Monitoring thrombin generation: Is addition of corn trypsin inhibitor needed?

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Summary

Thrombin generation monitoring has the potential to be used as a clinical diagnostic tool in the near future. However, robust pre-analytical conditions may be required, and one factor that has been reported is in-vitro contact activation that might influence in-vitro measurements of thrombin generation and thereby act as an unpredictable pre-analytical variable. The aim of the current study was to investigate the influence of contact activation and the necessity of corn trypsin inhibitor (CTI) to abolish contact activation in thrombin generation measurements at low tissue factor (TF) concentrations. Thrombin generation was performed using the calibrated automated thrombogram (CAT), thereby determining the endogenous thrombin potential (ETP), peak height, and the lag time, in plasma obtained from healthy volunteers. Addition of CTI after plasma preparation had no significant influence on thrombin generation triggered with 0.5 pM TF or higher, as demonstrated by unaltered ETP and lag time values between analyses with and without CTI. Addition of CTI before blood collection reduced thrombin generation triggered with 0.5 pMTF: both the ETP and peak height were significantly reduced compared to no CTI addition. In contrast, thrombin generation remained unaltered at a 1 pM TF trigger or above. This study demonstrates that addition of CTI after plasma separation is not necessary when triggering with TF concentrations of 0.5 pM and higher. Furthermore, it was demonstrated that it is not needed to pre-fill blood collecting tubes with CTI when measuring thrombin generation at TF concentrations of ≥1 pM.

Keywords

Thrombin generation, calibrated automated thrombogram, corn trypsin inhibitor

Introduction

Thrombin is an essential enzyme in the coagulation cascade (1) and changes in thrombin production may be associated with thrombotic as well as haemorrhagic conditions in patients (2–9). One of the more commonly used methods to assess thrombin generation in plasma is the Calibrated Automated Thrombogram (CAT, Thrombinoscope BV, Maastricht, The Netherlands). This method uses a low affinity fluorogenic substrate (Z-Gly-Gly-Arg-AMC), which is converted into a fluorescent signal by thrombin. The activity of thrombin is thus continuously registered over time, resulting in a characteristic thrombin generation curve. Over the past years this method was validated for pre-analytical conditions (10–12) and shown to exert good reproducibility and stability. One problem, however, might be the activation of factor (F)XII during blood drawing and/or during analysis. Recently, van Veen and Dargaud demonstrated the contribution of contact activation to thrombin generation analysis, especially at low concentrations of TF (10, 12), thereby introducing an unpredictable pre-analytical variable which increased inaccuracy and imprecision (13). The use of the specific FXIIa inhibitor corn trypsin inhibitor (CTI) attenuated the effects of contact activation observed during thrombin generation. The consequences of contact activation for clinical practice are considerable: addition of CTI to blood collecting tubes increases the costs and requires an additional tube besides the commonly used vacu-
tainer. Since contact activation can occur during venipuncture or in the thrombin generation analysis itself, we hypothesised that the blood collection procedure and the used reagents for thrombin generation are key determinants in in-vitro activation of FXII. In order to verify the previous results of Van Veen and Dargaud, as well as to determine the effects of blood collection and used reagents, we analysed thrombin generation under well defined conditions with and without CTI.

Methods

Experimental design

1. Effects of blood collection method
Venous blood from four healthy volunteers was simultaneously collected using a standard 21-gauge needle (BD, Plymouth, UK) for the right arm and a Winged Infusion Set or “butterfly”-needle equipped with a 30 cm long tubing (Push Button Blood Collection Set, BD) for the left arm.

2. Effects of CTI-addition to plasma
Venous blood from 12 healthy volunteers was collected in 3.2% (w/v) citrate using a 21-gauge needle and plasma was prepared as described below. Just before thrombin generation analysis CTI was added to the plasmas to obtain a final concentration of 25 or 50 µg/ml. Thrombin generation was analysed using 0.5, 1, 2, or 5 pM TF as trigger.

3. Effects of CTI-addition to venous blood
Venous blood from twelve healthy volunteers (the same subjects as above) was collected in 3.2% (w/v) citrate containing CTI (final concentration of 40 µg/ml in plasma) using a 21-gauge needle. Thrombin generation was analysed using 0.5, 1, 2, or 5 pM TF as trigger.

