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Simultaneous thrombin and plasmin generation capacities in normal and abnormal states of coagulation and fibrinolysis in children and adults ☆

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ABSTRACT

Introduction: Thrombin and plasmin are the key enzymes involved in coagulation and fibrinolysis, respectively. Plasma coagulative and fibrinolytic potentials in normal children and adults, and in representative pathologically altered hemostatic states, were evaluated via simultaneous assessment of thrombin and plasmin generation.
Materials and Methods: An assay of Simultaneous Thrombin and Plasmin generation (STP) was developed to measure thrombin and plasmin in plasma using individual fluorometric substrates. Coagulation is initiated with dilute tissue factor, phospholipid, and calcium in platelet-poor plasma; fibrinolysis is accelerated via tissue plasminogen activator (tPA). Abnormal states of hemostasis were investigated.
Results: STP assay reproducibility and normal adult and pediatric values for measured and calculated parameters have been established. Onset of both thrombin and plasmin generation was significantly delayed in children relative to adults ($p<0.001$) and the maximum amplitudes of thrombin and plasmin generation were less in children than adults ($p<0.01$). No significant differences were measured among pediatric age groups. The most profound impairments in thrombin generation were observed for extrinsic and common pathway factor deficiencies, with the exception of afibrinogenemia. Plasmin generation was severely impaired in deficiencies of fibrinogen and plasminogen as well as with decreased tPA reagent concentration and addition of aminocaproic acid. Plasmin generation was greatly enhanced by alpha-2-antiplasmin deficiency and excess tPA reagent.
Conclusion: Simultaneous assessment of thrombin and plasmin generation in plasma shows promise for affording an enhanced understanding of overall coagulative and fibrinolytic functions in physiological and pathologically altered states of hemostasis in children and adults.

Introduction

Abnormalities of clot formation or breakdown lead to variable clinical scenarios of hemorrhage, thrombosis, or both. Many laboratory tools are available that attempt to assess these processes from measuring individual coagulation factor activities to evaluating overall hemostatic function. Since the coagulation (clot formation) and fibrinolytic (clot dissolution) systems are interrelated, it would be advantageous to measure the key enzymes driving each system (i.e., thrombin and plasmin) simultaneously.

Global assays of coagulation and fibrinolysis used in clinical research include the thromboelastogram, the Overall Hemostatic Potential in Plasma (OHPP) assay, and the Clot Formation and Lysis (CloFAL) assay. The latter two assays are turbidimetric (i.e., register fibrin) and presently employ tissue factor for coagulation activation and tissue plasminogen activator for enhancement of fibrinolysis [1,2]. While thrombin generation assays such as the Calibrated Automated Thrombogram

Abbreviations: aPTT, activated partial thromboplastin time; AUC, area under the curve; $CaCl_2$, calcium chloride; CloFAL, Clot Formation and Lysis assay; CV, coefficient of variation; FVIII, factor VIII; IQR, interquartile range; Lag_P, plasmin lag time (time to start of plasmin generation); Lag_T, thrombin lag time (time to start of thrombin generation); MA, maximum amplitude; MA_P, maximum amplitude of plasmin generation; MA_T, maximum amplitude of thrombin generation; NaCl, sodium chloride; OHPP, Overall Hemostatic Potential in Plasma; PPP, platelet poor plasma; PT, prothrombin time; STP, Simultaneous Thrombin and Plasmin generation; TBS, Tris Buffered Saline; TF, tissue factor; tPA, tissue plasminogen activator; Vmax, maximum velocity of intensity over time; $V_P$max, maximum velocity of intensity of plasmin generation over time; $V_T$max, maximum velocity of intensity of thrombin generation over time.

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have been applied in a broad array of clinical research settings, fibrinolysis is not assessed by this method [3–5].

The purpose of this work was to investigate relative changes in thrombin and plasmin generation capacity simultaneously (i.e., in parallel) in the plasma of healthy children as compared to adults and in numerous pathologically altered states of coagulation and fibrinolysis. In doing so, a new global hemostasis method, the simultaneous thrombin and plasmin generation (STP) assay, is described.

Materials and Methods

Subject Groups

Collection of blood specimens and recruitment of participants for the standardization of new coagulation tests and the development and application of research tools to evaluate bleeding and thrombotic disorders were approved by the Colorado Multiple Institutional Review Board (#03-700). Signed informed consent was obtained prior to participation. All healthy individuals recruited for the establishment of normal values were screened by clinical history to exclude subjects with recent illness, prior abnormal bleeding or thrombotic events in the participant and first degree relatives, or recent use of antibiotic, anticoagulant, anti-platelet, or estrogen-containing medications. Samples from all healthy children and adults were confirmed to have prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen values in the respective normal ranges. Plasmas from healthy adults were obtained commercially (Core Set Adult Normals, George King Bio-Medical, Overland Park, KS).

Blood Collection and Sample Processing

Blood was collected with the subject at rest in the seated position by atraumatic peripheral venipuncture technique with minimal applied stasis into BD Vacutainer 3.2% buffered sodium citrate siliconized blood collection tubes (Becton-Dickinson, Franklin Lakes, NJ), after collection of the initial 1 mL of blood into a discard tube. As is the procedural standard in our laboratory, samples that were hemolyzed were deemed not acceptable for processing and further analysis. Specimens were centrifuged for 15 minutes at 4 °C and 2500×*g*, and the plasma supernatant was then centrifuged for an additional 15 minutes at the same settings to remove residual platelets. Plasma found to be icteric or lipemic was excluded from analysis in assays that measure optical density or fluorescence intensity. Platelet-poor plasma (PPP) samples were aliquoted into 1.5 mL copolymer polypropylene long-term freezer storage tubes with O-ring screw caps (USA Scientific, Ocala, FL) and stored at -70 °C until time of assay. Commercially obtained individual and pooled PPP specimens (George King Bio-Medical, Overland Park, KS) were collected and processed by a similar protocol.

