PART 12 Fibrinolysis Lab Investigation

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TOPICS COVERED

- ✓ Plasma Clot Lysis Time Induced by tPA
- ✓ Plasmin Generation Assay

Fibrinolysis is a series of enzymatic reactions that degrade insoluble fibrin and depends on the quantity and quality of various fibrinolytic enzymes, such as tissue-type plasminogen activator (tPA) and plasmin, their respective inhibitors, plasminogen activator inhibitor type 1 (PAI-1) and alpha-2-antiplasmin (α2AP), as well as the structure of the clot (Longstaff and Kolev, 2015). In a clinical setting, increasing the rate of fibrinolysis is used to reverse thrombotic occlusion (e.g. recombinant tPA for acute treatment of stroke, myocardial infarction, or pulmonary embolism), while lowering the fibrinolytic rate is used to reduce bleeding (e.g. tranexamic acid for acute treatment of stroke, myocardial infarction, or pulmonary embolism) (Draxler and Medcalf, 2015; Ilich et al, 2017; Kwaan et al, 2017). Recently, in hemophilia, assays measuring clot formation and fibrinolysis have been used to facilitate direct and functional comparisons between new and emerging hemostatic agents that present different mechanisms of action (Holle et al, 2024). However, global testing to identify an individual's fibrinolytic potential is not widely implemented. In contrast, basic fibrinolysis research is generally based on the quantification of the various fibrinolytic factors. Total concentrations are measured using antigen-based assays, and specific functional tests to determine their activity. Despite the variety of assays available, it remains a challenge to assign individual fibrinolytic factors that contribute to the overall fibrinolytic result due to the dynamic nature of the environment surrounding the clot (Longstaff, 2018). For decades, turbidity-monitored clot lysis assays have been used as a standard method to quantify the overall fibrinolytic potential of a sample, and variations of this global and simplistic assay have been developed to address the function(s) of fibrinolytic factors. In this context, two assays have been explored in the context of clinical practice and show promising results for use in assessing general fibrinolytic potential.

Plasma Clot Lysis Time Induced by tPA: Plasminogen activator-induced plasma clot lysis times are frequently reported to assess and quantify the general fibrinolytic property of a sample (Longstaff, 2018). This test is performed by simultaneously adding agonists to initiate coagulation (e.g. tissue factor and Ca2+) and fibrinolysis (e.g. tPA) to citrated plasma. Alternatively, clots can be generated first and then covered with tPA to mimic the clinical scenario in which tPA is infused to degrade existing ischemic thrombi (Longstaff et al, 2011). The simplicity of the reaction system and minimal sample processing make this assay an ideal method for investigating the susceptibility of plasma clots to fibrinolysis (Holle et al, 2024). The test is also sensitive to inhibitor molecules that target specific factors, such as PAI-1 and α2AP, which can be included to infer the respective roles of PAI-1 and α2AP in general fibrinolysis (Zheng et al, 2023).

Reagents and method:

- Phospholipids [4 μM final]
- $CaCl_2$ [10 mM final]
- Tissue factor [1:15,000 dilution of Innovin, 1 pM final tissue factor]
- HEPES-buffered saline (20 mM HEPES, pH7.4, 150 mM NaCl)
- tPA (0.5 μg/ml final)

1) Into a U-bottom 96-well plate, pipette 10 ul TF/PL/rtPA into the each well (reverse pipetting to the bottom of the well)
2) Add 40 ul of PPP into each well (reverse pipetting to the side of the well at the top)
3) Preheat the plate in incubator for 5 min
4) After 5 min preheating, use multichannel pipette to transfer 10 ul Ca, mix well and avoid creating bubbles.
5) Quickly put the plate into the plate reader and start reading
6) Monitor the reaction for 2 hours by measuring the turbidity at 405 nm every 12 seconds using a plate reader.

Plasmin Generation Assay: The plasmin generation assays developed by various groups share some common elements (Longstaff, 2018; Zheng et al, 2023). In general, procoagulant activity in the plasmin generation assay is initiated by the addition of exogenous tissue factor to the recalcified plasma, and fibrinolytic activity is triggered by the addition of exogenous tPA. Plasmin generation is detected through the cleavage of a fluorogenic substrate, and the parameters are defined from the accumulation of fluorescence or through a mathematical derivative of this fluorescence curve. Subtle differences between these assays include the concentrations of tissue factor and tPA used and whether diluted plasma is used or not. Some variations of these assays detect thrombin and plasmin simultaneously, while others carry out these measurements separately, but in parallel. Studies show that these assays are specific for plasmin and sensitive to α2AP, with the measured activity representing free plasmin. Plasmin generation is also sensitive to tranexamic acid. Considering the high concentrations of tPA required to trigger measurable plasmin generation, the test is not sensitive to plasma concentrations of PAI-1 (Miszta et al, 2021). However, the plasmin generation response to the addition of exogenous tPA is dose-dependent. A growing body of work suggests that plasmin generation assays, especially when used with thrombin generation and turbidity assays, produce a multidimensional impression of the integrated effects of procoagulant and fibrinolytic activities in health and disease (Miszta et al, 2021; Zheng et al, 2023).

Reagents and method:

- Tris-buffered saline (TBS), containing 66 mM of Tris and 130 mM NaCl
- 34 mM of $CaCl_2$
- 10 pM of tissue factor (lipidated recombinant human tissue factor, Innovin, Siemens Healthcare, Erlangen,Germany)
- 900 ng/ml of tPA (recombinant two-chain tissue plasminogen activator, Actylise, Boehringer Ingelheim, Biberach an der Riss, Germany)
- α-thrombin (BOC-VAL-PrO-ARG-MCA, Peptides International, 5 mg)
- Plasmin (BOC-GLU-LYS-LYS-MCA, Peptides International, 5 mg)

1) Prepare the reagent solution 34 mM of $CaCl_2$, 10 pM of tissue factor , and 900 ng/ml of tPA to the TBS solution. The final concentration of reactants in plasma were 5 pM TF and 450 ng/ml tPA.
2) Two substrates were prepared with a final concentration of 100 µM and used for the detection of the enzymes: α-thrombin and plasmin.
3) The first two wells of the plate (Grelner, 96-well, flat bottom, black clear) should be used as a blank, and the samples were run in duplicate in parallel rows for each substrate, avoiding potential interference and/or interaction in signal detection.
4) Add substrate solutions (20 µl) to the wells of the plate, followed by 90 µl of the samples and blank (TBS solution).
5) Using a multi-tip automated pipette, add 90 µl of the pre-warmed reaction solution (37°C for 3 min) to each well of the plate.
6) Finally, the plate should be read in the fluorometer wavelength of 340 nm excitation and 450 nm emission for 4 hours at 45-second intervals.
7) Data analysis could be performed using Microsoft® Excel® software. The curves for thrombin and plasmin should be generated by calculating the average at each time point for the duplicated

plasma wells, subtracting the reading values of the blank (for thrombin and plasmin separately). Parameters to be calculated in both assays, using Shiny app tools: onset (time to the inflection point before turbidity increase), maximum rate (slope of a line fitted to the maximum rate of turbidity increase using 5 to 10 points to determine the line), time to plateau/peak (time to the turbidity plateau [clot formation] or peak [fibrinolysis]), turbidity change (maximum clot turbidity less the starting turbidity), and area under the curve (AUC) (calculated as the sum of trapezoids formed by turbidity curves).

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