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Continuous thrombin generation in whole blood: new applications for assessing activators and inhibitors of coagulation

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Abstract

Hemostatic tests have been utilized to clarify the blood coagulation potential. The novel thrombin generation (TG) assay of this study provides explicit information and is the most physiologically-relevant hemostatic test *ex vivo*. We describe how this assay allows for TG under a number of relevant circumstances. First, whole blood (WB) from healthy individuals was analyzed $\pm$ 5 pM tissue factor (TF) and $\pm$ contact pathway inhibition. Without an exogenous initiator TG was decreased and delayed, but addition of 5 pM TF shortened the lag phase and increased peak thrombin. Additional experiments included fresh WB from a trauma patient analyzed for endogenous activity and TG from healthy donors subjected to TG antagonists which prolonged the lag phase whereas TG agonists consistently shortened the lag phase in a dose dependent manner. Lastly, platelet-poor plasma was reconstituted with packed red blood cells and TG was monitored in the presence and absence of both TF as an activator and PCPS as a phospholipid surface. Our data illustrate the potential that this continuous TG assay has in the evaluation of disorders relevant to blood coagulation and in the monitoring of treatments administered in response to these disorders.

1. Introduction

Altered hemostasis can lead to excessive bleeding or thrombosis, which is the most predominant cause of cardiovascular disease and contributes to the majority of death worldwide (1). Various tests have been utilized to evaluate hemostatic potential of both healthy individuals and patients, with plasma clotting tests such as aPTT (2), PT (3) and a derivative of the latter, INR (4), as the most widely used in clinical practice. However, these tests have at least two shortcomings: a) they stop at the inception of the propagation phase of

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thrombin generation (5); and b) blood cells and platelets are not present in these tests. Thrombin is a multifunctional and key enzyme in the process of blood coagulation that enhances its own generation via the feed-back activation of factors V, VII, VIII and XI (6). Thrombin also down-regulates the process of coagulation by cleaving protein C resulting in activated protein C, which cleaves factors Va and VIIIa and, as a consequence, shuts down further thrombin generation. In addition to these functions, thrombin increases clot stability by activating factor XIII and thrombin activatable fibrinolysis inhibitor. So, it is not surprising that a decreased thrombin potential correlates with bleeding tendencies (hemophilia) (5, 7) and an elevated potential correlates with a prothrombotic phenotype related to cardiovascular diseases (8, 9), trauma-induced coagulopathy (10), cancer (11), *etc.* To better evaluate hemostatic potential, new tests measuring thrombin generation in native or “synthetic” plasma were developed (12, 13). These tests provide more information related to the hemostatic potential than standard clotting time-based tests, but they were still quite distant from the physiologically-relevant hemostasis, primarily due to the absence of blood cells and platelets, both of which play an important role in TG and clot formation (14, 15). To further improve hemostatic tests and make them more physiologically relevant, methodologies were developed for the quantitation of TG in fresh whole blood. These thrombin generation assays provide explicit information and remain the most physiologically-relevant hemostatic tests *ex vivo*. One of the first such methodologies used fresh contact pathway-inhibited native blood triggered with low concentrations of relipidated TF (16). Over the past 2 decades, this methodology provided several new insights into the roles of various blood components on thrombin generation and clot formation [6], but it has been quite labor and time consuming. In the current study, a new, simple and robust thrombin generation assay in whole blood developed by Ninnivaggi and coworkers (17) was used for the evaluation of several hemostatic agents and for the characterization of some disorders of blood coagulation.

