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Activation state of platelet in experimental severe hemophilia A

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Factor VIII (FVIII) is a key protein of the coagulation cascade, where it acts as the cofactor of activated factor IX. Upon activation, FVIII dissociates from von Willebrand factor and binds to phospholipids at the surface of activated platelets to form the tenase complex, thus leading to the activation of factor X, to a burst of generation of thrombin and to the formation of fibrin that consolidates the platelet clot. Abnormalities in the gene encoding FVIII lead to haemophilia A (HA), a rare X-linked recessive hemorrhagic disorder that concerns one male in 5000. Depending on the residual activity of FVIII in plasma, HA is classified as severe (<1%), moderate (1 to 5%) or mild (5 to 25%). Although this classification correlates with the bleeding phenotype, about 10% of severe HA patients rarely bleed spontaneously, indicating that FVIII activity is not the only parameter defining the phenotypic severity of the disease.

In their recent publication, van Bladel et al investigated the basal state of activation and responsiveness of circulating platelets under conditions of abnormal haemostasis as a potential parameter influencing the bleeding phenotype of the patients. They found that the state of activation of platelets is up-regulated in patients with severe HA. The percentage of platelets expressing CD62P (P-selectin) at their surface was greater in patients with severe HA (15.9%) as compared to that in patients with mild/moderate HA (8.2%) and in healthy controls (6.4%). An earlier report had however failed to find signs of enhanced platelet pre-activation in patients with severe HA. In order to address the question in vivo in a system that is not ‘perturbed’ by the different genetic or environmental factors that are typical of the human population, we studied the state of activation and responsiveness of platelets from wild-type mice and FVIII-deficient (FVIII°) mice, an experimental model of severe haemophilia A. To this end, wild-type and FVIII° mice were crossed on the C57Bl/6 background in our laboratory to ensure the lowest genetic variability with the exception of the HA-causing gene alteration.
Blood was collected on ACD-C buffer (124 mM sodium citrate, 130 mM citric acid, 110 mM dextrose, pH 6.5) from 12-14-week old mice by intra-cardiac puncture. Animals were handled in agreement with local ethical authorities (ethical committee in animal experiment “Charles Darwin” Ce5/2010/045). Platelets were purified in the presence of inhibitors (0.3 U/ml apyrase and 100 nM prostaglandin E1) as described. The purity of platelets was greater than 99% as assessed by the expression of CD41 and size estimate (side scatter) by flow cytometry (data not shown). Platelets were then stimulated by the thrombin receptor-activating peptide TRAP-4. The state of activation of platelets was estimated by the surface expression of CD62P, of αIIbβ3 (GPIIb/IIIa) and by the surface binding of fibrinogen, measured by flow cytometry. Results are expressed as mean fluorescence intensities (MFI±SEM) on the total platelet population (Figure 1).

Unstimulated platelets demonstrated low levels of expression of CD62P, αIIbβ3 and of surface-bound fibrinogen, that did not differ between wild-type mice and FVIII° mice (wild-type: 85±20, 479±60 and 173±49 MFI, respectively; FVIII°: 71±9, 519±74, and 217±55 MFI respectively). Interestingly, similar levels of expression of CD62P and αIIbβ3 were measured on platelets in fresh blood (data not shown), suggesting that the purification procedure does not activate the platelets. In the case of wild-type mice, incubation of the platelets with TRAP-4 resulted in a drastic increase in the expression levels of CD62P, αIIbβ3 and in surface-bound fibrinogen (916±138, 3282±236, and 1714±182 MFI respectively; P<0.05 in all cases as compared to unstimulated platelets), indicating that the purified platelets were functional and susceptible to activation. The incubation of platelets from FVIII° mice with TRAP-4 yielded expression levels of CD62P, αIIbβ3 and levels of surface-bound fibrinogen (1151±186, 4529±851 and 2103±255 MFI, respectively) that were statistically identical to that induced by TRAP-4 on platelets from their wild-type littermates. Similar results were
obtained with different concentrations of TRAP-4 (Figure 1) and when thrombin was used instead of TRAP-4 (data not shown).

Taken together, our data indicate that the absence of pro-coagulant FVIII in un-manipulated adult animals is associated neither with alterations in the activation status of circulating platelets nor with their ability to be activated. In patients with severe HA, the most significant morbidity is the development of arthropathy in joints, that results from recurrent intra-articular hemorrhages. In our laboratory conditions, FVIII° mice do not bleed spontaneously and do not experience premature mortality. Indeed, FVIII° mice do not develop arthropathy unless bleeding is experimentally provoked in the articulations.8, 9 Whether the state of activation and responsiveness of platelets differs under conditions of chronic hemorrhages in haemophilic mice remains to be investigated.
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References

**Legend to Figure**

**Figure 1.** Basal activation status and responsiveness of platelets from FVIII-deficient mice (FVIII°) and their wild-type littermates (WT). Platelets were purified from the blood of WT (open bars) and FVIII° mice (full grey bars). Purified platelets were incubated alone or with 100 µM or 150 µM of TRAP-4 in tyrode buffer for 15 min at room temperature. The surface expression of CD62P (upper panel), αIIbβ3 (middle panel) and surface binding of fibrinogen (lower panel) were determined by flow cytometry using a PE-conjugated rat anti–mouse CD62P monoclonal antibody (Wug.E9), a PE-conjugated rat anti–mouse αIIbβ3 monoclonal antibody (JON/A), and oregon-green-labelled fibrinogen, respectively. Results (5 to 8 mice per group) are expressed as mean fluorescence intensities (MFI) ± standard error mean (SEM). Differences were compared statistically using the non-parametric Mann-Whitney test.