (10µM). Whole blood was then incubated with MMP-2 (50ng/ml) for 5 minutes and perfused for 5 minutes at 1600 sec\(^{-1}\).

A) Analysis of confocal sections corresponding to the initial layers (0-2µm) of platelets deposited on the coverslip surface.

B) Groups of 10 consecutive confocal sections (corresponding to a thickness of 2µm) were analyzed and the mean surface coverage was calculated for each. Data are expressed mean±SD (n=10). * p<0.05 vs Vehicle

Data are expressed as means±SD (n=10).
A. MMP-2
   Static Adhesion

B. Collagen
   Static Adhesion

C. MMP-2
   Adhesion under flow

D. Collagen
   Adhesion under flow
Suppl. Figure 3: Platelet adhesion to an active MMP-2-covered surface.

Washed platelets were layered over an active MMP-2 (100µg/ml)- or Type I fibrillar collagen (type I from equine tendon) (100µg/ml)-coated coverslip and let adhere for 60 minutes, then washed, fixed, permeabilized and stained with FITC-phalloidin. (200x final magnification).

A) Adhesion to active MMP-2 under static conditions.

B) Adhesion to type I fibrillar Collagen under static conditions.

C) Platelet deposition on active MMP-2 under flow conditions.

   Human whole blood, was perfused for 5 minutes on a coverslip coated with human recombinant active MMP-2. Platelet deposition on active MMP-2 (10µg/ml) immobilized on a glass coverslip, under flow conditions (3000 sec⁻¹, 5 min), and then fixed, permeabilized and stained with FITC-phalloidin (200x final magnification) was analyzed.

D) Platelet deposition on type I fibrillar collagen under flow conditions.

   Human whole blood, was perfused for 5 minutes on a coverslip coated with type I fibrillar collagen. Platelet deposition on type I fibrillar collagen (10µg/ml), immobilized on a glass coverslip, under flow conditions (3000 sec⁻¹, 5 min), and then fixed, permeabilized and stained with FITC-phalloidin (65x final magnification) was analyzed.
Suppl Figure 4: High shear stress-induced platelet activation: effect of ADP.

Citrated whole blood was preincubated for 2 min with ADP (6µM), prior to the filtration test.

A) Filter occlusion time (sec).

B) Platelets retained between 20 and 40 sec (% of total).

*p<0.05 vs Control;
§ p<0.05 vs 0-5 sec
Suppl. Figure 5: Platelet deposition on immobilized collagen (type I from equine tendon) under flow conditions is inhibited by RGD.

Effect of MMP-2 (50ng/ml) and/or dRGDW (200 µM) (preincubation 2min at 37°C) on human platelet deposition on collagen at 3000 sec⁻¹ shear rate in whole blood. After shear coverslip were fixed and stained (May-Grünwald/Giemsa).

Representative images of platelet adhesion on collagen.

A) Control

B) MMP-2 (50ng/ml)

C) dRGDW (200µM)

D) dRGDW + MMP-2
Suppl. Figure 6: Pretreatment with active MMP-2 of immobilized collagen (type I from equine tendon) does not affect platelet deposition under flow conditions.

Effect of the pretreatment of a collagen coated surface (5 min, 37°C, at a flow rate of 580 µl/min) with MMP-2 (50ng/ml), Inhibitor II (10µM), the combination of the two or their respective vehicles. Platelet deposition was then carried out at 3000 sec⁻¹ shear rate in whole blood. Coverslip were fixed and stained (May-Grünwald/Giemsa).