2. Materials and methods

2.1 Materials

Pooled citrate platelet-poor multi-donor plasma (MDP) was prepared in-house using healthy donors (18). Trypsin inhibitor from corn (CTI; prevents contact pathway initiation of coagulation) was prepared as previously described (19, 20). Phospholipid vesicles (PCPS) composed of 25% dioleoyl-*sn*-glycero-3-phospho-L-serine and 75% of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (both from Avanti Polar Lipids, Inc; Alabaster, AL) were prepared as previously described (21). Inhibitory monoclonal anti-TF (α TF-5; prevents binding of TF to FVIIa), anti-FXIa (α FXIa-2; inhibits FIX activation by FXIa) and anti-FIXa (α FIXa-91; inhibits FX activation by FIXa) antibodies were produced and characterized in-house (22, 23). Inhibitory polyclonal anti-FVIII (α FVIII; inhibits FX activation by the FVIIIa/FIXa complex) was obtained from Haematologic Technologies, Inc. (Essex Junction, VT). Anticoagulants used were rivaroxaban and dabigatran (Alsachim; Illkirch Graffenstaden, France), fondaparinux (GlaxoSmithKline; Research Triangle Park, NC), heparin (Sigma; St. Louis, MO) and bivalirudin (Medicines Company; Boston, MA). The fluorogenic substrate used was Z-GGR-rhodamine (P₂Rho; from Diagnostica Stago; Asnieres sur Seine, France). TF, FIXa and FXIa activity in blood was calculated from

calibration curves developed with human FIXa, human FXIa and relipidated TF₁₋₂₆₃ (19) (all from Haematologic Technologies, Inc.; Essex Junction, VT). BSA-5 buffer (20 mM HEPES, 140 mmol/L NaCl, 0.02% NaN₃ and 5 g/L bovine serum albumin (BSA) at pH 7.35), BSA-60 buffer (20 mM HEPES, 0.02% NaN₃ and 60 g/L BSA at pH 7.35), calibrator buffer (20 mM HEPES, 100 mM sodium citrate, 0.02% NaN₃ and 60 g/L BSA at pH 7.4) and 1M CaCl₂ were all supplied by Diagnostica Stago (all reagents from Sigma-Aldrich; St. Louis, MO). Additionally, α 2-macroglobulin-thrombin complex for use as a calibrator (prepared by Synapse Research Institute (24), microtiter plates (both flat and round-bottom 96-well polystyrene plates; Thermo Fischer Scientific; Waltham, MA), Whatman 589/1 filter paper (Dassel, Germany) and the plate reader (Ascent reader, Thermolabsystems OY; Helsinki, Finland) were all provided by Diagnostica Stago. The thrombin generation assay was performed as previously described (17) and modified for the assays below.

2.2 Whole Blood Thrombin Generation Assay

The study was approved by the University of Vermont Committee on Human Research. A written consent was obtained from all blood donors. This assay was modified from one previously published (17). In this study, each application of the assay required a different treatment of blood, however after treatment all thrombin generation experiments were done as follows; aliquots of blood were mixed with a solution of substrate (1.8 mM P₂Rho substrate in BSA-60 buffer) at a ratio 3:1. In parallel, activator/calibrator solution was prepared (300 nM calibrator in a calibrator buffer and 15 pM TF and 50 mM Ca²⁺ in BSA-5 buffer). Forty μ l of the blood/substrate mixture was then added to a preheated (37C) round-bottom microtiter plate, followed by the addition of 20 μ l of activator/calibrator solution. Final concentrations of reagents in blood are: 450 μ M P₂Rho, 100 nM calibrator, 5 pM TF and 16.7 mM CaCl₂. The mixture was immediately transferred to the preheated flat-bottom microtiter plate containing filter paper, in triplicate, and mineral oil (40 μ L) was added on the top. The plate was then placed in the plate reader and substrate hydrolysis was monitored at 485 nm excitation and 538 nm emission wavelengths for 50 minutes. The derived data was analyzed for thrombin generation curves using the analysis platform provided by Diagnostica Stago.

2.3 Thrombin Generation in Blood from Healthy Donors

Blood was collected from 10 healthy donors (5 male; 5 female) into 3.2% sodium citrate and kept at room temp. Two citrate tubes were collected per donor at the time of draw, with 1 tube remaining citrate only and the other receiving 0.1 mg/mL CTI before the TG assay. TG in both citrate only and citrate/CTI blood, was monitored either in the absence or in the presence of an exogenous TF. For TG in the absence of TF, TF was omitted from the activating solution and replaced with corresponding volume of HBS (20 mM HEPES, 150 mM NaCl, pH 7.4). Each blood sample was subjected to both activating solutions, for a total of 4 conditions per donor. The rest of the assay was performed as described above.

