Measuring Fibrinolysis

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Abstract

Physiological fibrinolysis under normal conditions progresses slowly, in contrast to coagulation which is triggered rapidly to stop bleeding and defend against microbial invasion. Methods to detect fibrinolysis abnormalities are less simple and poorly standardized compared with common coagulation tests. Fibrinolysis can be accelerated by preparing euglobulin from plasma to reduce endogenous inhibitors, or by adding plasminogen activators to normal plasma. However, these manipulations complicate interpretation of results and diagnosis of a "fibrinolysis deficit." Many observational studies on antigen levels of fibrinolysis inhibitors, plasminogen activator inhibitor 1 or thrombin-activatable fibrinolysis inhibitor, zymogen or active enzyme have been published. However, conclusions are mixed and there are clear problems with harmonization of results. Viscoelastic methods have the advantage of being rapid and are used as point-of-care tests. They also work with whole blood, allowing the contribution of platelets to be explored. However, there are no agreed protocols for applying viscoelastic methods in acute care for the diagnosis of hyperfibrinolysis or to direct therapy. The emergence of SARS-CoV-2 and the dangers of associated coagulopathy provide new challenges. A common finding in hospitalized patients is high levels of D-dimer fibrin breakdown products, indicative of ongoing fibrinolysis. Well-established problems with D-dimer testing standardization signal that we should be cautious in using results from such tests as prognostic indicators or to target therapies.

Keywords

- fibrinolysis
- biomarkers
- standardization
- thrombolytics
- antifibrinolytics

Introduction

A simplified outline of fibrinolysis is presented in ►Fig. 1 and more detailed reviews can be found elsewhere.[1,2] By necessity, in response to vascular damage, coagulation must be rapid to reduce the dangers of bleeding and prevent entry of pathogenic microorganisms. Subsequently, fibrinolysis takes place slowly under normal circumstances, over hours and days as vessels are repaired. These considerations explain why there are many relatively simple, rapid, well-standardized tests to measure blood clotting, which can identify defects in coagulation pathways. However, fibrinolysis is more difficult to measure for diagnostic purposes and methods are more cumbersome, so the role of fibrinolysis in the hemostatic balance may be underestimated.

Fibrinolysis assays are needed to study antifibrinolytic therapy and also in the development and quality control of thrombolytic drugs. Diverse approaches are available to assess fibrinolysis in healthy or sick populations to identify factors that may be involved in regulation or dysregulation, and hyperfibrinolysis or fibrinolysis resistance.

The National Institute for Biological Standards and Control (NIBSC) is a World Health Organization (WHO) collaborating center with the responsibility to generate, store and distribute biological standards. A major part of our portfolio covers diagnostic and drug-related standards in hemostasis, including fibrinolysis[3] and reviews on the ways in which our biological standards can be used have been published elsewhere.[4,5]

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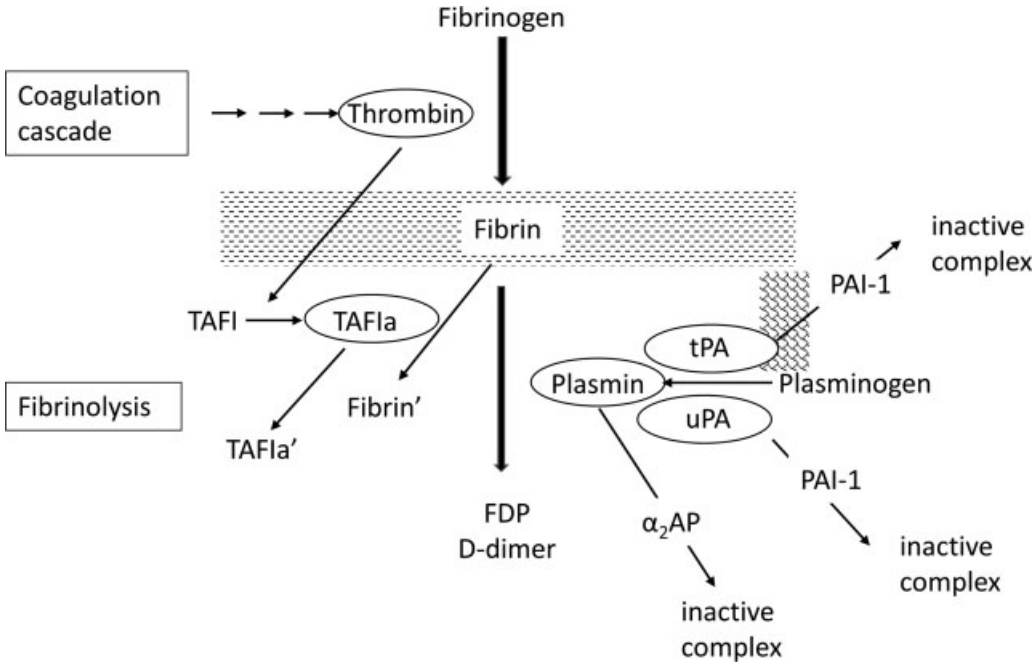

