

ORIGINAL ARTICLE

Development of a calibrated automated thrombography based thrombin generation test in mouse plasma

S. N. TCHAIKOVSKI,* B. J. M. VAN VLIJMEN,† J. ROSING* and G. TANS*

*Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht; and †Eindhoven Laboratory for Experimental Vascular Medicine, Department of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, The Netherlands

To cite this article: Tchaikovski SN, van Vlijmen BJM, Rosing J, Tans G. Development of a calibrated automated thrombography based thrombin generation test in mouse plasma. *J Thromb Haemost* 2007; 5: 2079–86.

Summary. *Background:* Mouse models have become increasingly important in thrombosis research. However, only a limited number of assays are available for assessment of the coagulation system in mouse plasma. *Objectives:* To quantify tissue factor-initiated thrombin generation in murine platelet-rich and platelet-free plasma and to develop a test for measurement of resistance to activated protein C (APC) in mouse plasma. *Methods:* Thrombin generation was monitored with calibrated automated thrombography (CAT) using a low-affinity fluorogenic substrate for thrombin. *Results:* To overcome the higher activity of coagulation inhibitors in mouse plasma as compared with human plasma, the reaction temperature was lowered to 33 °C and the assay was carried out at a 2-fold higher final plasma dilution (1:3) than commonly used for CAT in human plasma. This increased the endogenous thrombin potential (ETP) 4- to 5-fold and enabled reliable measurement of thrombin generation in both platelet-free and platelet-rich mouse plasma. For the APC resistance measurement, the reaction conditions were further optimized with respect to tissue factor, phospholipid, APC and CaCl₂ concentrations. The test was validated using plasma of mice with different genetic background with respect to the factor V Leiden mutation (FV Leiden). Mice homozygous for FV Leiden had higher APC sensitivity ratios (mean 5.46; 95% CI 4.88–6.03) than heterozygous FV Leiden mice (mean 4.21; 95% CI 3.53–4.89) and than wild-type mice (mean 2.71; 95% CI 2.15–3.27). *Conclusions:* We have established reaction conditions for measurement of thrombin generation and APC resistance in mouse plasma. This assay enables evaluation of the coagulation system and the function of the protein C system in mouse models.

Keywords: activated protein C resistance, mouse, thrombin generation.

Introduction

Venous thromboembolism (VTE) is a complex disease which results from the interplay between inherited predisposition and environmental factors [1]. The multifactorial origin of VTE, for example, the numerous acquired and congenital risk factors involved, makes the use of animal models in thrombosis and hemostasis research inevitable. Particularly, mouse models are of interest because of the ease of breeding, the availability of the entire genome and the potential for generating genetically modified animals. Various transgenic mouse strains [2] are available for investigation of the (patho)physiology of *in vivo* thrombus formation and clot lysis and for evaluation of new therapeutic agents. Especially, factor V Leiden (FV Leiden) mice may become a useful model to study the interaction between genetic and acquired risk factors in the development of VTE [3,4]. These mice carry the murine equivalent of human FV Leiden [5], a single point mutation in the factor V (FV) gene [6], which is the most prevalent risk factor for VTE in the Caucasian population [7]. The FV Leiden mutation renders FVa less susceptible to inactivation by the natural anticoagulant, activated protein C (APC) [8].

Despite the availability of numerous assays for evaluation of the coagulation system in humans, methods used in mice are restricted to evaluation of induced thrombosis, determination of coagulation factor levels or clotting times [2,9]. However, measurement of thrombin generation, which is an overall functional test, sensitive for both prothrombotic and haemophilic phenotypes [10,11], is currently recognized as an important tool in hemostasis research. The recent development of calibrated automated thrombography (CAT) allows assessment of thrombin generation in clotting (human) plasma with or without platelets. In this paper, we describe the development and optimization of CAT for evaluation of thrombin generation in platelet-free and platelet-rich mouse plasma and we define test conditions for assessment of APC resistance in mouse plasma.

Correspondence: Jan Rosing, Department of Biochemistry, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands.
Tel.: +31 43 3881678; fax: +31 43 3884159; e-mail: j.rosing@bioch.unimaas.nl

Received 1 May 2007, accepted 18 July 2007

Materials and methods

Materials

Ancrod was from NIBSC, Hertfordshire, UK; ecarin from Pentapharm, Basel, Switzerland; the chromogenic thrombin substrate D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide (S2238) from Chromogenix, Mölndal, Sweden and the fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland. A phospholipid emulsion composed of phosphatidylcholine, phosphatidylserine and sphingomyelin (Phospholipid-TGT) was provided by Rossix, Mölndal, Sweden. Human recombinant tissue factor (hrTF) was from Dade Innovin[®], Behring, Germany. Mouse thromboplastin [12] was a gift of R. van Oerle. One unit of mouse thromboplastin equals the activity of 1 pM hrTF (Innovin) in bovine FX activation by human recombinant FVIIa.

Human APC was from Enzyme Research Laboratories, Kordia, Leiden, The Netherlands. Mouse APC was a gift of Dr H. Spronk. The thrombin calibrator and PPP-Reagents were from Thrombinoscope BV, Maastricht, The Netherlands.

Animals

Mice carrying the FVL mutation (R504Q) were described by Cui *et al.* [5]. Factor V R504Q mice were backcrossed to C57Bl/6 J mice (Jackson Laboratories, Bar Harbor, ME, USA) for at least eight generations (N8), and N8 heterozygous mice were inter-crossed to produce homozygous ($n = 16$, 11 males and 5 females, age 7–14 weeks), heterozygous ($n = 18$, 12 males and 6 females, age 7–14 weeks), and wild-type ($n = 18$, 12 males and 6 females, age 7–14 weeks) littermates. The FVL genotype was confirmed by polymerase chain reaction analysis of tail DNA.