2.4 Thrombin Generation Initiator Responses

Blood from healthy individuals was collected into 3.2% sodium citrate and kept at room temp. CTI was added at a final 0.1 mg/mL concentration and 6 aliquots were made. FXIa was added to these aliquots to a final 0, 1, 5, 10, 50 and 100 pM concentrations, with HBS

added to remain volumetrically equivalent across all samples. The assay was then performed as described above with no TF in the activating solution. For TF and FIXa titrations, experiments were done the same way with the exception of protein concentrations used. TF was used at 0, 0.5, 1, 2.5 and 5 pM and FIXa at 0, 15, 31, 125, 500 and 1000 pM concentrations.

2.5 Thrombin Generation in Blood from Trauma Patients

This experiment is a modification of previously published assays in plasma (10) and is based on the response of the lag phase of thrombin generation in CTI-treated fresh whole blood to the addition of inhibitory monoclonal antibodies to FXIa, FIXa and TF. Blood was collected from a trauma patient at the University of Vermont Medical Center (UVMMC) into 3.2% sodium citrate, CTI was added to collected blood to achieve 0.1 mg/mL and the blood was kept at room temp. The antibodies used in this experiment, α FXIa-2, α FIXa-91 and α TF-5 were all added directly to the wells of the preheated round-bottom plate that the blood sample would go in, achieving a final concentration in blood of 0.1 mg/mL for all antibodies. The addition of antibodies to the plate was performed just prior to the addition of blood to prevent evaporation. The rest of the assay was performed as described above with no TF in the activating solution. Endogenous concentrations of TF, FXIa and FIXa were calculated from corresponding calibration curves constructed by titrating purified proteins into fresh blood from healthy individuals.

2.6 Thrombin Generation Antagonist Responses

Blood from healthy individuals was collected into 3.2% sodium citrate and kept at room temp. CTI was added to the blood tube to achieve 0.1 mg/mL and 4 aliquots were made. These aliquots were treated directly with various thrombin generation antagonists at final concentrations in blood equivalent to concentrations used in published pharmacokinetic studies (when needed, HBS was added to maintain an equivalent volume): 5.6 μ M bivalirudin, 0.31 μ M rivaroxaban, 0.39 μ M dabigatran and the last aliquot (control) lacked any TG antagonist. The assay was then performed as described above using TF-containing activator. In a second experiment, different thrombin generation antagonists were utilized, i.e. 0.4 μ M fondaparinux and 0.4 U/mL unfractionated heparin. The assay was then performed as described above (separate experiments were necessary due to limited number of conditions within assay).

2.7 Thrombin Generation in “Induced” Hemophilia

Blood from healthy individuals was collected into 3.2% sodium citrate and kept at room temp. CTI was added to blood to achieve a final concentration of 0.1 mg/mL, followed by the addition of either α FIXa-91 at a final 0.1 mg/mL concentration (“induced” hemophilia B) or α FVIII at a final 0.3 mg/mL concentration (“induced” hemophilia A). HBS was added to the control aliquot. The assay was then performed as described above using TF-containing activator.

2.8 Thrombin Generation in pRBC/MDP

pRBC were prepared at UVMMC as described previously (25) and provided by Dr. Fung. PPP, freshly thawed for 3 minutes at 37°C, was then mixed with the pRBC at a 1:1 ratio. In parallel, blood from a healthy individual was collected into 3.2% citrate and kept at room temp. The activating solution for this experiment was altered, so that there were 4 activating solutions in total; TF + CaCl₂, CaCl₂, TF + CaCl₂ + PCPS, CaCl₂ + PCPS (with the PCPS at 20 µM). These solutions were mixed with whole blood and pRBC/PPP at various combinations, making 6 different mixtures: whole blood, pRBC/MDP, pRBC/MDP + PCPS, whole blood + TF, pRBC/MDP + TF and pRBC/MDPP + PCPS + TF. The rest of the assay was performed as described above.

