Usefulness of Thrombin Generation

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Abstract

Thrombin generation (TG) is a global coagulation procedure meant to continuously monitor thrombin formation and decay upon exposure of platelet-poor plasma to exogenous triggers such as tissue factor, phospholipids, and calcium chloride. The procedure can also be performed in platelet-rich plasma by omitting exogenous phospholipids. TG is thought to mimic more than other coagulation procedures the process that occurs in vivo. Over the years, TG has been used to investigate coagulation mechanisms, which were not completely understood, or to investigate hyper- or hypocoagulability in clinical conditions known to be at increased risk of thrombosis or hemorrhage, respectively. More recently, TG has been employed as a laboratory tool to assess the risk of recurrent venous thromboembolism or to assess the risk of arterial thromboembolism in specific clinical settings or in the general population. The article reviews the value and limitations of TG.

Keywords

- arterial thrombosis
- coagulation factors
- Hemostasis

Introduction

Thrombin is the key enzyme of coagulation, responsible for the fibrinogen to fibrin conversion. Beside this paramount function, thrombin mediates many other mechanisms, which are instrumental for hemostasis, such as the activation of platelets, protein C, factors VIII, V, XI, XIII, and the thrombin activatable fibrinolysis inhibitor. Furthermore, thrombin mediates mechanisms which are apparently well beyond hemostasis. For example, thrombin regulates the vascular tone and permeability, the inflammatory response,[1,2] the liver parenchymal extinction, subsequent to the progression of fibrosis in patients with cirrhosis,[3,4] and many others. It is therefore not surprising that the quantification of thrombin generation (TG) in clotting blood or plasma has attracted the attention of researchers over time.

In the past, thrombin was measured as its ability to clot whole blood or plasma in the early global procedures of coagulation, namely prothrombin time (PT) and activated partial thromboplastin time (APTT). Much more attention to the TG, evaluated with different procedures, came about in the early 1950s when MacFarlane and Biggs[5] performed experiments of in vitro TG measurement. They sought to activate coagulation in whole blood or plasma by triggers such as cephaline or tissue extracts from human or animal origin and calcium chloride. Following the activation, small amounts of the clotting mixture were transferred at sequential time points into a series of test tubes containing a fibrinogen solution. The clotting times of the fibrinogen solution were inversely related to the thrombin activity generated at specific time points and were used to interpolate thrombin activity from a dose–response curve. Thrombin activity was then used to construct TG curves generated over time. The typical pattern was described by the increasing thrombin activity soon after the coagulation ignition, followed by a peak and declining activity as soon as the naturally occurring anticoagulant mechanisms started to inhibit TG. However, the procedure was soon abandoned because it was deemed too complex for routine use. Many years later it was resumed by Hemker et al,[6] who made substantial changes (reviewed in Tripodi[7]). The endpoint for the measurement of thrombin activity switched from clotting fibrinogen to cleavage of a chromogenic substrate. This allowed the procedure to be performed in clinical chemistry

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analyzers. However, one of the limitations of the procedure was the presence of fibrin that was generated during clot formation. The potential interference brought about by the turbidity of fibrin on the analyzer optical system was resolved either by defibrination of the plasma prior to testing or by including in the system an inhibitor to fibrin polymerization. Other key changes have been introduced over the years, namely the substitution of the chromogenic in favor of a fluorogenic substrate and the use of a computer software coupled with a dedicated fluorimeter. The former allowed the procedure to be performed in platelet-poor or platelet-rich plasma without defibrination and the latter allowed the continuous monitoring of TG in a completely automated fashion for many samples at the same time. Currently, the procedure is designed to operate on a microplate-based assay and has been extensively used over the last two decades as a multipurpose laboratory tool. A new TG procedure has been recently introduced (i.e., ST Genesia), which is designed to assess TG under strict control of temperature and volumes, ensuring easy and reliable performance even in less specialized laboratories.[8] The practice will inform on its suitability as a laboratory tool for patient management. Finally, a TG procedure for testing whole blood has been developed, but not yet thoroughly evaluated.[9]