Blood collection and plasma preparation
Venous blood from the left and from the right arm of four healthy volunteers was collected in 3.2% (w/v) citrate using a 21-gauge needle (BD) or a Winged Infusion Set (BD) through venipuncture. The first 10 ml of venous blood were discarded. Platelet-poor plasma (PPP) was prepared by two centrifugation steps: the first at 2,000 x g for 10 min. Plasma aliquots were snap-frozen in liquid nitrogen and stored at −80°C until use. All samples were thawed at 37°C for 15 min before analysis and analysed batch-wise. CTI was obtained from Haematologic Technologies, Inc. (Essex Junction, VT, USA) and added to the 3.2% (w/v) citrate collection tubes to obtain a final concentration of 40 µg/ml in plasma. To study the effect of CTI addition during thrombin generation, CTI was added to plasma before analysis to obtain final concentrations of 25 or 50 µg/ml. Kaolin was added to 3.2% (w/v) citrate tubes to obtain a final concentration of 1 mg/ml after plasma collection.

Normal pool plasma and FXII-deficient plasmas
Normal pooled plasma was prepared at the departments of Haematology and Clinical Chemistry of the Maastricht University Medical Center, The Netherlands, by pooling plasma from 85 healthy volunteers not using any medication. FXII-deficient plasma was either obtained from the Maastricht University Medical Center (indicated as FXII-deficient plasma [1]) or George King Bio-Medical Inc. (Overland Park, KS, USA; indicated as FXII-deficient plasma [2]) and were both from patients with a congenital FXII-deficiency. FXI-deficient plasma was obtained from George King Bio-Medical Inc.

Thrombin generation
Thrombin generation in tissue factor (TF)-triggered platelet poor plasma was measured by means of the CAT method (Thrombinscope BV) as described previously (14), which makes use of a low affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) to continuously monitor thrombin activity in clotting plasma. In order to correct for inner-filter effects and substrate consumption, each thrombin generation measurement was calibrated against the fluorescence curve obtained in the same plasma with a fixed amount of thrombin-α2-macroglobulin complex (Thrombin Calibrator, Thrombinscope BV), as recommended by the manufacturer. Fluorescence was read in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 filter set, and thrombin generation curves were calculated with the Thrombinscope software (Thrombinscope BV).

 Unless stated otherwise, thrombin generation was determined under several experimental conditions (final plasma con-
centrations): 0.5, 1, 2, or 5 pM TF and 4 µM phospholipids. The TF triggers and phospholipids were obtained from Thrombinscope BV. Three parameters were derived from the thrombin generation curves: lag time, peak height and endogenous thrombin potential (ETP, area under the curve).

**FXIIa- C1-esterase inhibitor complex measurement**
Complexes of FXIIs and FXIa with their natural inhibitor C1 esterase inhibitor, respectively, were measured with enzyme-linked immunosorbent assays (ELISAs), using monoclonal antibodies (mAb) and as described previously (15). In brief, mAb F3 was used to recognise FXII, mAb XI-5 to FXI, and mAb KOK 12 specific for complexed C1 esterase inhibitor. Appropriate secondary antibodies were biotinylated using EZLink N-hydroxysuccinimide ester-biotin according to instructions from the manufacturer (Pierce, Rockford, IL, USA). Absorbance was read at 450 nm on an EL 808 Ultra microplate reader (Bio-tek Instruments Inc., Winooski, VT, USA). Results were expressed as a percentage of activated normal pooled plasma. Normal pooled EDTA plasma was maximally activated at 37°C by incubation with an equal volume of 0.2 mg/ml dextran sulfate (Mr 500 000; Sigma Chemical Co., St Louis, MO, USA) to obtain reference curves for the FXIIa:C1 esterase inhibitor ELISA, whereas kaolin (final concentration 5 mg/ml)-activated EDTA plasma was used for the FXIa:C1 esterase inhibitor ELISA. Activation was stopped by adding three volumes of phosphate-buffered saline (PBS) containing 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) and 0.05% (w/v) polybrene (Sigma Chemical Co.). Kaolin was removed by centrifuging the reaction mixture for 5 min at 13,000 x g. No signal was detected in ELISAs performed on activated FXII- or FXI-deficient plasmas.

