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Review Article

Global haemostasis assays, from bench to bedside

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ABSTRACT

Bleeding and thrombosis are the ultimate clinical outcomes of aberrations in the haemostatic process. Haemostasis prevents excessive blood loss due to the effort of various compartments like the vasculature, blood cells, coagulation and fibrinolysis. The complexity of all processes involved makes the diagnosis of aberrations difficult, cumbersome and expensive. A single assay to detect any factor disturbing this haemostatic balance with high sensitivity and specificity would be of great value, especially if the outcome of this assay correlates well with clinical outcome. Despite years of research, such an assay is not yet available; however, some interesting candidates are under development and combine the effects of various compartments. This review describes the development of global haemostasis assays and summarizes the current state of the art of these haemostasis assays covering thrombin and plasmin generation, turbidity and thromboelastography/thromboelastometry. Finally, we discuss the applicability of global assays in clinical practice and we provide a future perspective on the ongoing development of automation and miniaturisation as it is our belief that these developments will benefit the standardization of global haemostasis assays.

Contents

Abbreviations: α, angle that assesses rate of clot formation; α2M, α2-macroglobulin; AMC, 7-amino-4-methylcoumarine; AT, antithrombin; AUC, area under the curve; APC, activated protein C; aPTT, activated partial thromboplastin time; A10, the amplitude after ten minutes; A20, the amplitude after twenty minutes; C, continuous measurement; CLT, clot lysis time; CT, clotting time; EA, enzyme activity; ECLT, euglobulin clot lysis time; ETP, endogenous thrombin potential; F, fixed end-point measurement; FV, factor V; FVII, factor VII; FVIIa, activated factor VII; FVIII, factor VIII; FX, factor X; FIX, factor IX; FXI, factor XI; FXII, factor XII; Factor XIII, FXIII; FLT, fibrin lysis time; FnDP, fibrin degradation products; GFC, global fibrinolytic capacity; HA, hemophilia A; HB, hemophilia B; HMWK, high molecular weight kininogen; K_{cat}, hydrolysis rate; K_M, Michaelis-Menten constant; MA, maximal amplitude; LMWH, low-molecular-weight-heparins; NHA, Novel Hemostasis Assay; OHP, Overall Hemostasis Potential; PAI-1, plasminogen activator inhibitor-1; POC, point-of-care measurement; ROTEM, rotational thromboelastometry; T, turbidity; TAFI, thrombin activatable fibrinolysis inhibitor; TAT, thrombin-antithrombin; TEG, thromboelastography; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TG, thrombin generation; TM, thrombomodulin; TMA, time to maximal amplitude; tPA, tissue-type plasminogen activator; UFH, unfractionated heparin; V, visoelasticity; WB, whole blood.

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Introduction

Damage to a blood vessel triggers a complex series of events designed to prevent blood loss and, eventually, restore the function of the vascular system. Nature provided the haemostatic system to assure adequate blood flow. This prevents a prolonged arrest of blood supply while causing damage to surrounding tissues and organs. The haemostatic system depends on the interaction between the vessel wall, platelets and pathways of coagulation and fibrinolysis. When blood vessels are damaged vasoconstriction temporarily decreases blood flow, followed by platelet adhesion to the exposed collagen fibres, platelet aggregation and formation of a primary platelet plug. Coagulation stabilizes the platelet plug due to the formation of a fibrin network. After reconstruction of the endothelium, the fibrin clot is lysed to ensure normal blood flow.

The formation of fibrin is orchestrated by a cascade of proteolytic reactions initiated by the exposure of tissue factor. The cascade is started by binding of FVII(a) to TF and subsequently activation of FIX and FX [1]. FXa has a dual function as it inhibits the TF-VII complex by forming a ternary complex with TFPI, thereby preventing further initiation [2] and by binding to factor V(a) to form the prothrombinase complex at a phospholipid surface exposed by activated platelets. The prothrombinase complex is able to convert prothrombin to thrombin. Factor IXa forms a similar complex with factor VIII(a), called the tenase complex, also able to activate factor X.

Another pathway initiating the coagulation cascade is the contact activation pathway. Contact activation is initiated when plasma comes in contact with a negatively charged surface [3]. Contact activation leads to autoactivation of FXII, and subsequently to activation of FXI in the presence of both high molecular weight kininogen (HMWK) and kallikrein. FXIa together with its cofactor FVa is able to activate factor IX [4]. Contact activation is downregulated by α1-antitrypsin that inhibits FXIIa and proteolysis of HMWK through FXIa.