Unresolved Issues

Although there are still unresolved issues concerning the choice and standardization of reagents, assay reproducibility, and result expression, TG is now ready for search of indications stemming from clinical trials. Currently, there are different commercial TG methods under different brand names and their results are hardly comparable.[10] This means that results obtained in patient plasma can be hardly compared with the traditional upper and lower limits of the reference interval, as it occurs for many other laboratory tests. Until these issues will be resolved, *it should be recommended* to perform case–control studies in which samples from controls undergo exactly the same testing procedure as the patient samples. The above design *should be recommended* especially whenever the procedure is performed in the presence of thrombomodulin (TM; meant to optimally activate protein C) or corn trypsin inhibitor (CTI; meant to quench the accidental activation of contact coagulation factors). The addition of TM or CTI may variably affect the results of TG parameters, depending on the assay condition and/or the batch or reagents. It is therefore important that results from patients and controls be confronted under the same experimental conditions.

Significance of the Thrombin Generation Parameters

The composite pattern of TG is expressed by the thrombogram and it is defined (among others) by the following parameters (►Fig. 1 and ►Table 1). (1) The lag time, which is a measure of the time (minutes) elapsing from the ignition of coagulation to the initial thrombin appearance. The lag time can be regarded as the traditional plasma clotting time and is expected to be shortened or prolonged in those conditions characterized by hyper- or hypocoagulability. (2) The peak thrombin, which represents the highest thrombin concentration (nM) that can be reached for the testing plasma under the specified experimental conditions. Peak thrombin is expected to be high or low in those conditions characterized by hyper- or hypocoagulability. (3) The time to reach the peak, which is a measure of the time (minutes) needed to reach the peak. This parameter is expected to be shortened or prolonged in those conditions characterized by hyper- or hypocoagulability. (4) The area under the curve, also called endogenous thrombin potential (ETP), which represents the net amount of thrombin that the test plasma can generate under the experimental conditions as a result of the two opposing coagulation drivers operating in plasma (i.e., pro- and anticoagulants). ETP is expected to be low or high in those conditions characterized by hypo- or hypercoagulability.

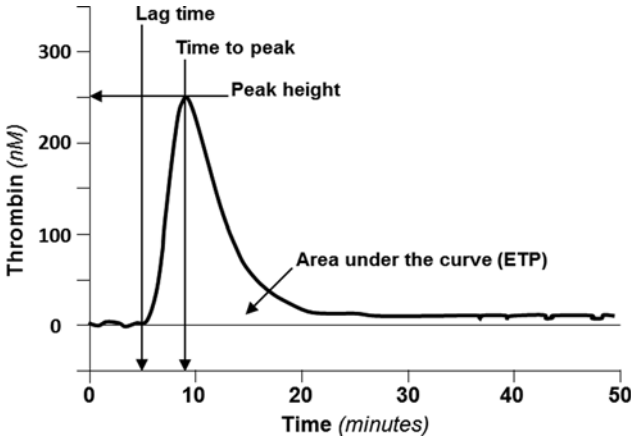

Fig. 1 Main parameters of the thrombin generation curve (see the text for more details).

Test Plasma and Reagents Needed for Thrombin Generation

The preparation of test plasma and reagents used for TG may considerably affect the results of the procedure.[11] Therefore, great care should be exerted for their appropriate definition.

Test Plasma

Residual platelets in plasma, especially after freezing–thawing, may have considerable detrimental effects on TG parameters, as platelets upon fragmentation expose negatively charged phospholipids, which may considerably increase the procoagulant driving force of the system. Plasma for TG should be prepared under careful standardized centrifugation to get residual platelets as low as possible. Double centrifugation has been advocated, but cannot be used on a regular basis, as it is not the standard practice in general laboratories that work by automated procedures. Furthermore, many samples prepared for general purpose via the standard centrifugation cannot be later used for TG. An acceptable compromise would be the blood centrifugation at 3,000 *g* for 15 minutes (controlled room temperature). This procedure would allow getting plasma with minimal residual platelets. Supernatant plasma