Fig. 1 Outline of the fibrinolysis system. The pathway of fibrin formation and degradation is shown by *heavy arrows*. Fibrin is a substrate (surface) for reactions and a substrate (target for enzymes) for plasmin.[1] Fibrin degradation products (FDP) are heterogeneous in size[88] and expose binding sites for D-dimer antibodies. Enzymes are in ovals and include the plasminogen activators, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). tPA activity is stimulated by binding to fibrin, where the finger domain is dominant,[11,89] whereas uPA is generated from an inactive zymogen scuPA (not shown) by the action of plasmin. Serpin inhibitors, PAI-1 and α_2-antiplasmin (α_2-AP) form inactive complexes to reduce fibrinolysis. Thrombin-activatable fibrinolysis inhibitor (TAFI, also known as procarboxypeptidase U, or CBP2 gene product) is activated by thrombin (and plasmin, not shown), to the active form (TAFIa). This enzyme modifies fibrin (shown as Fibrin′) to remove C-terminal lysines, which is less effective at binding plasminogen and plasmin and more resistant to lysis. TAFIa is thermally unstable and degrades to an inactive form, shown as TAFIa′. Other components that are involved include α_2-macroglobulin, a broad specificity inhibitor and thrombomodulin which has a role in regulating the activation of TAFI.[90] Additional factors that can impair fibrinolysis include variants of fibrinogen such as γ′-fibrinogen which affects clot architecture and fibrinogen-binding sites to make more resistant clots[91] and FXIII which creates a more resistant clot by cross-linking fibrin chains and α_2-AP to fibrin.[92,93] The incorporation of cells into clots can also delay fibrinolysis. Platelets cause clot retraction and release PAI-1[94]; and red blood cells can interact with fibrin,[95] and also become compressed during clot retraction to form an impermeable barrier which delays clot lysis.[96] PAI-1, plasminogen activator inhibitor 1.

Assays for Thrombolytic Proteins

Thrombolytics such as tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) are serine proteases that transform plasminogen into plasmin. Microbial plasminogen binding and activating proteins such as streptokinase and staphylokinase have no intrinsic protease activity but in practice, experimentally their reaction kinetics look like other plasminogen activators and they can be analyzed in the same way (although the details of the kinetic mechanisms may be complicated[6]). In practice, investigations on enzyme mechanism and regulation, or determination of specific activity or concentration will be performed in vitro in purified systems of proteins, or in plasma-based systems and can be optimized over a chosen thrombolytic enzyme range to give the most robust results. Activities of fibrinolytic proteins are often determined relative to the WHO International Standard as a primary calibrator.[3] Examples are available in publications of the development of fibrinolysis as per WHO International Standards (e.g., Locke et al[7]).

The simplest methods for following proteolytic activity involve optical monitoring of amidolytic substrates made of peptides linked to a chromophore or fluorophore. These types of substrates are not so useful when used directly on an enzyme of interest, but are valuable to study linked reactions that generate plasmin, for example. Active-site titration of serine proteases is a subgroup of chromogenic/fluorogenic assays and is useful to establish molar concentrations of active enzymes, including thrombolytic enzymes and thrombin.[8] Early fibrin-based methods for measuring plasminogen activator activity used fibrin plates, but have been superseded by microtiter plate-based methods.[9] This approach can be adapted to internal lysis (in which plasminogen activator is mixed with fibrinogen, plasminogen and thrombin to form a clot that is subsequently lysed evenly throughout), or external lysis (where activator is added to the top of a preformed clot). Internal lysis is related to normal hemostasis, while superficially added activator more closely reflects the situation during thrombolytic therapy. It is possible to combine fibrin-based methods and chromogenic substrate-linked measurement of plasminogen activation to precisely follow plasmin generation in the presence of fibrin.[10,11] In this way, rates of plasmin generation in SI units (e.g., pM/s) for tPA, uPA and streptokinase can be compared in the same format. Comparison of WHO assigned international units (IU) for these activators is not so useful as the units are unrelated. The long-established European Pharmacopoeia method for assaying alteplase

(tPA) activity,[12] in which clot lysis time is determined by passage of a ball through the clot in a tube or release of trapped bubbles from the fibrin network, is simple and reliable. More recently, the "Halo" method has been published that uses a small volume of whole blood, clotted in a ring or halo around the edge of a microtiter-plate well.[13] Lysis is followed by monitoring increasing absorbance as the clot breaks down and products released into solution.