Collection and handling of plasma samples

For tail blood collection, restrained mice were placed under a heating lamp (37 °C). The tail was cut 1 cm from the tip. Within 10–15 s, 200 µL blood was collected in Eppendorf tubes (first droplet discarded), 180 µL blood was pipetted to 20 µL 3.2% (w/v) trisodium citrate. Plasma was prepared by centrifugation at 21 000× *g* for 10 min at room temperature (RT), snap-frozen, and stored at –80 °C prior to analysis.

Mouse pooled normal plasma (MPNP) was prepared as follows. Mouse blood was collected as previously described [13] with minor modifications. After i.p. anaesthesia with Nembutal[®] (CEVA; Sante Animale, Libourne, France) 200 mg kg⁻¹, the abdominal cavity was opened and phlebotomy of the vena cava inferior was performed. Blood was drawn into a syringe, containing 3.2% sodium citrate (9:1 vol). Platelet-free plasma (PFP) was prepared by centrifuging twice at 21 000× *g* for 10 min at RT after which the supernatant plasmas of 10 C57Bl/6 healthy mice (5 males and 5 females, 8–14 weeks old) were pooled. Blood samples containing a visible fibrin clot were rejected. MPNP was snap-frozen and stored at –80 °C until use.

Platelet-rich plasma (PRP) was prepared by consecutive centrifugations (3 min at 280× *g* and 1 min at 625× *g*) at RT without a brake and was used within 2 h.

Human pooled normal plasma (HPNP) was prepared by pooling plasma of 87 healthy donors free of medication (53 male and 34 female, mean age 38.6 years) as previously described [14].

Thrombin generation

Thrombin generation was measured in duplicate via CAT in a Fluoroskan Ascent[®] reader (Thermo Labsystems, Helsinki, Finland; filters 390-nm excitation and 460-nm emission) using the fluorogenic substrate Z-Gly-Gly-Arg-AMC [15]. Thrombin generation curves and the area-under-the-curve (endogenous thrombin potential, ETP) were calculated using the Thrombinoscope[™] software (Thrombinoscope BV, Maastricht, The Netherlands) and thrombin calibrator to correct for inner filter and substrate consumption effects [15]. As mouse thrombin converts the fluorogenic substrate (420 µM) at a 20% lower rate than human thrombin (data not shown), thrombin concentrations and ETP calculated in mouse plasma were corrected for this difference in activity. Alternatively, the correct amount of thrombin generated in mouse plasma will be calculated by the Thrombinoscope[™] software after increasing the calibrator activity by 20% in the program settings. Thrombin generation was initiated by addition of either hrTF or mouse thromboplastin in the presence of calcium (Ca²⁺) and phospholipids as described under Results. APC resistance was assessed by quantification of the effect of added APC (human or mouse) on the ETP. Normalized APC sensitivity ratios (nAPCsr) were defined as the ratio of the ETP's determined in the presence (ETP_{+APC}) and absence of APC (ETP_{-APC}) normalized against the same ratio determined in MPNP in the same experiment.

$$\text{nAPCsr} = \frac{\text{Sample ETP}_{+APC} / \text{Sample ETP}_{-APC}}{\text{MPNP ETP}_{+APC} / \text{MPNP ETP}_{-APC}}$$

Prothrombin activation and subsequent thrombin inactivation

Human and mouse plasma were defibrinated with Ancrod for 10 min at 37 °C, diluted in Buffer A (25 mM HEPES, 175 mM NaCl, 5 g L⁻¹ bovine serum albumin, pH 7.7 at RT) and prothrombin was activated by addition of Ecarin (final concentration 6.5 U mL⁻¹) in the presence of 5 mM CaCl₂ at 37 °C at 1:50 final plasma dilution. The amount of Ecarin added was sufficient to completely activate prothrombin within 30 s. At various time intervals, aliquots of the reaction mixture were diluted twenty-fivefold in ice-cold buffer containing 25 mM HEPES (pH 7.9 at RT), 175 mM NaCl, 60 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 g L⁻¹ ovalbumin. Thrombin amidolytic activity was subsequently determined at 37 °C using S2238 and expressed as percentage of the activity present at 30 s activation.

Results

Reduction of assay volume

Thrombin generation is routinely performed in a total volume of 120 μL , which consists of 80 μL plasma and 40 μL buffer containing phospholipids, TF, fluorogenic substrate and CaCl_2 [15]. Additionally, analysis of thrombin generation requires for each plasma sample calibration of fluorogenic substrate conversion with a thrombin calibrator to correct for 'inner filter effects', substrate consumption, state of the thrombinoscope filters and lamp, and plasma colour. When thrombin generation is assessed in the presence of APC (APC resistance test) a minimum of 240 μL plasma is required for an APCsr determination. With small animals such as mice this represents a great part of the total blood volume. Thus, initial experiments were aimed at reduction of the assay volume. The APC resistance test in HPNP in twice-reduced assay volumes (60 μL total volume containing 40 μL plasma) was compared with the same measurement in 120 μL (80 μL plasma). The decrease in reaction volumes resulted in $\sim 10\%$ reduction of the $\text{ETP}_{-\text{APC}}$ and the $\text{ETP}_{+\text{APC}}$, but the APCsr was not affected. The intra-assay coefficients of variation (human plasma) were 4% and 5% ($\text{ETP}_{-\text{APC}}$), 10% and 9% ($\text{ETP}_{+\text{APC}}$) and 7% and 11% (APCsr) for assay volumes of 120 and 60 μL , respectively ($n = 32$). All further thrombin generation measurements were performed in 60 μL reaction volume.

