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Monitoring platelet dependent thrombin generation in mice

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ABSTRACT

Calibrated automated thrombin generation assay was adapted to measure thrombin generation in platelet rich plasma from mice. Vena cava phlebotomy appeared the best technique for blood sampling. The concentration-effect curves of tissue factor and platelet count have been determined. Corn trypsin inhibitor 2 μM inhibits contact activation effectively. Recombinant human thrombomodulin does not inhibit thrombin generation in mouse plasma but activated protein C (20 nM) does. Thrombin generation was dose dependently diminished by low molecular weight heparin and increased by high concentrations of exogenous factor VIII i.e. the assay can detect both hypo- and hypercoagulability.

Introduction

Laboratory mice have become an important and frequently used model for studying haemostasis. It has been previously shown that the routine laboratory assays used to assess human coagulation, e.g. prothrombin time, activated partial thromboplastin time, are also suitable to assess murine coagulation [1]. However, the correlation of these assays with the clinical risk of bleeding or thrombosis appears weak explaining the growing interest of physicians for global haemostasis assays such as thrombin generation test (TGT) [2]. Recently, Tchaikovski et al. [3] developed a calibrated automated thrombography -based thrombin generation assay in mouse plasma enabling the detection of factor V Leiden, oral contraception and pregnancy-induced hypercoagulability in mouse plasma.

In vivo thrombin generation is strongly dependent upon platelet procoagulant functions. Interactions between platelets and the clotting system escape our attention when clotting is studied in platelet poor plasma (PPP). Platelet rich plasma (PRP), however, is the optimal material to explore the haemostatic capacity of patients with inherited and acquired platelet disorders. Platelets are also intimately involved in several steps of thrombin generation induced by recombinant activated factor VII (rFVIIa) in haemophiliacs with inhibitors. We, therefore employed the Calibrated automated thrombin generation assay (CAT) to measure thrombin generation in mouse PRP and optimized the assay conditions to detect hypo- and hypercoagulability in mouse PRP.

Materials and methods

Animals and blood sampling

Experiments have been performed in twenty four female mice of the C57BL/6 strain weighting approximately 25 grams and 8-12 weeks of age. The mice had free access to water and diets. The animals were housed in usual cages in an environment with controlled temperature at 20 ± 2 °C.

Mouse blood was collected from the inferior vena cava (IVC) under isoflurane 5% anesthesia, unless otherwise indicated. As previously described, the abdominal cavity was opened and 180 μL 3.2% sodium citrate was i.v. administrated in the IVC, using a 23 gauge needle and 1 mL plastic syringe, 15-20 seconds prior to blood drawing from the same vein into the syringe [4].

Platelet rich plasma (PRP) was prepared immediately after blood drawing by centrifugation at 200×*g* during 3 minutes at room temperature. Platelet poor plasma (PPP) was obtained by double centrifugation: samples were centrifuged at 2500×*g* during 15 minutes at room temperature, subsequently plasma was centrifuged at 10,000×*g* for 5 minutes to remove platelets and cell fragments. Mouse platelets were counted with a Coulter ACT diff analyzer (Beckman Coulter, Mijdrecht, The Netherlands). PRP was prepared immediately after blood drawing and was used within 2 hours.

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Table 1
Normal thrombin generation values obtained in platelet rich- and platelet poor plasmas from 15 female mice.

N = 15	PRP	PPP
Lag time (min)	2.8 ± 0.35	1.84 ± 0.4
ETP (nM.min)	638 ± 145	583 ± 102
Peak IIa (nM)	84 ± 30	129 ± 18
Time to peak (min)	5.9 ± 0.9	3.6 ± 0.4
Intra-assay CV (%) (n = 3)	8.2 ± 2.7	10.7 ± 1.9

Calibrated Automated Thrombin generation assay (CAT) reagents

Recombinant human tissue factor Innovin® was obtained from Siemens (Marburg, Germany). The phospholipid vesicles were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA) and consisted of 20 mol% phosphatidylserine (PS), 20 mol% phosphatidylethanolamine (PE) and 60 mol% phosphatidylcholine (PC) and were prepared by extrusion method [5,6]. Hepes-buffered saline contained 20 mM Hepes, 140 mM NaCl and 5 mg/mL bovine serum albumin (BSA), pH 7.35 [7]. This buffer was stored at -20 °C until use. A fresh mixture of fluorogenic substrate and $CaCl_2$ was prepared before each experiment. Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). The mixture of 2.5 mM fluorogenic substrate and 100 mM $CaCl_2$ was prepared using buffer containing 20 mM Hepes and 60 mg/mL BSA, pH 7.35. The Calibrator with the activity of 600 nM human thrombin was obtained from Thrombinoscope BV (Maastricht, The Netherlands). Transparent, round bottom microtiter 96-well plates (Thermo Fisher Scientific Immulon, Helsinki, Finland) were used. Activated protein C (APC) was purified according to Regnault et al. [8], recombinant human thrombomodulin (TM) preparation was a kind gift of Asahi (Japan). Corn trypsin inhibitor (CTI) was obtained from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Recombinant factor VIII (Advate®) was obatined from Baxter Healthcare (Vienna, Austria) and low molecular weight heparin, enoxaparin (Lovenox®) was obtained from Sanofi Synthelabo (Paris, France).

