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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 ± 5 pM tissue factor (TF) and ± 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.

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 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.

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Materials and methods

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 (αTF-5; prevents binding of TF to FVIIa), anti-FXIa (αFXIa-2; inhibits FIX activation by FXIa) and anti-FIXa (αFIXa-91; inhibits FX activation by FIXa) antibodies were produced and characterized in-house [22,23]. Inhibitory polyclonal anti-FVIII (αFVIII; 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 (P_2Rho; 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 $TF_{1–263}$ [19] (all from Haematologic Technologies, Inc.; Essex Junction, VT). BSA-5 buffer (20 mM HEPES, 140 mmol/L NaCl, 0.02% NaN_3 and 5 g/L bovine serum albumin (BSA) at pH 7.35), BSA-60 buffer (20 mM HEPES, 0.02% NaN_3 and 60 g/L BSA at pH 7.35), calibrator buffer (20 mM HEPES, 100 mM sodium citrate, 0.02% NaN_3 and 60 g/L BSA at pH 7.4) and 1M $CaCl_2$ were all supplied by Diagnostica Stago (all reagents from Sigma-Aldrich; St. Louis, MO). Additionally, α2-macroglobulin-thombin 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, Finlad) were all provided by Diagnostica Stago. The thrombin generation assay was performed as previously described [17] and modified for the assays below.

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 P_2Rho 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 Ca^{2+} in BSA-5 buffer). Forty μl of the blood/substrate mixture was then added to a preheated (37C) round-bottom microtiter plate, followed by the addition of 20 μl of activator/calibrator solution. Final concentrations of reagents in blood are: 450 μM P_2Rho, 100 nM calibrator, 5 pM TF and 16.7 mM $CaCl_2$. The mixture was immediately transferred to the preheated flat-bottom microtiter plate containing filter paper, in triplicate, and mineral oil (40 μL) 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 min. The derived data was analyzed for thrombin generation curves using the analysis platform provided by Diagnostica Stago.

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.

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.

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, αFXIa-2, αFIXa-91 and αTF-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.

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 μM bivalirudin, 0.31 μM rivaroxaban, 0.39 μM 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 μM 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).

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 αFIXa-91 at a final 0.1 mg/mL concentration (“induced” hemophilia B) or αFVIII at a final 0.3 mg/mL concentration (“induced” hemophilia A).