Measurement of the ETP in mouse plasma

Fig. 1 shows time courses of thrombin generation obtained in human and mouse PFP using commercially available reagents (PPP reagents, Fig. 1A–C) or a coagulation trigger used in the thrombin generation-based APC resistance test for human plasma [16] (13.6 μM TF, 30 μM phospholipid, Fig. 1D). Thrombin generation in mouse plasma showed substantially shorter lag times and lower amounts of thrombin (reduced peak height and ETP) than in human plasma and proved unsuitable for accurate measurement of thrombin generation and APC resistance. The virtual absence of a lag time before the onset of thrombin generation (Fig. 1B–D) triggered at high TF concentrations (5, 13.6 and 20 μM) precluded calculation of thrombin generation curves and ETPs using the ThrombinoscopeTM software. At low TF concentrations (Fig. 1A), thrombin formation was too low to reliably calculate the ETP and determine effects of anticoagulants (e.g. APC).

The low amounts of free thrombin formed in mouse plasma likely result from higher activities of coagulation inhibitors (e.g. antithrombin) in mouse than in human plasma. This was confirmed by the experiment presented in Fig. 2, which shows disappearance of thrombin activity with time in human and murine plasma after complete and rapid activation of plasma prothrombin with Ecarin. The amidolytic activity generated in mouse and human plasma was fully inhibited by hirudin indicating that thrombin was the only coagulation factor contributing to S2238 conversion. As judged from the initial

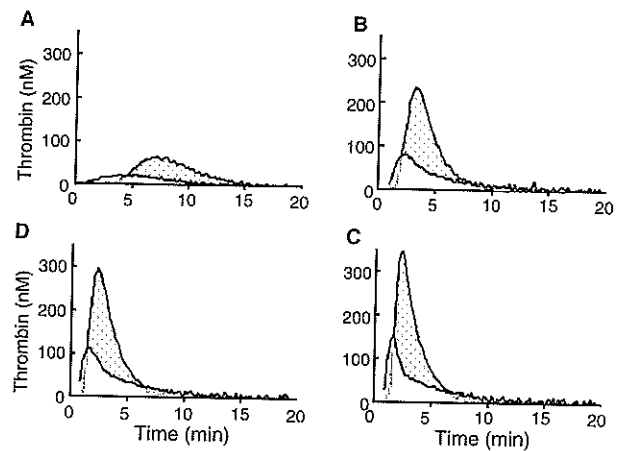


Fig. 1. Calibrated automated thrombography (CAT) in human and in mouse plasma. Thrombin generation in \square mouse and \blacksquare human plasmas was measured by CAT at 37 $^{\circ}\text{C}$ in 60 μL reaction mixtures, containing 40 μL plasma and 10 μL (A) PPP-Reagent LOW (1 μM TF/4 μM phospholipids), (B) PPP-Reagent (5 μM TF/4 μM phospholipids), (C) PPP-Reagent HIGH (20 μM TF/4 μM phospholipids) or (D) 13.6 μM human recombinant tissue factor/30 μM phospholipids. Thrombin generation was initiated with 16.4 mM CaCl_2 and 0.42 mM fluorogenic substrate (final concentrations). As the ThrombinoscopeTM software could not calculate the thrombin generation curves in mouse plasma all curves were calculated from the fluorescence data as previously described [33].

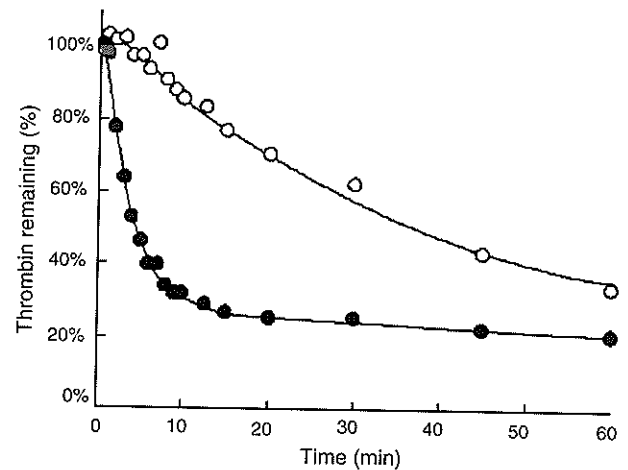


Fig. 2. Inhibition of thrombin in diluted human and mouse plasmas. Plasma prothrombin was fully activated in 1:50 diluted defibrinated human (O) and mouse (●) plasmas by addition of Ecarin to result in complete activation within 30 s. The time course of inhibition of thrombin amidolytic activity by the plasma inhibitors was followed in time and expressed as percentage of the activity determined at 30 s.

slopes of the inactivation time courses, thrombin inhibition was ~ 9 -fold faster in murine than in human plasma.