**Statistical analysis**
The data obtained for the thrombin generation experiments are expressed as median with interquartile range (IQR). Differences in thrombin generation parameters between the groups treated with or without CTI were calculated with a non-parametric paired test (Wilcoxon signed rank test). A two-tailed probability value (p) of p< 0.05 was considered statistically significant. Statistical analysis was performed using the Graph Pad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

**Results**

**Effects of blood collection method**
Inter-laboratory variations in thrombin generation measurements can be the result of differences in equipment (including filter settings and temperature), reagent batches, as well as preanalytical variables such as the method used for venous blood drawing. Using two different needle systems venous blood was simultaneously obtained from four healthy volunteers. Thrombin generation triggered with 5 pM TF in the prepared PPP was not different between the two collection methods. For the needle system (21-gauge needle) a median ETP of 1,171 nM.min (IQR: 1,158–1,321) was observed whereas for the Winged Infusion Set the ETP was 1,219 nM.min (IQR: 1,179–1,352), differences not significant. Also, the peak height and lag time did not differ between the two systems (data not shown). For the 1 pM TF trigger, however, both the ETP and peak height were significantly increased for plasmas obtained with the Winged Infusion Set compared to the needle system (Fig. 1). On average, the ETP increased by almost 15% or 151 nM.min whereas the peak height increased by 24% more thrombin (38 nM) by using a low TF trigger in the thrombin generation assay.

The differences between the two blood drawing systems are most likely due to enhanced contact activation induced by the 30 cm long tubing in the Winged Infusion Set. In order to find out whether CTI selectively inhibits FXIIa and no other enzymes of the coagulation cascade, 0, 25 or 50 µg/ml CTI was added to normal pool plasma and FXII-deficient plasmas. Subsequently, thrombin generation was triggered with 1 or 5 pM TF. No differences in thrombin generation curves were observed for both triggers in the absence or presence of 25 or 50 µg/ml CTI, suggesting selectivity of CTI for FXIIa. Since no differences were observed between 25 or 50 µg/ml CTI a “safe” concentration of 40 µg/ml CTI (which is two times the concentration previous used [10, 12, 13]) was chosen for all further experiments.
Activation of FXII during blood collection

To demonstrate activation of FXIIa during collection of blood the contact activation complexes FXIIa-C1 esterase inhibitor and FXIa-C1 esterase inhibitor were analysed in plasmas obtained from blood collected in 3.2% (w/v) citrate blood collection tubes with and without 40 µg/ml CTI. Plasma collected without CTI from twelve healthy volunteers contained 0.16% (IQR 0.14–0.16) FXIIa-C1 esterase inhibitor complexes and this level was comparable to the concentrations in plasmas collected in the presence of CTI (0.16% IQR: 0.14–0.18). FXIa-C1 esterase inhibitor complexes, however, were decreased in plasmas containing CTI (0.17% IQR: 0.14–0.18) vs. 0.27% IQR: 0.22–0.33, p=0.01; Fig. 2).

To show that the presence of CTI in a venous blood collection tube effectively inhibits FXIIa, kaolin (1 mg/ml) was added to the tubes in order to activate FXII and thrombin generation was recorded subsequently. Kaolin was found to be a strong initiator of thrombin generation since the lag times shifted from 10.3 min (IQR 8.1–12.2) towards 2.6 min (IQR 2.4–2.9, p<0.01). The ETP and peak height increased as well upon addition of kaolin to venous blood collection tubes. The ETP increased by almost 16% from 1,386 nM.min (IQR 1,109–1,448) to 1,603 nM.min (IQR 1,386–1,764) upon kaolin addition. The effect was even stronger for the peak height with an increase of 40% from 297 nM (IQR 226–354) to 415 nM (IQR 403–442). Combined addition of kaolin and CTI to the blood collection tubes reduced thrombin generation to a level observed for citrated plasma alone. The lag time prolonged to 8.3 min (8.0–13.6) and both ETP and peak height reduced to 1,490 nM.min (IQR 1,129–1,590, p<0.01 compared to kaolin) and 326 nM (IQR 209–365, p<0.01 compared to kaolin), respectively.