Calibrated Automated Measurement of Thrombin Generation (CAT) in mice

Thrombin generation was measured using a Fluoroscan Ascent® fluorometer (Thermolab systems OY, Helsinki, Finland), equipped with a dispenser. Fluorescence intensity was detected at wavelengths of 390 nm (excitation filter) and 460 nm (emission filter). A dedicated software program, Thrombinoscope® version 3.0.0.29 (Thrombinoscope bv, Maastricht, The Netherlands) enabled the calculation of thrombin activity against the calibrator (Thrombinoscope bv, Maastricht, The Netherlands) and displayed thrombin activity with the time. All experiences were carried out in duplicate at 37 °C and the measurements usually lasted 60 minutes.

The most important parameters that can be derived from CAT are lag time, endogenous thrombin potential (ETP) corresponding to the area under the CAT curve, peak height of thrombin corresponding to the maximal amount of thrombin that can be generated by the plasma sample during the thrombin burst.

Data analysis

Data were expressed as mean ± SD. Groups were compared using Mann Whitney test (Instat, GraphPad, CA, USA). An a priori α of $p \leq 0.05$ was considered statistically significant.

Results

The effect of sample dilution on thrombin generation measurement in mouse PRP

Thrombin generation was measured at 37 °C, triggered by 1 pM TF and using PRP diluted at 1:1, 1:2, 1:3, 1:4 and 1:6 in Hepes-buffered saline. Thrombin generation in 1:1 and 1:2 diluted PRP was hardly detectable with very low peak thrombin and ETP, and a reliable calculation of ETP was impossible. Higher peak thrombin and ETP results were obtained using higher dilutions of PRP. In most of the experiments, ETP was directly calculated by the Thrombinoscope software when thrombin generation test was performed in PRP diluted at 1:6.

Table 1 shows normal thrombin generation results triggered with TF 1 pM in mice PRP and PPP (n = 15) diluted at 1:6, as well as the intra-assay coefficient of variation on the basis of ETP, determined in 3 different PRP samples measured 6 times in a single run.

The effect of tissue factor concentration on thrombin generation measurement in mouse PRP

Thrombin generation in PRP was determined in the presence of increasing concentrations of TF at 0.25, 0.50, 1, and 2 pM (final concentrations) using PRP $150x10^9$/L at 37 °C (n = 6 mice). The PPP samples from the same mice were also tested using the same TF concentrations and with a final phospholipid concentration of 4 μM. A dose-dependent increase of peak thrombin and ETP was observed in both PRP and PPP associated with a decrease of the lag time (Fig. 1). In

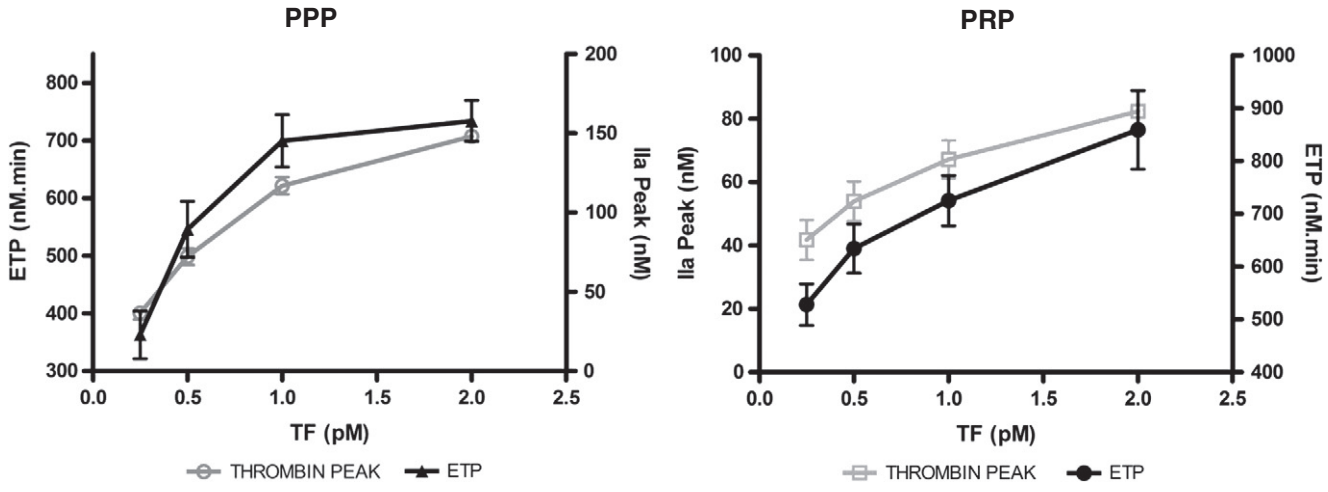

Fig. 1. Effect of tissue factor concentration on ETP and thrombin peak in platelet rich and platelet poor plasma samples.