To overcome the inhibitory activity and to increase the amount of thrombin generated, thrombin generation experiments were performed at higher plasma dilutions and at lower reaction temperatures. Fig. 3A shows the effect of mouse plasma dilution on thrombin generation. The peak height and the ETP (Fig. 3A) increased at higher dilutions, showing an optimum at final plasma dilutions of 1:3 and 1:4. Fig. 3B shows

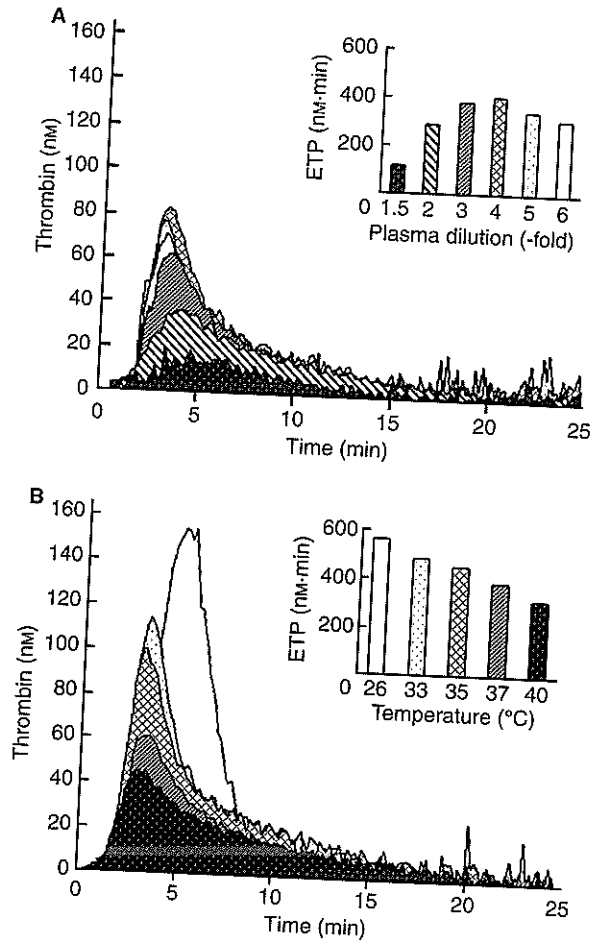


Fig. 3. Effect of plasma dilution and temperature on thrombin generation in mouse plasma. (A) Thrombin generation was measured by calibrated automated thrombography (CAT) with 40, 30, 20, 15, 12 and 10 μL mouse plasma in a total assay volume of 60 μL . Thrombin generation was initiated at 37 $^{\circ}\text{C}$ in the presence of 1 μM hrTF, 16.4 mM CaCl_2 , 4 μM phospholipids, 0.42 mM fluorogenic substrate (final concentrations). Inset: endogenous thrombin potential (ETP) as a function of plasma dilution. (B) Thrombin generation was measured by CAT in reaction mixtures (60 μL) containing 20 μL mouse plasma and 1 μM hrTF, 16.4 mM added CaCl_2 , 4 μM phospholipids, 0.42 mM fluorogenic substrate at temperatures of: 26, 33, 35, 37, and 40 $^{\circ}\text{C}$, respectively. Inset: ETP as function of temperature.

that lowering temperature also increased the ETP. From these experiments the combination of final 1:3 dilution (20 μL mouse plasma in 60 μL total reaction volume) and a reaction temperature of 33 $^{\circ}\text{C}$, together resulting in a 4- to 5-fold higher ETP and a prolonged lag time, were chosen for further optimization of thrombin generation.

Fig. 4 shows that increasing amounts of hrTF increased thrombin generation and reduced the lag time. The effects on the lag time and the ETP were similar when thrombin generation was triggered in mouse plasma with increasing amounts of mouse thromboplastin (data not shown). As 6 μM hrTF yielded a sufficiently high ETP and a reliably measurable lag time (~ 1 min), this TF concentration was chosen for the thrombin generation-based APC resistance test.

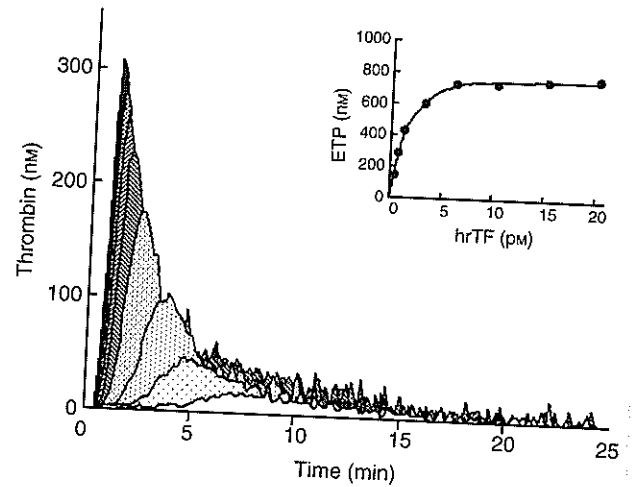


Fig. 4. Effect of tissue factor concentration on thrombin generation in mouse plasma. Thrombin generation was initiated by 0.2, 0.5, 1, 3, 6, 10, 15 and 20 μM human recombinant tissue factor (hrTF) at 33 $^{\circ}\text{C}$ in 60 μL reaction mixtures containing 20 μL mouse plasma and 16.4 mM CaCl_2 , 4 μM phospholipid, 0.42 mM fluorogenic substrate. Inset: endogenous thrombin potential values plotted as function of hrTF concentrations.