3. Results

3.1. Thrombin generation in blood from healthy donors

Blood from 10 healthy donors (5 males and 5 females) was collected into sodium citrate and processed in the presence and in the absence of CTI (in the latter case CTI was added prior to the assay), recalcified and thrombin generation was measured in the presence and absence of an exogenous activator, TF (Fig. 1; the data presented are averages for 10 donors). The propagation phase of thrombin generation in blood collected into citrate started after 10.1 min of the lag phase and the maximum concentration of active thrombin observed was 60 nM. When the same blood was processed in the presence of CTI, the lag phase was prolonged to 17.5 min and the maximum concentration observed was significantly reduced to 20 nM. An addition of 5 pM relipidated TF to both anticoagulation conditions led to a significant enhancement and almost identical profiles of thrombin generation, with the lag phase decreased to 5.5 min and the peak thrombin increased to 103–105 nM.

When thrombin generation profiles were compared for individual donors, a pronounced inter-individual variability was observed. For example, the lag phase for blood drawn into citrate and processed without CTI or TF varied from as short as 9.3 min for the Donor 4 to as long as 22.5 min for the Donor 1. Processing blood in the presence of CTI did not greatly change thrombin generation parameters for Donor 1, but dramatically prolonged thrombin generation for Donor 4, with the lag phase extending to 17.9 min. The most dramatic effect of CTI addition to citrated blood was observed for Donors 3 and 5 where there was no thrombin generation observed in 50 min.

For blood triggered with 5 pM TF, not only did the lag phase of thrombin generation became shorter for all donors, but inter-individual variability dropped dramatically as well. However, variability of the peak thrombin value (from ~80 nM for Donor 9 to 232 nM for Donor 4) remained quite pronounced.

3.2. Response to varying concentrations of initiators

The response of thrombin generation in CTI-treated citrated blood from healthy donors to varying concentrations of relipidated TF, FXIa and FIXa was evaluated. For TF, thrombin generation parameters, primarily the lag phase, responded in a concentration-dependent manner. In blood triggered with 0.5 pM TF, the lag phase of thrombin generation decreased

from 11.5 min (observed in the absence of an exogenous TF) to 8.8 min (Fig. 2A). A sequential increase in TF concentration led to a corresponding sequential decrease in the lag phase. At 5 pM TF (the highest TF concentration tested), the lag phase was 4.9 min. The response of peak thrombin concentration was affected to a lesser extent to these varying TF concentrations. For example, the peak thrombin concentration in the absence and in the presence of 0.5 pM TF was the same (72 nM). Even at the highest TF concentration tested (5 pM), this thrombin generation parameter was higher than that observed in the absence of TF by only 43% (103 nM vs. 72 nM, respectively).

Similar to TF, thrombin generation in CTI-treated citrated blood from several healthy donors was initiated with purified FXIa. Thrombin generation parameters analyzed (lag phase and peak thrombin) correlated with the concentration of exogenous FXIa added to blood (Fig. 2B). For example, the lag phase sequentially decreased with an increasing FXIa concentration from 13.8 min in the absence of exogenous FXIa to 3.9 min in the presence of 50 pM FXIa (almost 4-fold increase). A further increase in FXIa concentration had almost no effect on thrombin generation parameters. The least pronounced effect of varying FXIa concentration was observed for the peak thrombin value in which only a 2.4-fold increase was observed when FXIa increased from 0 to 50 pM.

Thrombin generation triggered with FIXa was less sensitive to the initiator concentration than thrombin generation triggered with relipidated TF or FXIa. As a consequence, higher molar concentrations of FIXa were required to achieve a lag phase similar to those observed in TF and FXIa-initiated reactions. For example, only 5 pM TF or 10 pM FXIa were required to provide an approximately 6 min lag phase, whereas for FIXa-triggered thrombin generation the concentration of this enzyme was increased to 500–1000 pM to lead to a similar lag phase (Fig. 2C). On the other hand, peak thrombin concentration was higher at these FIXa concentrations (240 nM for 1000 pM FIXa) than those observed for TF and FXIa initiated reactions (103 nM for 5 pM TF; 98 nM for 50 pM FXIa). With decreasing FIXa concentration, the lag phase steadily increased in a concentration-dependent manner through the entire range of FIXa concentrations tested. The maximum thrombin decreased in a dose-dependent manner until the concentration of FIXa reached 31 pM, at which point a further decrease in FIXa concentration had almost no effect on any parameters of thrombin generation.