can be stored frozen for later use, if the storage temperature is −70°C or below. Frozen plasma must be thawed rapidly (immersion in a water bath at 37°C for 1–2 minutes) and homogenized by gentle inversion before testing. In special situations, when one wishes to investigate the role played by the platelet procoagulant activity, platelet-rich plasma can be used with addition of tissue factor, but not of exogenous negatively charged phospholipids.[12] On these occasions, strict standardization of platelet counts in platelet-rich plasma is paramount and it is also recommended to run control samples from healthy subjects along with patient samples.

Triggers

Coagulation ignition is performed by small amounts of tissue factor and negatively charged phospholipids. At variance with the PT and APTT, the concentrations of these triggers should be maintained as low as possible to mimic in vitro as much as possible the conditions operating in vivo. The most used tissue factor (final) concentrations are 1 or 5 pM and the source is (diluted) thromboplastin from relipidated recombinant tissue factor. Higher concentrations can be used in special situations (heparins or other anticoagulants) when the procoagulant force of the plasma is expected to be relatively small. Negatively charged phospholipids are usually a blend of synthetic phospholipids at concentrations ranging from 1 to 4 μM. It should be realized that the source and concentration of triggers may have considerable effect on TG parameters and therefore strict adherence to standardization and/or case–control studies should be considered to compare results over time.

Addition of other Agents

TG can be measured in the presence of TM.[13] TM is the physiological activator of protein C, which is relatively abundant in endothelial cells and much less in plasma. Therefore, TG does not fully account for the anticoagulant activity of protein C, as this naturally occurring anticoagulant cannot be optimally activated in the absence of TM. This may be detrimental in some clinical conditions where protein C is greatly reduced. Typical examples are the congenital deficiency of protein C as well as its acquired deficiency due to liver cirrhosis (see below). In both conditions, the relevant anticoagulant driving force of protein C would be undetected in the absence of TM. Therefore, the addition of soluble TM in the test system is beneficial for the proper assessment of the balance between pro- and anticoagulants. However, the optimal amount of TM to be added to the test system is *difficult to determine* as in vivo TM is located on endothelial cells and its density/concentration is not accurately known. A pragmatic solution could be to add an amount of TM, which is able to reduce the ETP of the pooled normal plasma by 50%. The correspondent TM concentration to achieve this reduction varies from lot to lot and should be evaluated for each lot of TM used over time.[13] Activated protein C (APC) can also be added to the TG system to help in increasing the sensitivity to acquired plasma APC resistance.[14]

Another important component of the TG procedure could be CTI. CTI is able to inhibit the contact phase of coagulation, thus avoiding the contribution to TG that could come about from an accidental activation of contact coagulation factors (e.g., factor XII, pre-kallikrein, or high-molecular-weight kininogen). This activation may be variably occurring when plasma samples are stored frozen for later use. CTI should be added directly in the tube used for blood collection,[15] as later addition to plasma could be ineffective. The value of adding CTI has been debated in the literature (reviewed in Tripodi et al[16]) and was abandoned because it deemed difficult in practice and because of the relatively high cost. Perhaps, as mentioned above a more pragmatic solution could be running case–control studies in plasma without addition of CTI, assuming that contact activation (if any) would affect the control and patient samples to the same extent.

Result Expression

Results of TG parameters are usually expressed as time (minutes) for lag time and time to peak, nM thrombin for peak, and nM thrombin × minutes for the area under the curve (ETP). These result expressions are poorly standardized and may vary owing to small methodological variations and/or changing reagent lots that may occur over time. Attempts to standardize this step have been done by expressing results relative to a pooled normal plasma taken as a standard and tested along with the patient plasma.[17] International standards have not yet been provided. As mentioned, the safest way to proceed for a fair comparison of results obtained over time is running case–control studies.