Effects of CTI addition to plasma on thrombin generation

In order to demonstrate whether CTI addition to plasma influenced thrombin generation under conditions using the stan-
The corresponding ETP values obtained for analysis with CTI added to plasma were 1,132 nM.min (IQR 739–1,517), 1,358 nM.min (IQR 971–1,646), 1,417 nM.min (IQR 1,093–1,688), and 1,585 nM.min (IQR 1,200–1,821). Peak height values obtained in analyses with and without the addition of CTI followed the same pattern as for ETP, with increasing maximum thrombin formation with increasing TF concentrations and no differences between measurements in the absence or presence of CTI (Fig. 3B).

Effects of CTI-addition to venous blood on thrombin generation

Since the presence of CTI during blood collection could efficiently inhibit contact activation, the influences of CTI addition to blood collection tubes was determined through thrombin generation under conditions of the default TF triggers. CTI was added to plasmas from 12 healthy volunteers and thrombin generation was triggered with 0.5, 1, 2, and 5 pM TF. For all TF triggers the measurement with CTI added to plasma was in accordance with the analysis without CTI added. To allow for intra-individual comparison of analyses without and with CTI, an acceptable within individual variability of 10% was arbitrarily chosen based on inter-assay and inter-individual variations published previously (11, 14). Both the ETP and peak height from thrombin generation with CTI were within this 10% range of the values obtained without CTI (Fig. 3). Overall, ETP and peak height on average increased with increasing concentration TF trigger and were not different between analysis with or without CTI. ETP values in the absence of CTI were 1,126 nM.min (IQR 731–1,464), 1,281 nM.min (IQR 904–1,570), 1,369 nM.min (IQR 1,015–1,663), and 1,602 nM.min (IQR 1,214–1,823) for 0.5, 1, 2, and 5 pM TF, respectively (Fig. 3A).
(40 µg/ml) was added to 3.2% (w/v) citrate blood collection tubes and plasma was obtained from 12 healthy volunteers. Thrombin generation was triggered with 0.5, 1, 2, or 5 pM TF and compared between plasmas derived from collection tubes with and without CTI (Fig. 4). For the higher TF concentrations thrombin generation was comparable between the two collection tubes. ETP values in the absence of CTI were 1,281 nM.min (IQR 904–1,570), 1,369 nM.min (IQR 1,015–1,663), and 1,602 nM.min (IQR 1,214–1,823) for 1, 2, and 5 pM TF, respectively. Addition of CTI to blood collection tubes hardly altered these values: 1259 nM.min (IQR 885–1,620), 1,333 nM.min (IQR 1,036–1,693), and 1,662 nM.min (IQR 1,241–1,873) for 1, 2, and 5 pM TF, respectively. For the 0.5 pM TF analysis, however, eight out of 12 analyses showed a lower ETP and peak height for thrombin generation in plasmas derived from collection tubes containing CTI (Fig. 4A). On average the ETP at 0.5 pM TF reduced with 28% from 1,126 nM.min (IQR 731–1,464) to 808 nM.min (IQR 367–1,414) upon addition of CTI to the collection tube. Whereas the peak height for the 1, 2, and 5 pM TF analyses followed the same pattern as the ETP, peak height measured at 0.5 pM TF trigger showed a reduction of 55% from 162 nM (IQR 85–253) to 74 nM (IQR: 29–182) when CTI was present during blood drawing (Fig. 4B).

Discussion

In recent literature it has been argued that CTI can abrogate the influence of in-vitro contact activation on thrombin generation (10, 12, 13). In this study the addition of CTI to a sample after plasma preparation had no influence on thrombin generation, whereas the presence of CTI during blood collection only altered thrombin generation triggered at TF concentrations below 1 pM.

In the first part of this study the use of a Winged Infusion Set for blood collection resulted in markedly increased thrombin generation, most likely through contact activation since the presence of CTI in the blood collection tubes reduced the FXIIa-C1 esterase inhibitor complexes. This was surprising because this needle system had been previously used assuming that it would provide a rather “atraumatic” sampling condition. Especially thrombin generation conducted at 1 pM TF was altered when blood was collected using the Winged Infusion Set. Due to the presence of a 30 cm long tubing between the collecting and delivering needle activation of blood coagulation FXII is likely to occur. As reported previously, one of the potential problems with the thrombin generation test in routine clinical practice will be the interference of the contact system (16). Considering our data, the use of the standard needles used for vacuum blood collection appears to be satisfactory.