Based on observed correlations between TF, FXIa and FIXa concentration and thrombin generation parameters, corresponding calibration curves were built for use in the quantitation of these 3 proteins in blood from trauma patients (see section 3.3).

3.3. Trauma patient blood

Fresh citrate blood from a trauma patient was recalcified and tested for endogenous procoagulant activity-related thrombin generation in the presence of CTI. After a 4.4 min lag phase, thrombin generation entered the propagation phase (Fig. 3) reaching a maximum concentration of active thrombin of 213 nM. The addition of an inhibitory monoclonal anti-TF antibody had almost no effect on parameters of thrombin generation, suggesting that there is no functional TF in the blood sample analyzed. In contrast, the addition of an inhibitory monoclonal anti-FXIa antibody prolonged the lag phase to 5.6 min and led to a

decreased maximum thrombin concentration of 150 nM. Based on the FXIa calibration curve (see section 3.2), the concentration of FXIa in this blood sample was 29.5 pM. Further addition of an inhibitory monoclonal anti-FIXa antibody completely suppressed thrombin generation. Based on the FIXa calibration curve, the concentration of FIXa in this blood sample was 800 pM.

3.4. Antagonists of thrombin generation

Several anticoagulants at pharmacologic concentrations were evaluated for their effect on 5 pM relipidated TF-triggered thrombin generation in contact pathway-inhibited citrate blood. The concentrations of anticoagulants used are matching those recommended for their clinical application in patients and approved by the FDA. Blood from a healthy donor was treated with 0.5 U/ml unfractionated heparin and 0.4 μ M fondaparinux. Heparin had a minor effect on parameters of thrombin generation by slightly prolonging the lag phase and minimally affecting the maximum concentration of active thrombin (Fig. 4a). In contrast, fondaparinux more than doubled the lag phase and significantly suppressed the peak thrombin value.

Two reversible direct thrombin inhibitors (bivalirudin and dabigatran) and one FXa inhibitor (rivaroxaban) were also evaluated for their effect on thrombin generation in whole blood from a healthy donor (Fig. 4B). Bivalirudin at 5.6 μ M prolonged the lag phase of thrombin generation from 6.8 min to 11.0 min, but did not suppress the peak thrombin value. In contrast, the peak thrombin value was slightly increased in the presence of bivalirudin (from 79 to 88 nM). Dabigatran at 0.4 μ M concentration was more efficient than bivalirudin as it more than doubled the duration of the lag phase (from 6.8 to 17.3 min) and slightly suppressed the peak thrombin (from 79 to 63 nM). Rivaroxaban at 0.3 μ M concentration produced an intermediate effect by prolonging the lag phase to 12.2 min and decreasing the peak thrombin value to 68 nM.

3.5. “Induced” hemophilia blood

Hemophilia A and hemophilia B was “induced” in blood from a healthy volunteer via the addition of inhibitory antibodies to FVIII and FIX, respectively. In both cases, relipidated TF-initiated thrombin generation was significantly suppressed (Fig. 5). In “induced” hemophilia A and B blood peak values of thrombin were reduced from 103 nM of normal blood to 36 nM and 27 nM, respectively.

3.6. pRBC

Thrombin generation in platelet poor multi-donor plasma (MDP)-reconstituted with pRBC (50/50) was compared with thrombin generation in whole blood. In the absence of TF, no thrombin generation was observed in reconstituted pRBC (Fig. 6). In contrast to pRBC, thrombin generation in CTI whole blood started after 16.2 min lag phase and the highest concentration of active thrombin observed was 136 nM.

When 5 pM relipidated TF was added to reconstituted pRBC, detectable thrombin generation started after 8.1 min of the lag phase and the peak value of active thrombin

measured was 175 nM. Thrombin generation in TF-triggered whole blood showed a shorter lag phase (6.5 min) and a slightly higher peak thrombin value (195 nM).

An addition of PCPS to the MDP-reconstituted pRBC had no effect to thrombin generation either in the presence or the absence of TF.

4. Discussion

Several years ago, a new methodology for the evaluation of thrombin generation in whole blood was reported by Ninivaggi and coworkers (17). In that paper (and several more published studies) this methodology is used to measure thrombin generation in blood from patients with hemophilia A, taking vitamin K antagonists (26) and patients undergoing cardiothoracic surgery (27). In the current study, an effect of CTI, several initiators and inhibitors of coagulation and reconstituted blood components were evaluated for their effect on thrombin generation in whole blood.