Although it is relatively simple to generate reproducible time courses of data to study the activity of thrombolytic enzymes using these methods, the analysis of the resulting data is the next challenge. It is common in fibrinolysis assays to report lysis times, usually as time to 50% lysis, but how this is calculated is not always explained. Alternatively, zymogen activation rates determined with chromogenic substrates require the use of time squared plots, which can be tedious to generate and analyze. To standardize calculations of lysis times and zymogen activation rates and improve reproducibility, several online apps have been developed and published in association with ISTH/SSC (International Society on Thrombosis and Haemostasis/Scientific and Standardization Committee) Subcommittee on Fibrinolysis.[14,15] These apps are freely available and run in a computer browser without downloading any software (see also Longstaff[16] for summaries, links to apps and detailed instructions).

Diagnostic Methods

Functional Tests

Functional tests begin with the problem mentioned above that fibrinolysis without any stimulation is slow. To speed up the process, it is common to either add tPA to plasma to stimulate the generation of plasmin, or to remove inhibitors, by for example preparing the euglobulin fraction from plasma. A long-established functional method for measuring fibrinolysis in subject samples is to determine euglobulin clot lysis times (ECLTs), and this approach has been reviewed previously.[5,17,18] The general approach is time-consuming and difficult to automate. The euglobulin fraction is reported to have a greater than 90% reduction in α_2-antiplasmin, but there is also significant depletion of plasminogen activator inhibitor 1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI).[19] It is observed that the ECLT is strongly influenced by PAI-1 (and free tPA) concentrations. Recently an updated method has been published[20] where samples received added fibrinogen and ovalbumin to increase the clot turbidity. These authors also explored a fibrinolysis resistance test using added tPA which identified samples with high free active PAI-1, but also showed some sensitivity of lysis times to TAFI levels.

When normal plasma is used to investigate fibrinolysis, it is common to add significant amounts of extraneous tPA (from 200 to 700 ng/mL, see Table 2 in Longstaff[18]). To generate a clot, $CaCl_2$ is added as a minimum, and often thrombin and/or tissue factor (TF) with phospholipids may also be used. Attempts have been made to establish a standardized method for this procedure to improve reproducibility.[21] A commonly used output is 50% lysis time where lysis is followed optically, and analysis can be facilitated by online apps.[14] A thorough review of data from clot lysis studies has been presented to explore the relationship of fibrinolytic potential and risk for arterial and venous thrombosis.[22] Conclusions from many studies are not always strong or consistent in identifying molecular risk factors in different populations. However, an important observation is that hypofibrinolysis, especially in combination with hypercoagulability, can constitute an increased thrombosis risk.

An interesting development in this area is methods to simultaneously measure generation of both thrombin and plasmin during plasma clotting and lysis.[23] However, the methods proposed so far (reviewed in Longstaff[18]) have not become established, possibly because they are technically difficult to perform and analyze and no commercial equipment or software is available, in contrast to popular thrombin-generation platforms.

Antigen Assays

Antigen tests for plasma proteins involved in fibrinolysis are common and relatively simple to perform, even in large population studies. Many studies have been organized to investigate variations in circulating tPA, PAI-1 and TAFI, including free-active/inactive/latent and inhibited tPA–PAI-1 complex forms, to look for associations with arterial or venous thrombosis.[24,25]

Reduced levels of fibrinolysis inhibitors are not diagnosed as often as coagulation deficiencies, but when found often lead to increased bleeding risk.[17] Alternatively, high circulating PAI-1 and TAFI may indicate a "fibrinolysis deficit" and thrombosis risk. There are many large-scale population studies involving PAI-1 and TAFI, but results are not consistent.[26] Standardization of assay methods is poor in this area and it is not possible to directly compare absolute values of analytes from studies using different methods for PAI-1 antigen or activity or tPA antigen.[27,28] Generally, elevated PAI-1 is associated with cardiovascular disease, metabolic syndrome, diabetes, obesity, senescence and as a prognostic marker for several cancers.[29] It is likely that harmonization of results from different methods could be improved with common standards.[30,31] However, although there are WHO International Standards for tPA antigen in plasma and PAI-1 activity, they are not so popular because they are labeled in IU, while commercial methods report results in ng/mL. Unfortunately, the origin of the commercial kit standards is not consistent, and each "ng" is different. To establish a firm basis for reported ng, we at NIBSC are pursuing isotope dilution mass spectrometry using ^{13}C-labeled recombinant proteins as a way of establishing real gravimetric concentrations of plasma analytes. Work on TAFI and PAI-1 antigens is underway. In addition to these considerations, pre-test processing issues further complicate PAI-1 measurements and make tPA activity measurements unreliable. Factors such as diurnal and seasonal variations,[32] and release of PAI-1 from platelets during venepuncture must be considered.[33] Historically, a source of variation in TAFI assays has been the Thr325Ile polymorphism, which affects TAFI activation and stability.[34,35] It has been proposed that TAFIa is a

more important biomarker than zymogen,[36] but measurements require very sensitive methods.[37–39]