Simultaneous Thrombin and Plasmin generation (STP) procedure

Frozen PPP aliquots are thawed in a 37 °C water bath for 3 minutes; comparison of freeze-thawed vs. fresh platelet-poor plasma specimens from the same person revealed no differences in the STP curves. Reactant solution is prepared as described previously for the CloFAL assay developed in our laboratory [2]. Briefly, lipidated recombinant human tissue factor (TF) (American Diagnostica Inc, Stamford, CT), non-lipidated recombinant human TF (American Diagnostica Inc), and recombinant two-chain tissue plasminogen activator (tPA) (American Diagnostica Inc) are added to a stock solution of Tris-buffered saline (TBS; 66 mM Tris, 130 mM NaCl, pH = 7.0) containing 34 mM $CaCl_2$, to a concentration of 10 pM of TF (made from 8.7 pM non-lipidated TF and 1.3 pM lipidated TF) and 900 ng/mL of tPA. Final concentrations are 5 pM TF and 450 ng/mL tPA after addition of reactant solution to plasma samples, as described below. The assay blank consists of TBS alone. The reactant solution is freshly prepared from stored stock solutions of TBS, aliquoted TF, and aliquoted tPA just prior to assay. Final concentrations of TF and tPA ranging from 0-10 pM and 0-900 ng/mL, respectively, were investigated during assay development, with ultimate concentrations of these reagents (as well as ratio of lipidated to non-lipidated TF) selected based upon empiric experiments designed to optimize precision and analytical sensitivity to pathologically and physiologically altered states of coagulation and fibrinolysis, as previously performed with the CloFAL assay [2,6,7].

Two fluorometric substrates are used for the detection of the key enzymes, thrombin and plasmin. Boc-Val-Pro-Arg-MCA [t-Butyloxycarbonyl-L-Valyl-L-Prolyl-L-Arginine-4-Methyl-Coumaryl-7-Amide] is a fluorometric substrate for α-thrombin (Peptide Institute Inc, Osaka, Japan; 10 mM stock solution prepared according to manufacturer instruction) [8] and Boc-Glu-Lys-Lys-MCA [t-Butyloxycarbonyl-L-Glutamyl-L-Lysyl-L-Lysine-4-Methyl-Coumaryl-7-Amide] is a fluorometric substrate for plasmin (Peptide Institute Inc, Osaka, Japan; 10 mM stock solution) [9]. Stock solutions of each fluorometric substrate are stored at -20 °C for up to one month. With each respective stock solution of fluorometric substrates, a fresh working solution of 1,000 μM concentration is made with dilution of the stock solution in TBS. Final well concentration of each fluorometric substrate is 100 μM.

For each assay, samples are run in duplicate with the initial two wells dedicated as a blank (TBS alone with fresh reactant solution) followed by duplicate wells of plasma samples. For each sample, the duplicates are run in parallel rows where the reactant solution in the first row contains thrombin substrate and that in the second row contains plasmin substrate. Therefore, four wells in total are dedicated to each sample where two represent the thrombin generation and the other two represent plasmin generation. In this manner, thrombin and plasmin reagent wells are kept separate, avoiding concerns of potential interference/interaction in signal detection.

Using a multi-tip automated pipette, 90 μL of fresh reactant solution is added to each sample well in a flat-bottom, black polystyrene, 96-well Costar assay plate (Corning Inc, Corning, NY). Following the addition of reactant solution to each well, 20 μL of appropriate fluorometric substrate is added to each well. For each sample, one row of the 96-well plate contains 20 μL per well of Boc-Val-Pro-Arg-MCA thrombin fluorometric substrate and the second row contains 20 μL per well of Boc-Glu-Lys-Lys-MCA plasmin fluorometric substrate, such that these enzymes of thrombin and plasmin are being detected simultaneously for each sample run in parallel. Once the assay plate wells contain the reactant solution and appropriate fluorometric substrate, the plate is then placed in a microplate fluorometric spectrophotometer (Synergy 2, BioTek Instruments Inc, Winooski, VT) where it is prewarmed at 37 °C for 3 minutes. At the end of the 3 minutes, 90 μL of blank or plasma sample to be analyzed is dispensed into each of four wells (two in the top row with thrombin substrate and two in the next row with plasmin substrate). The plate is then read with fluorescent excitation wavelength of 360 nm and emission wavelength of 460 nm at 45 second intervals for 4 hours, following an initial 5 second mixing step prior to the first reading.

The fluorescent spectrophotometer interfaces with a computer such that all of its operations, including continuous analysis of fluorescence using Gen-5™ PC software, are automated. At the end of the 4 hour reading time, the data generated in the Gen-5™ software is exported to a Microsoft Excel spreadsheet where the data is further analyzed. Fluorescence data for each sample are blanked by subtracting the fluorescent reading for each sample well minus that of the blank well for thrombin and plasmin separately. Blanked fluorescence data are then averaged at each time point for the duplicate plasma wells containing reagent.

Unique thrombin and plasmin generation curves are produced (see also Fig. 1). Each curve has an initial baseline fluorescent intensity, followed by a rise in fluorescent intensity (slope phase) to a