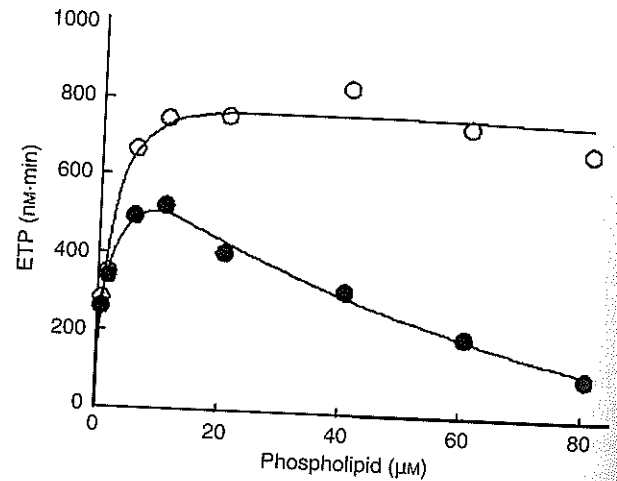


Fig. 5. Effect of phospholipid concentration on thrombin generation in the absence and presence of activated protein C (APC) in mouse plasma. Thrombin generation was measured by calibrated automated thrombography at 33 $^{\circ}\text{C}$ in 60 μL reaction mixtures containing 20 μL mouse plasma, 6 μM hrTF, 16.4 mM CaCl_2 , phospholipid concentrations as indicated in the figure and 0.42 mM fluorogenic substrate with (●) or without (○) 25 nM of human APC present.

CAT-based APC resistance test in mouse plasma

Fig. 5 shows the effect of increasing phospholipid concentrations on the ETP in MPNP. In the absence of APC, the ETP increased at increasing phospholipid concentration with an optimal plateau between 10 and 60 μM phospholipid. The data obtained in the presence of 25 nM APC illustrate that APC hardly inhibited thrombin formation at low phospholipid and that higher phospholipid concentrations were needed to

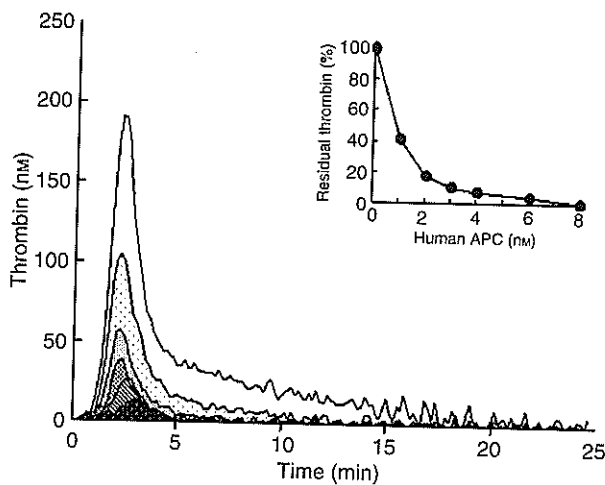


Fig. 6. Effect of the concentration of human activated protein C (APC) on thrombin generation in mouse plasma. Thrombin generation was measured by calibrated automated thrombography at 33 °C in 60 μ L reaction mixtures containing 20 μ L mouse plasma, 6 pM human recombinant tissue factor (hrTF), 8.2 mM CaCl_2 , 60 μ M phospholipids, 0.42 mM fluorogenic substrate and amounts of human APC indicated in the inset of panel. Inset: the residual endogenous thrombin potential (ETP) determined in the presence of APC was calculated as percentage of the ETP determined in the absence of APC and is shown as function of APC concentration.

increase the sensitivity to APC. In the final optimization step, the CaCl_2 concentration was decreased to 8.2 mM (added concentration) to match the decrease in citrate concentration as a result of the lower amounts of plasma present in the assay (data not shown).

Fig. 6 shows down-regulation of thrombin generation in mouse plasma initiated with 6 pM hrTF by varying amounts of human APC. Approximately 4 nM APC was necessary to achieve 90% inhibition of the ETP in mouse plasma, which is comparable to the amount of APC used in the thrombin generation-based APC resistance test in human plasma [17,18]. A similar experiment using mouse thromboplastin (0.25 U) and murine APC showed the same pattern of inhibition (data not shown).

Resistance to APC in plasma of FVL mice

The APC resistance test was validated using plasma samples of mice with different genetic background with respect to the FV Leiden mutation. Fig. 7 shows that nAPCs_r values were low in wild type mice (mean 2.71; 95% CI 2.15–3.27), higher in FV Leiden heterozygous mice (mean 4.21; 95% CI 3.53–4.89) and further increased in FV Leiden homozygous mice (mean 5.46; 95% CI 4.88–6.03). The differences between all groups were significant according to one-way ANOVA followed by *post hoc* analysis with Bonferroni's correction for multiple comparisons ($P \leq 0.01$). The higher APC resistance in the test samples as compared with the reference MPNP may be explained by differences in blood sampling techniques (Materials and methods).

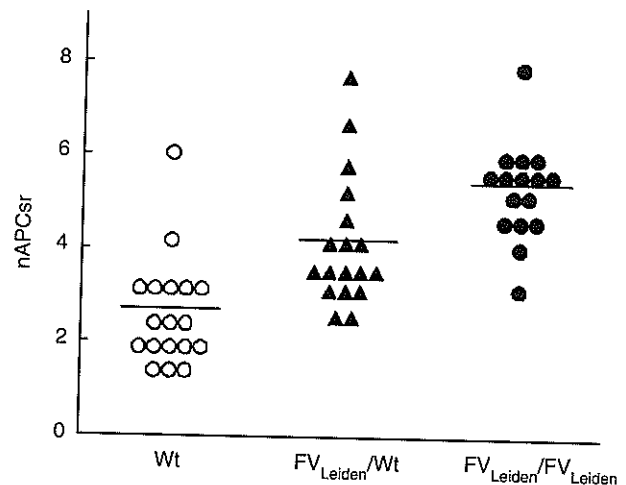


Fig. 7. Activated protein C (APC) resistance in wild-type, heterozygous and homozygous factor V Leiden (FV Leiden) mice. Thrombin generation was measured in plasma from wild-type (WT), heterozygous FVL/WT and homozygous (FVL/FVL) for FVL mice at 33°C in the absence and presence of 5 nM human APC in 60 μ L reaction mixtures containing 20 μ L mouse plasma, 6 pM hrTF, 8.2 mM CaCl_2 , 60 μ M phospholipids and 0.42 mM fluorogenic substrate. The nAPCs_r was calculated as described under Materials and methods. Solid lines indicate the mean nAPCs_r.