Interest in PAI-1 and TAFI as drug targets to modulate hemostasis has raised awareness of the importance of robust assay methods.[40–42] The idea of inhibiting PAI-1 activity is interesting in the context of aging as a mutation in the SERPINE1 gene resulting in around 50% reduced circulating PAI-1 is associated with longevity in animal models and a population of Amish in the United States.[43]

Hyperfibrinolysis and Hypofibrinolysis

Moore and colleagues[44] have attempted to define or clarify different types of pathological fibrinolysis observed clinically, for instance in trauma but also in surgery and disseminated intravascular coagulation (DIC). In addition to hyperfibrinolysis, there are varieties of fibrinolysis resistance (classically identified in ECLT assays) including hypofibrinolysis (a failure to trigger fibrinolysis after clotting) and fibrinolysis shutdown (where there is a rebound of increased PAI-1 activity and antigen after the triggering of coagulation and early release of tPA). The timing of fibrinolysis resistance and concepts such as occult fibrinolysis provide further complications.[44]

Hyperfibrinolysis in acute situations such as trauma or surgery is a life-threatening situation requiring rapid tests to direct treatment such as plasma or clotting factor replacements, or possibly with antifibrinolytics such as tranexamic acid (TXA). Instances where markers of fibrinolysis are elevated include trauma, DIC (where there is a fibrinolytic phenotype),[45] acute promyelocytic leukemia, liver damage, congenital abnormalities and surgical procedures (reviewed in Kolev and Longstaff[17]). Common markers for ongoing fibrinolysis would be elevated D-dimer,[46] raised tPA or decreased PAI-1, reduced plasminogen, reduced α_2-antiplasmin and elevated plasmin-α_2-antiplasmin (P-AP) complexes. Assays for these proteins are time-consuming, with the possible exception of point-of-care tests for D-dimer. However, D-dimer tests are approved for excluding thrombosis and the accurate measurement of high D-dimer levels is complicated by the low specificity of these tests and poor standardization.[47,48] Where hyperfibrinolysis is detected there is a high risk of death. For instance, in trauma only a minority of patients display a hyperfibrinolysis phenotype but mortality in this group is very high.[49,50] The underlying fibrinolysis imbalance is likely dominated by an increase in tPA resulting in plasmin generation with concomitant consumption of inhibitors PAI-1 and α_2-antiplasmin.[51,52] The potential for hyperfibrinolysis to be associated with thrombotic complications has been highlighted previously.[22]

Viscoelastic methods potentially have a role in diagnosing hyperfibrinolysis and fibrinolysis resistance as they are capable of generating results more rapidly than other available methods.[53] In particular, rapid thrombelastography (r-TEG) has been developed to speed up clotting by stimulating both intrinsic and extrinsic coagulation using kaolin and TF activators, and making tests available in cartridge form with potential for improved reliability.[54] The fundamentals of viscoelastic methods have been reviewed elsewhere[55,56] and involve the analysis of clot formation and lysis by physical measurement of blood viscosity and clot strength. In practice, the common platforms rotational thromboelastometry (ROTEM) and TEG provide an array of parameters from multiple variations of clotting tests and there is no agreed way of implementing results from these tests, although attempts are being made to develop optimized algorithms.[57,58] While the speed of testing is attractive, sensitivity and specificity may be an issue. A study by Raza and colleagues tested samples from trauma patients and found high P-AP complexes and D-dimer in samples where ROTEM did not detect ongoing fibrinolysis.[49] It has been proposed that occult (local) fibrinolysis or the earlier production of long-lived D-dimer or P-AP complexes could be responsible for this discrepancy between fibrinolysis biomarkers and viscoelastic methods.[44]