Addition of CTI to plasma has been shown to reduce or overcome the influence of contact activation on thrombin generation (10, 12, 13). Using 5 pM TF as trigger, Luddington and Baglin observed an almost two-fold reduction in ETP upon addition of CTI to the blood collection tube (13). This effect abolished at a TF concentration of 15 pM, suggesting a larger influence of contact activation at lower TF levels. Furthermore, Van Veen et al. observed an average reduction in ETP of 343 nM.min upon addition of CTI to plasma and using a TF trigger of 1 pM (12). Even the use of a higher TF trigger (5 pM) did not completely attenuate the effects of contact activation on thrombin generation. And last, Dargaud et al. used the addition of CTI to plasma to reduce the inter-laboratory variations in thrombin generation. In their study, addition of CTI to the collection tubes reduced the ETP on average with 178 nM.min at a 1.5 pM TF trigger (10). Again, the use of prefilled CTI/citrate tubes is feasible under research conditions, but major drawbacks exist for application in a hospital laboratory. First of all, currently most hospitals use 3.2 or 3.8% (w/v) citrate Vacutainer blood collection tubes for coagulation assays. Second, there is a significant cost issue. Currently, CTI is sold for a list price of around €100 (app. $150 or £70) for 1 mg. Pre-filling 4.5 ml tubes to a final plasma concentration of 40 µg/ml (used in this paper) will cost around €15 (app. $22 or £12) per tube. In conclusion, the use of pre-filled CTI blood collection tubes is not preferred given the major logistic and financial consequences.

In contrast to the described inhibition of contact activation through addition of CTI and the beneficial effects on thrombin generation (10, 12, 13), no influence on thrombin generation was observed when CTI was added to plasma. Using blood collection tubes pre-filled with CTI, however, thrombin generation triggered with low TF (0.5 pM) was reduced, but this reduction was negligible or absent at higher TF concentrations. These data suggest that the extrinsic pathway can overrule the contact activation pathway in thrombin generation when triggered with 1 pM TF or higher. The differences between our results and the data reported by Van Veen and Dargaud might stem from differences in the used plasma preparations and reagents used for thrombin generation. With regard to the used TF reagents a special note should be made. For all experiments conducted within our laboratory the available CAT-reagents from Thrombinoscope (Maastricht, The Netherlands) were obtained and one batch of reagents was used to avoid differences between batches of reagents. In the absence of an international TF standard, however, it is hard to claim that a given TF concentration is really the absolute concen-
tation. Differences between laboratories might therefore stem from variations in both the TF sources and given concentrations. One striking difference between used protocols is the centrifugation method: in one paper plasma was obtained through double centrifugation at 2,000 x g for 10 min at room temperature (RT) (12) and in another by double centrifugation at 3,000 x g for 15 min at RT (10). Within our laboratory blood is first centrifuged at 2,000 x g for 15 min and the obtained plasma is centrifuged a second time at 11,000 x g for 10 min (both at RT). The second step at a higher speed appears to be essential to remove remaining micro particles which possibly facilitate activation of FXII (Trappenburg, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands, personal communication). Secondly, the source of phospholipids and TF to trigger thrombin generation could be major determinants in variations in thrombin generation. We therefore agree with Van Veen et al. (12) and with Dargaud et al. (10) that there is a lack of standardisation among different groups in studies on thrombin generation, which needs to be solved. Standardisation without the introduction of pre-filled CTI blood collection tube is preferred since additional tubes will lead towards increasing costs and more complicated logistics in a clinical setting.

Our study showed that there is no significant influence of contact activation on thrombin generation by means of the CAT at a TF trigger of 1 pM or above. On the basis of these results and previous data (11), we propose that a standard thrombin generation protocol should include the following: 1: no addition of CTI during or after blood drawing, 2: venipuncture through a 21-gauge needle and blood collection using 3.2%(w/v) citrate vacutainer (with the first 10 ml of venous blood discarded), 3: uniform plasma preparation at high speed centrifugation, 4: uniform reagents, and 5: normalisation against an international reference plasma.

References