CTI, an inhibitor of FXIIa (20), efficiently prevents thrombin generation in PCPS-containing plasma from healthy individuals in the absence of TF (10) because TG is triggered by the contact pathway. Based on the previously published data (16) and on the data of this study, CTI inhibits thrombin generation in whole blood as well. In contrast, thrombin generation in blood triggered with 5 pM TF was not affected by the presence of CTI. These results are in an agreement with previously published studies indicating that at a relatively fast onset of TF-initiated thrombin generation, i.e. at picomolar concentrations of membrane bound TF, contact pathway plays no detectable role in thrombin generation in blood or plasma (28, 29).

Both TF and FXIa efficiently initiated thrombin generation providing similar profiles at equimolar concentrations. It was shown in a previously published study that TF in a complex with FVIIa is required only for the initiation of thrombin generation, not playing any significant role during the propagation phase (30). On the other hand, inhibition of the FVIIa/TF complex by the major regulator of it, tissue factor pathway inhibitor, is efficient only in the presence of FXa (31), i.e. during the propagation phase of thrombin generation. These observations explain the high efficiency of TF in triggering processes leading to blood coagulation. As for the efficiency of FXIa, it can be explained by its relatively slow inhibition by serine protease inhibitors present in blood and plasma (18). FIXa can be inhibited in blood more rapidly than FXIa (32), leading to its lower efficiency in initiating thrombin generation, consistent with this study.

In several previously published studies we observed an endogenous procoagulant activity present in plasma of cardiovascular and trauma patients and in plasma of patients with inflammatory diseases (10, 18, 33–35). This activity is caused by the presence of either functional TF or active FXIa or FIXa, or by any combination of these 3 proteins. No endogenous procoagulant activity was observed in plasma from healthy individuals. In contrast to plasma, thrombin generation was observed in CTI-containing whole blood from healthy individuals (see Fig. 1), however in trauma patient blood thrombin generation occurred after a shorter lag phase and reached a higher maximum thrombin concentration than in blood from healthy donors (compare thrombin generation profiles in the absence of

exogenous TF presented in Figs. 1 and 3). A more efficient thrombin generation in trauma patient blood is caused primarily by a higher FXIa concentration (unpublished observation). The mechanism leading to an elevated FXIa generation in trauma patient blood is yet unclear, but preliminary data suggest that it could be related to the presence of histones and polyphosphates circulating in trauma patient blood (36–38).

A comparison of unfractionated heparin and fondaparinux shows that the latter is a more efficient inhibitor of thrombin generation in whole blood than the former. It is consistent with previously published studies and the knowledge that fondaparinux is primarily an AT-III-dependent inhibitor of FXa and FIXa (39–41). As a consequence of the inhibition of these two enzymes, a significant suppression of thrombin generation during the propagation phase and a delayed onset of this process would occur. In contrast, the primary target of unfractionated heparin at the concentrations used is thrombin, which would lead to a delay in the onset of the propagation phase until all inhibitor is consumed, thus having little effect on the propagation phase itself. This effect is similar to that observed for relatively low concentrations of another thrombin inhibitor bivalirudin (42). All three direct thrombin and FXa inhibitors showed an expected effect (41, 43) by prolonging the lag phase of thrombin generation.

Hemophilia A and hemophilia B involves a deficiency of FVIII and FIX, respectively, which leads to significantly impaired FXa generation and, consequentially, to a decreased concentration of the prothrombinase complex. The efficiency of thrombin generation is impaired, primarily affecting a propagation phase of the process and having little effect on the initiation/lag phase (5, 44). Profiles of thrombin generation observed in this study at hemophilia A and hemophilia B-representing conditions are quite similar to those observed in minimally altered whole blood from a severe hemophilia A patient (5) or in “induced” hemophilia B blood (44).