After initial formation of a minute amount of thrombin, propagation of coagulation occurs by activation of FXI (positive feedback pathway) and simultaneously by the activation of cofactors FV and FVIII. FXIa activates FIX, and FIXa forms together with FVIIIa the tenase complex, which activates FX and ultimately results in a burst of thrombin generation [5].

Termination of coagulation is mediated by antithrombin (AT) that binds after exposure of the reactive site loop to the active centre of thrombin or factor Xa and realises a covalent binding. AT is also able to inhibit other serine proteases, albeit at a lesser extent. The binding of thrombin to its receptor thrombomodulin (TM) regulates termination of coagulation by allowing the conversion of protein C in activated protein C (APC). Subsequently, APC inactivates FV and FVIII together with its cofactor protein S.

After the damaged tissue is repaired, clot degradation occurs to allow sufficient blood flow. Both tissue-type plasminogen activator (tPA) and plasminogen bind to the fibrin fibers followed by the formation of plasmin. Plasmin will lyse the clot. Excess levels of plasmin will be inhibited by a number of natural inhibitors; α1-antitrypsin, α2-antiplasmin, C1-inhibitor, AT and α2-macroglobulin (α2M). By the continuous degradation of fibrin, new C-terminal lysine residues become available creating a growing surface for extra stimulation of the formation of plasmin. This enhanced stimulation is inhibited by the activation of thrombin activatable fibrinolysis inhibitor (TAFI).

Thromboplastin generation test

In 1953, MacFarlane and Biggs [6] presented a method for the measurement of TG in recalcified plasma, and at the same time Pitney and Dacie [7] studied TG in whole blood. Later that year, Biggs and Douglas [8] presented the thromboplastin generation test, sensitive to differentiate between haemophilia A (HA) and B (HB). The thromboplastin generation assay made use of aluminium hydroxide treated plasma, normal serum and platelets. Treatment of plasma with aluminium hydroxide resulted in removal of prothrombin, factor VII and IX and serum was devoid of factor V and VIII. Mixing aluminium hydroxide treated plasma with patient serum resulted for a HB patient in reduced thromboplastin generation whereas a HA patient demonstrated a normal thromboplastin generation. These tests were based on the ideas of Brinkhous and Herbert who developed a two-stage prothrombin assay [9,10] to describe time-dependent formation of thrombin. Every 30 s 100 μL of blood was taken from a blood sample and incubated with a fibrinogen solution. In this second stage, the clotting time of the fibrinogen solution was measured and thrombin concentration was deduced. A short clotting time corresponded to a high amount of thrombin in the sample.

Chromogenic thrombin generation

The two-stage prothrombin assay had two major drawbacks: It was a laborious and time-consuming test and lacked accuracy due to sub-sampling of the plasma in a fibrinogen solution. To overcome these problems several different synthetic substrates were developed and tested. Hemker and Béguin were the first who reported the use of a chromogenic substrate to determine thrombin generation in plasma [11]. This substrate was split by thrombin and released *p*-nitroaniline that was measured spectrophotometrically.

The introduction of these small synthetic substrates to determine the thrombin potential raised a few problems. Under physiological circumstances, the conversion of prothrombin to thrombin and subsequent feedback activation immediately leads to mechanisms to control thrombin formation. One of these mechanisms involved, is the covalent binding of thrombin to α2M. This interaction inhibits the activity of thrombin towards it natural substrates, but still allows the conversion of small, synthetic substrates [12,13] leading to residual substrate conversion. Thus, the final concentration of the thrombin-α2M complex is proportional to the total thrombin generation. Two methods were developed to overcome this undesired residual conversion. On the one hand, the residual activity in the TG curve was corrected by mathematical processing and on the other hand, addition of hydroxylamine or a metalloprotease resulted in the removal of α2M activity [14].

Another drawback of using synthetic substrates is that the substrates emit a signal with a wavelength in the visual spectrum. Consequently, defibrinated and platelet-poor plasma is required, because the change in turbidity during coagulation interferes with the emitted signal of the split product. Defibrinated plasma was obtained by incubating plasma with reptilase, an enzyme that directly converts fibrinogen to fibrin without activating any other coagulation factor. The solidified fibrin can then be removed from the plasma. A consequence of this treatment is that approximately 30% of the thrombin formed is complexed to α2M whereas in the presence of fibrin this is less than 5%. Addition of Gly-Pro-Arg-Pro-peptide may also be used to prevent polymerization of the fibrinogen monomers.