An interesting and controversial aspect of these discussions is how to use antifibrinolytic therapies, particularly TXA to reduce bleeding in surgery[59] or trauma. Several large-scale clinical trials have demonstrated that TXA given early in trauma is safe and effective[60] and pre-hospital treatment is recommended in Europe. In some quarters there are concerns that adding antifibrinolytics to a situation where there may be fibrinolysis resistance is potentially dangerous and could lead to thrombotic complications, including widespread vascular microthrombosis, organ failure and death. Thus, it is proposed that rapid testing, by viscoelastic methods, particularly rapid TEG, should be used to target only those patients who would benefit from antifibrinolytic treatment. However, several large clinical trials of TXA in trauma,[61] postpartum hemorrhage[62] and traumatic brain injury[63] observed no increases in thromboembolic complications,[60] and there is a lack of evidence of disperse microvascular thrombi.[64,65] On the other hand, the HALT-IT trial, which failed to show benefit of TXA in the treatment of gastrointestinal bleeding, did observe an increased risk of venous thromboembolic events (deep vein thrombosis plus pulmonary embolism) in the TXA treatment group.[66] The authors speculated that the increased risk may be due to disturbed hemostasis in the liver cirrhosis and variceal bleeding patients that made up half the subjects in the study, and/or the high dose of TXA used (4 g over 24 hours), which may also explain an observed increase in seizures. The conclusions from many studies and systematic reviews seem to be that more research is needed before there is sufficient confidence in viscoelastic methods for routine diagnostic testing, although there are promising signals of benefit in situations to reduce blood component use.[67–69] Hard evidence from randomized controlled trials to support the application of viscoelastic methods in targeting antifibrinolytic therapy is lacking.[50,70] It is argued that without this evidence the established risks of TXA treatment delay should outweigh theoretical but unproven risks of non-targeted treatment.

As TXA treatment is delayed, it becomes progressively less effective and after 3 hours benefit is lost[60] and the dangers appear to be excess bleeding, not thrombosis. There are few

studies of uPA in pathological fibrinolysis, but Hijazi and colleagues[71] have identified the slow development of a peak of uPA after several hours in a mouse model of brain injury, following the earlier rise and decay of tPA. We have shown in vitro that TXA plus urokinase stimulates plasmin generation to aggravate consumption of α_2-antiplasmin, which is often reduced in trauma patients, to allow the fibrinolytic system to go unchecked. The consequences are destruction of fibrinogen and clotting factors, which could contribute to a bleeding pathology.[72] There are little data available on changes in uPA or uPA–PAI complexes in trauma patients and currently tests for these analytes are poorly standardized so that estimates of gravimetric or molar concentrations in patient plasma are unreliable.

Covid-19

Coagulopathy was soon observed to be a life-threatening complication of infection with SARS-CoV-2 in hospitalized patients.[73,74] Venous thromboembolic events are noted in many patients in intensive care and a high proportion of patients are diagnosed with pulmonary embolism.[75,76] Postmortem investigations have identified widely distributed microvascular thrombi in the lungs, heart, kidney, liver, skin and fat.[77] Biomarkers in severely ill patients include raised fibrinogen and D-dimer, which can reach very high levels,[77,78] often >2 µg/mL, many times over the routine cut-off used to exclude a diagnosis of thrombosis (0.5 µg/mL). D-dimer has been investigated as a marker to predict mortality and manage patient care and direct anticoagulant treatment.[74,79] Reporting results and standardization of D-dimer testing protocols is an area of concern in Covid-19 patients and is particularly important if it is to be linked to patient care.[80] Given the prevalence of thrombotic complications in Covid-19 patients in intensive care, it is not surprising that thrombolytic therapy with tPA is being considered for seriously ill patients via the intravenous route[81] or in nebulizer form.[82] Other factors that may influence coagulation and fibrinolysis in Covid-19 patients may include neutrophil extracellular trap (NET) formation, which stimulates coagulation and retards fibrinolysis[83,84] and NETs have been observed in some early studies with samples from Covid-19 patients.[77,85,86] There is interest in changes in PAI-1 levels during Covid-19 infection, and there are early reports of fibrinolysis resistance assessed by antigen studies and viscoelastic methods.[85,87]

Conclusions

There is a long history of fibrinolysis research and assay method development, but no simple direct methods to establish something like a fibrinolysis capacity or a measurement of fibrinolysis resistance on par with prothrombin time or activated partial thromboplastin time, for example. Many problems associated with poor standardization and assay variability remain unresolved. Rapid universal methods would be useful in situations such as trauma, DIC and surgery, but there is a need for large-scale randomized trials to establish safety and efficacy. A better understanding is needed of changes in fibrinolysis following infection with agents causing hemorrhagic diseases, and the emergence of SARS-CoV-2 coagulopathy highlights the need for ongoing research to improve the measurement of fibrinolysis.

Conflict of Interest

The authors declare that they have no conflict of interest.

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