Ninivaggi and coworkers demonstrated that TF-induced thrombin generation in RBC reconstituted with PPP is similar to that observed in whole blood (17). The data from our study support this observation. These results from both studies are somewhat surprising: although the RBC can support TF-induced thrombin generation, the presence of platelets enhances this process (15). This apparent contradiction could be explained by the observation that relatively low concentrations of platelets (approximately 5% of mean physiologic) is required to normalize clotting of whole blood (45). The concentration of residual platelets present in PPP could be substantial enough to enhance thrombin generation in reconstituted RBC. In contrast to this observation from Ninivaggi *et al.*, we did not observe any increase in thrombin generation when phospholipid vesicles were added. It is quite possible that the difference in phospholipid compositions used in these 2 studies led to a different effect regarding thrombin generation.

In summary, the whole blood thrombin generation assay is more physiologically relevant than the widely used plasma thrombin generation assay in that it uses whole blood containing all proteins and cellular components rather than a lipid-reconstituted plasma system. Additionally, it spans a wider range of blood coagulation processes than viscoelastometric assays. This thrombin generation assay allows for the determination of

hemostatic potentials of healthy individuals, both endogenous and triggered by exogenous initiators, and for the assessment of endogenous procoagulant activities in trauma patient blood along with the sources of it. The assay could be also used for the characterization of an effect of certain protein deficiencies and that of various anticoagulants on thrombin generation. However, it has to be emphasized that this assay requires some handling and training along with further comparison with established assays prior to clinical implementation for the uses described in this manuscript.

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Figure 1. Thrombin generation (TG) in blood from healthy individuals (each TG trace represents the average from 5 males and 5 females) collected into citrate with or without CTI and with or without exogenous 5 pM TF initiation.

Figure 2.

Thrombin generation in citrated blood containing CTI from healthy individuals (each panel represents a different healthy donor) initiated with varying concentrations of TF (A), FXIa (B), and FIXa (C).

Figure 3.

Thrombin generation in citrated blood containing CTI from a trauma patient (TP) in the absence and presence of 0.1 mg/mL inhibitory monoclonal antibodies α TF-5, α FXIa-2 and α FIXa-91, with a corresponding healthy donor control (HD). Endogenous FXIa and FIXa concentrations of 29.5 pM and 800 pM, respectively, were calculated based on calibration curves built from titrating these proteins into blood from healthy donors.

Figure 4.

Thrombin generation in citrated blood containing CTI from healthy individuals (each panel represents a different healthy donor) treated with 5 pM TF and **A**). 0.4 U/ml unfractionated heparin and 0.4 μ M fondaparinux and **B**). 5.6 μ M bivalirudin, 0.31 μ M rivaroxaban and 0.39 μ M dabigatran.

Figure 5. Thrombin generation in “induced” hemophilia A and B (healthy donor blood with the addition of 0.3 mg/mL α FVIII and 0.1 mg/mL α FIX, respectively) citrated blood containing CTI initiated with 5 pM TF.

Figure 6. Thrombin generation in citrated whole blood and reconstituted pRBC (MDP +/- PCPS) in the absence (**A**) and presence (**B**) of 5 pM TF.

Thrombin generation (TG) parameters for all experimental TG traces presented in Figures 1–6 (analyzed using software provided by Diagnostica Stago). With the exception of Figure 1, all traces represent an average curve of 3 identical runs (“triplos”) and the corresponding average parameters with standard deviations are displayed here. In Figure 1, each TG trace represents the average of 10 healthy donor triplos and the corresponding average parameters with standard deviations are displayed here.