Thrombin generation in platelet-rich mouse plasma

Thrombin generation under the modified conditions (1:3 diluted plasma, 33 °C) was measured in the presence of platelets (mouse PRP) without phospholipids added. Reliable thrombin generation curves were obtained at a final platelet concentration of $100 \times 10^9 \text{ L}^{-1}$ after triggering coagulation with 2.5 pM hrTF in the presence of 8.2 mM of CaCl_2 (Fig. 8). Thrombin formation was platelet-dependent as under these conditions no thrombin was generated in PFP (data not shown). The peak height of the thrombin generation curve (~ 100 nM thrombin) was twice as high as that reported by Leon *et al.* [19] who measured thrombin generation in mouse PRP triggered with ~ 0.7 pM hrTF (1:12 000 dilution of Innovin) at $300 \times 10^9 \text{ platelets L}^{-1}$. In our hands, a higher platelet count in the assay mixture resulted in a further increase of the peak height but also induced scattering (data not shown).

Summary of the methodology for measurement of thrombin generation via CAT in mouse plasma

Thrombin generation in mouse PFP: 20 μ L PFP is mixed with 20 μ L hrTF/phospholipid mixture in buffer A and thrombin generation is initiated at 33 °C with 20 μ L fluorogenic substrate/ CaCl_2 mixture. Final concentrations: 33.3% mouse PFP, 6 pM hrTF, 60 μ M phospholipid, 8.2 mM CaCl_2 and 0.42 mM fluorogenic substrate. Comparable thrombin generation curves were obtained when coagulation was triggered with five times concentrated PPP-Reagent LOW yielding final concentrations of 5 pM TF and 20 μ M phospholipid.

Thrombin generation in mouse PRP: 20 μ L PRP is mixed with 20 μ L hrTF in buffer A and thrombin generation is

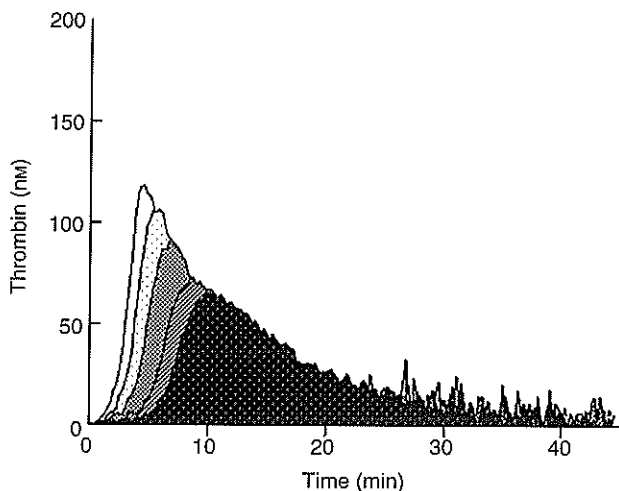


Fig. 8. Effect of varying tissue factor concentration on thrombin generation in platelet-rich mouse plasma. Thrombin generation was initiated by: 0.5; 1; 2.5; 5 and 10 pM hrTF at 33 °C in reaction mixtures containing 8.2 mM CaCl_2 , 0.42 mM fluorogenic substrate and 33% platelet-rich plasma (final platelet concentration $100 \times 10^9 \text{ L}^{-1}$).

initiated at 33 °C with 20 μL fluorogenic substrate/ CaCl_2 mixture. Final concentrations: 33.3% mouse PRP, $100 \times 10^9 \text{ L}^{-1}$ platelets, 2.5 pM hrTF, 8.2 mM CaCl_2 and 0.42 mM fluorogenic substrate.

Thrombin generation-based APC resistance test in mouse PFP: 20 μL PFP is mixed with 10 μL hrTF/phospholipid mixture in buffer A and 10 μL human APC or buffer A and thrombin generation is initiated at 33 °C with 20 μL fluorogenic substrate/ CaCl_2 mixture. Final concentrations: 33.3% mouse PFP, 6 pM hrTF, 60 μM phospholipid, 8.2 mM CaCl_2 and 0.42 mM fluorogenic substrate \pm 4 nM human APC.

Discussion

Use of mouse models is widespread in thrombosis research [20,21]. However, a limited number of assays are available for overall evaluation of the coagulation system in mouse plasma. Here we describe the development of a thrombin generation assay in platelet-free and platelet-rich mouse plasma using CAT. Additionally, we have developed a thrombin generation-based test, which enables evaluation of APC resistance and the function of the protein C system in mice.

For reliable measurement of thrombin generation in the presence of an inhibitor (e.g. an anticoagulant drug or APC) it is essential that substantial amounts of thrombin are formed in the absence of inhibitor. The conditions for thrombography developed for human plasma yielded in mouse plasma low thrombin generation curves at the trigger usually employed in human plasma (1 pM TF, Fig. 1A) and extremely short lag times at higher trigger concentrations (5, 13.6 and 20 pM TF, Fig. 1B–D) which made it impossible to reliably calculate the ETP. The low thrombin generation can be explained by a high activity of naturally occurring coagulation inhibitors (e.g. antithrombin) present in mouse plasma which results in much

faster inhibition of thrombin in mouse than in human plasma (Fig. 2). As the amount of free (measurable) thrombin present at any time point in plasma is the resultant of the amounts of thrombin formed and inhibited, an increase of the ETP may be achieved either by increasing the rate of thrombin formation or slowing down its inhibition. Increasing thrombin formation by increasing the coagulation trigger proved unsuitable as lag times in murine plasma became too short to reliably calculate the ETP (Fig. 1B–D). A decrease of thrombin inhibition was achieved by lowering the reaction temperature to 33 °C and measuring thrombin generation at a higher plasma dilution. This resulted in sufficiently long lag times and large amounts of thrombin formed (cf. Fig. 3). It is interesting to note that plasma dilution, which decreases the amount of prothrombin available for activation, is accompanied with an increase in the ETP (Fig. 3A). This is explained by the observation that at low TF concentrations the percentage of prothrombin that is activated during thrombin generation increases upon plasma dilution presumably as a result of reduced inhibition of TF–FVIIa and FXa–FVa by TFPI and antithrombin, respectively (unpublished observations). As dilution of plasma also results in a proportionally slower inhibition of thrombin by plasma protease inhibitors (e.g. antithrombin), the ETP, which is defined as the time integral of thrombin (thrombin formed \times time it is active), is increased when plasma is diluted.

Generally, conditions of a thrombin generation-based APC resistance test for human plasma are adjusted in such a way that added APC inhibits thrombin generation in HPNP \sim 90%. This provides a wide range for evaluation of an individual's resistance to APC: from plasmas more sensitive than HPNP (thrombin generation is inhibited $>$ 90% by APC) to a 10 times higher resistance to APC than HPNP (no inhibition of thrombin generation by APC).

However, in agreement with previous reports [22,23], a relatively low susceptibility of thrombin generation in mouse plasma to human APC was observed. To avoid using high amounts of APC we have optimized the assay conditions to improve the efficacy of APC. As expected, increasing phospholipid concentration enhanced the activity of APC, while thrombin generation in the absence of APC was not affected up to 60 μM phospholipids (Fig. 5). The low affinity of APC for negatively charged phospholipids [24] can account for the increasing efficacy of APC at higher phospholipid concentrations [18,25]. Furthermore, the concentration of Ca^{2+} in the assay was changed. As APC-catalyzed inactivation of FVa which has a sharp Ca^{2+} optimum and is inhibited at high Ca^{2+} concentrations [26], the 2-fold reduction of the amount of plasma used in the assay which caused a proportional decrease of sodium citrate concentration likely resulted in an excess of Ca^{2+} and subsequent inhibition of APC. Lowering CaCl_2 concentration 2-fold greatly increased sensitivity to APC and enabled inhibition of thrombin generation by 90% with APC amounts, comparable to those used in the thrombin generation-based APC resistance test in human plasma [17,18].

We have demonstrated that APC resistance in mouse plasma can be assessed by evaluation of the effect of human APC on

thrombin generation triggered with human recombinant TF (Fig. 6). Human and mouse APC showed a similar efficacy to down-regulate thrombin generation in mouse plasma triggered with either hrTF or mouse thromboplastin (data not shown), which confirms that human TF is fully functional in murine FX activation [27].

The thrombin generation-based APC resistance test showed a differentially impaired response to APC in plasma of FV Leiden heterozygous and homozygous mice as compared with wild-type mice (Fig. 7). Thus, the newly developed assay allows evaluating (dys)function of the protein C system in mouse. It should be emphasized, however, that possible species specificity in the regulation of the protein C system should be taken in to account. In human plasma APC resistance is not only caused by FV Leiden, but is also associated with elevated plasma levels of the procoagulant proteins prothrombin [28] and FVIII [29] or decreased levels of anticoagulant proteins protein S and TFPI [30], conditions that have been shown to be associated with an increased risk for VTE in humans [31,32]. Although, there are limited data available on the modulation of the protein C system in mouse [3,4], the variation of the nAPCsr in mice with the same genetic background (FV or FV Leiden) shows that, like in humans, other factors (e.g. gender and age) contribute to the level of APC resistance.

We believe that the assays described here may become a useful tool in the evaluation of the overall state of the coagulation system and of the function of the protein C pathway in mouse plasma.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