Table 1

Figure	Condition	Concentration	Lag Phase (min)	Max FIIa (nM)	AUC (nM * min)
Fig 1	Citrate	11 mM	10.1 ± 4.4	60.3 ± 49.9	970 ± 585
	Citrate + TF	11 mM + 5 pM	5.5 ± 0.6	102.6 ± 49.6	1041 ± 636
	CTI/Citrate	0.1 mg/mL/11 mM	17.5 ± 8.7	20.2 ± 13.3	232 ± 202
	CTI/Citrate + TF	0.1 mg/mL/11 mM + 5 pM	5.5 ± 0.4	105 ± 49.2	984 ± 589
Fig 2A	TF Titration	0 pM	11.5 ± 0.7	72 ± 8	607 ± 25
		0.5 pM	8.8 ± 0.0	72 ± 12	560 ± 88
		1 pM	7.5 ± 0.2	88 ± 5	610 ± 14
		2.5 pM	6.3 ± 0.4	96 ± 6	650 ± 15
		5 pM	4.9 ± 0.2	103 ± 7	647 ± 20
Fig 2B	FXIa Titration	0 pM	13.8 ± 1.0	43 ± 3	589 ± 27
		1 pM	12.8 ± 0.4	62 ± 2	618 ± 72
		5 pM	8.7 ± 0.6	103 ± 4	733 ± 27
		10 pM	5.4 ± 0.4	118 ± 15	683 ± 8
		50 pM	3.9 ± 0.4	134 ± 5	681 ± 16
Fig 2C	FIXa Titration	100 pM	4.0 ± 0.2	139 ± 3	748 ± 20
		0 pM	17.1 ± 1.3	116 ± 5	964 ± 101
		15 pM	15.8 ± 1.4	106 ± 12	834 ± 76
		31 pM	14.0 ± 1.9	100 ± 19	770 ± 85
		125 pM	9.9 ± 0.3	151 ± 11	907 ± 94
Fig 3	Healthy Donor (HD) Trauma Patient (TP) TP + α-TF	500 pM	7.2 ± 1.5	190 ± 15	1114 ± 50
		1000 pM	6.0 ± 0.6	248 ± 18	1307 ± 60
		N/A	5.0 ± 0.3	112 ± 3	987 ± 39
		N/A	3.6 ± 0.3	217 ± 20	1491 ± 66
		0.1 mg/mL	3.5 ± 0.1	217 ± 9	1440 ± 52

Figure	Condition	Concentration	Lag Phase (min)	Max FIIa (nM)	AUC (nM * min)
	TP + α -XI	0.1 mg/mL	4.7 $\pm$ 0.4	159 $\pm$ 10	1513 $\pm$ 182
	TP + α -IX	0.1 mg/mL	>50	0	0
	No Anticoagulant	N/A	12.3 $\pm$ 0.3	167 $\pm$ 12	928 $\pm$ 42
Fig 4A	Heparin	0.5 U/mL	9.3 $\pm$ 0.7	162 $\pm$ 7	945 $\pm$ 47
	Fondaparinux	0.4 uM	22.0 $\pm$ 1.0	58 $\pm$ 13	519 $\pm$ 93
	No Anticoagulant	N/A	5.9 $\pm$ 0.2	80 $\pm$ 2	473 $\pm$ 6
Fig 4B	Bivalirudin	5.6 uM	10.4 $\pm$ 0.3	87 $\pm$ 6	503 $\pm$ 15
	Rivaroxaban	0.31 uM	11.7 $\pm$ 0.9	73 $\pm$ 2	523 $\pm$ 10
	Dabigatran	0.39 uM	16.2 $\pm$ 0.9	64 $\pm$ 4	500 $\pm$ 3
	Normal Blood A	N/A	4.8 $\pm$ 0.4	71 $\pm$ 11	626 $\pm$ 55
Fig 5	Normal Blood B	N/A	5.3 $\pm$ 0.3	109 $\pm$ 8	637 $\pm$ 47
	"Induced" Hemophilia A	N/A	5.4 $\pm$ 0.2	37 $\pm$ 4	425 $\pm$ 22
	"Induced" Hemophilia B	N/A	7.2 $\pm$ 0.4	30 $\pm$ 4	547 $\pm$ 50
	Whole Blood	N/A	10.7 $\pm$ 4.7	141 $\pm$ 8	1795 $\pm$ 48
Fig 6A	pRBC/MDP	N/A	>50	0	0
	pRBC/MDP + PCPS	N/A	>50	0	0
	Whole Blood + TF	5 pM	5.3 $\pm$ 0.6	204 $\pm$ 13	2140 $\pm$ 87
Fig 6B	pRBC/MDP + TF	5 pM	7.4 $\pm$ 0.4	180 $\pm$ 7	1837 $\pm$ 66
	pRBC/MDP + PCPS + TF	5 pM	6.6 $\pm$ 0.9	162 $\pm$ 15	1698 $\pm$ 135