- Rosendaal FR. Venous thrombosis: a multicausal disease. *Lancet* 1999; **353**: 1167-73.
- Emeis JJ, Jirouskova M, Muchitsch EM, Shet AS, Smyth SS, Johnson GJ. A guide to murine coagulation factor structure, function, assays, and genetic alterations. *J Thromb Haemost* 2007; **5**: 670-9.
- Yin ZF, Huang ZF, Cui J, Fiehler R, Lasky N, Ginsburg D, Broze GJ Jr. Prothrombotic phenotype of protein Z deficiency. *Proc Natl Acad Sci USA* 2000; **97**: 6734-8.
- Eitzman DT, Westrick RJ, Bi X, Manning SL, Wilkinson JE, Broze GJ, Ginsburg D. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. *Circulation* 2002; **105**: 2139-42.
- Cui J, Eitzman DT, Westrick RJ, Christie PD, Xu ZJ, Yang AY, Purkayastha AA, Yang TL, Metz AL, Gallagher KP, Tyson JA, Rosenberg RD, Ginsburg D. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. *Blood* 2000; **96**: 4222-6.
- Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-7.
- Franco RF, Reitsma PH. Genetic risk factors of venous thrombosis. *Hum Genet* 2001; **109**: 369-84.
- Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004-8.
- Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* 2005; **115**: 3355-62.
- Chantarangkul V, Clerici M, Bressi C, Giesen PL, Tripodi A. Thrombin generation assessed as endogenous thrombin potential in patients with hyper- or hypo-coagulability. *Haematologica* 2003; **88**: 547-54.
- Al Dieri R, Peyvandi F, Santagostino E, Giansily M, Mannucci PM, Schved JF, Beguin S, Hemker HC. The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding. *Thromb Haemost* 2002; **88**: 576-82.
- de Waard V, Hansen HR, Spronk HH, Timmerman JJ, Pannekoek H, Florquin S, Reitsma PH, ten Cate H. Differential expression of tissue factor mRNA and protein expression in murine sepsis. The role of the granulocyte revisited. *Thromb Haemost* 2006; **95**: 348-53.
- Schnell MA, Hardy C, Hawley M, Propert KJ, Wilson JM. Effect of blood collection technique in mice on clinical pathology parameters. *Hum Gene Ther* 2002; **13**: 155-61.
- Rosing J, Tans G, Nicolaes GA, Thomassen MC, van Oerle R, van der Ploeg PM, Heijnen P, Hamulyak K, Hemker HC. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. *Br J Haematol* 1997; **97**: 233-8.
- Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoort R, Lecomte T, Beguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb* 2003; **33**: 4-15.
- Brugge JM, Tans G, Rosing J, Castoldi E. Protein S levels modulate the activated protein C resistance phenotype induced by elevated prothrombin levels. *Thromb Haemost* 2006; **95**: 236-42.
- Brugge JM, Simioni P, Bernardi F, Tormene D, Lunghi B, Tans G, Pagnan A, Rosing J, Castoldi E. Expression of the normal factor V allele modulates the APC resistance phenotype in heterozygous carriers of the factor V Leiden mutation. *J Thromb Haemost* 2005; **3**: 2695-702.
- Curvers J, Christella M, Thomassen LG, de Ronde H, Bertina RM, Rosendaal FR, Tans G, Rosing J. Effects of (pre-) analytical variables on activated protein C resistance determined via a thrombin generation-based assay. *Thromb Haemost* 2002; **87**: 483-92.
- Leon C, Ravanat C, Freund M, Cazenave JP, Gachet C. Differential involvement of the P2Y1 and P2Y12 receptors in platelet procoagulant activity. *Arterioscler Thromb Vasc Biol* 2003; **23**: 1941-7.
- Leadley RJ Jr, Chi L, Rebello SS, Gagnon A. Contribution of in vivo models of thrombosis to the discovery and development of novel antithrombotic agents. *J Pharmacol Toxicol Methods* 2000; **43**: 101-16.
- Dorffler-Melly J, Schwarte LA, Ince C, Levi M. Mouse models of focal arterial and venous thrombosis. *Basic Res Cardiol* 2000; **95**: 503-9.
- Katsuura Y, Mochizuki T, Tamura M, Hoshida S, Kiyoki M, Nakagaki T, Miyamoto S. Species specificity of anticoagulant activity of activated human protein C: involvement of factor V as well as protein S. *Thromb Res* 1996; **82**: 147-57.
- Yang TL, Cui J, Rehumtulla A, Yang A, Moussalli M, Kaufman RJ, Ginsburg D. The structure and function of murine factor V and its inactivation by protein C. *Blood* 1998; **91**: 4593-9.
- Nelsetuen GL, Kisiel W, Di Scipio RG. Interaction of vitamin K dependent proteins with membranes. *Biochemistry* 1978; **17**: 2134-8.
- Smirnov MD, Esmon CT. Phosphatidylethanolamine incorporation into vesicles selectively enhances factor Va inactivation by activated protein C. *J Biol Chem* 1994; **269**: 816-9.
- Bakker HM, Tans G, Janssen-Claessen T, Thomassen MC, Hemker HC, Griffin JH, Rosing J. The effect of phospholipids, calcium ions and protein S on rate constants of human factor Va inactivation by activated human protein C. *Eur J Biochem* 1992; **208**: 171-8.
- Petersen LC, Norby PL, Branner S, Sorensen BB, Elm T, Stennicke HR, Persson E, Bjorn SE. Characterization of recombinant murine factor VIIa and recombinant murine tissue factor: a human-murine species compatibility study. *Thromb Res* 2005; **116**: 75-85.

- 28 Tripodi A, Chantarangkul V, Mannucci PM. Hyperprothrombinemia may result in acquired activated protein C resistance. *Blood* 2000; **96**: 3295–6.
- 29 Laffan MA, Manning R. The influence of factor VIII on measurement of activated protein C resistance. *Blood Coagul Fibrinolysis* 1996; **7**: 761–5.
- 30 de Visser MC, van Hylckama Vlieg A, Tans G, Rosing J, Dahm AE, Sandset PM, Rosendaal FR, Bertina RM. Determinants of the APTT- and ETP-based APC sensitivity tests. *J Thromb Haemost* 2005; **3**: 1488–94.
- 31 de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood* 1999; **93**: 1271–6.
- 32 Tans G, van Hylckama Vlieg A, Thomassen MC, Curvers J, Bertina RM, Rosing J, Rosendaal FR. Activated protein C resistance determined with a thrombin generation-based test predicts for venous thrombosis in men and women. *Br J Haematol* 2003; **122**: 465–70.
- 33 Hemker HC, Wielders S, Kessels H, Beguin S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. *Thromb Haemost* 1993; **70**: 617–24.