Use of Thrombin Generation

As mentioned, over the last two decades TG attracted the attention of many. Searching PubMed with the key word "*thrombin generation*" yields nearly 7,500 items. Close examination reveals that they are dealing with a certain number of fields for which TG was successfully used. Not all of them are however related to significant applications on the direct management of patients. In the following paragraphs, I discuss the merits and limitations on the application of the procedure (►Table 2).

Investigation of Mechanisms of Thrombogenesis

As mentioned, thrombin plays a key role in clot formation in vitro and in vivo. Therefore, the use of TG may help in understanding the process of thrombogenesis in many clinical conditions that were for long time associated with an increased risk of thrombosis, but for which there was no clear demonstration of plasma hypercoagulability. This is not surprising as the conventional procedures used to assess coagulation such as the global tests PT and APTT or the measurement of the individual components of the coagulation balance (either pro-, anticoagulants, or both) present with obvious limitations. PT and APTT are static tests that are unable to account for the entire process from TG to decay, based on the two opposing drivers (pro- and anticoagulants). On the other hand, the measurement of the individual components (pro- and anticoagulants) can tell us whether or not one or more individual components are deranged, but not if the balance between the

two is or is not perturbed. This may occur especially in clinical conditions associated with acquired hemostasis disorders when the activities of both pro- and anticoagulants are variably reduced or increased. Indeed, TG has been successfully used to unveil plasma hypercoagulability in many conditions that have for long time been associated with an increased risk of thrombosis, especially venous thrombosis. Among them, one can mention diabetes,[18] obesity,[19] Cushing syndrome,[20] fatty liver,[21,22] inflammatory bowel disease,[23] and many others for which it has been shown that TG is increased in spite of the fact that PT, APTT, and the individual components of coagulation are near normal. Furthermore, TG procedures performed in the presence/absence of TM has been instrumental in dismantling the old dogma that depicted liver cirrhosis as the prototype of the acquired hemorrhagic disease.[24,25] Thanks to TG, we now know that cirrhosis is associated with an acquired coagulopathy characterized by rebalanced but unstable coagulation that (depending on the circumstances and risk factors) may tip toward thrombosis or hemorrhage.[26] TG has also been used to help in understanding mechanisms of thrombogenesis in special situations. For example, Miyawaki et al[27] have used TG to investigate a prothrombin variant associated with thrombosis. They found that the start tail (i.e., duration of thrombin-generation activity), assessed from the area under the TG curve, was impaired (extended) in the carriers of the mutation.

Investigation of Hemorrhagic Conditions

TG helped in understanding the relative contribution of coagulation factor to TG and fibrin formation. A situation where the basic tests of coagulation (PT and APTT) or the measurement of the individual coagulation factors cannot help, as coagulation is a tight integrated system that requires full cooperation of many factors to generate thrombin and to convert fibrinogen into fibrin.[28]

Monitoring Procoagulant Agents

A good example in this context is hemophilia. Up to 30% of hemophiliacs may develop during their life specific inhibitors to factor VIII or IX. When the inhibitor titer is relatively high, they cannot be treated with conventional coagulation factor concentrates and benefit from infusion of prohemostatic agents (either activated prothrombin complex concentrates or recombinant activated factor VII) collectively called bypassing agents. Monitoring such drugs by the postinfusion measurement of the individual coagulation factor VIII or IX does not make sense as bypassing agents exert their hemostatic efficacy with little modification of these factors. TG procedures would instead be the candidate monitoring systems.[29] TG procedures have also been found useful in the context of hemophilia treatment in special conditions.[30]

Evaluation of the Risk Recurrence of Venous Thromboembolism

The risk of recurrent venous thromboembolism (VTE) after a first episode is relatively high, especially for unprovoked events. The optimal duration of the secondary prophylaxis in these patients is paramount and should be established by careful considerations of the benefit/risk ratio, which include clinical as well as laboratory considerations. Among the latter, D-dimer has been established over the last two decades as one of the laboratory tools that can be used to assess the risk of VTE recurrence after discontinuation of anticoagulation.[31] The TG procedure also proved to be a good predictor of recurrent VTE[32,33] and can therefore be used alone or in combination with D-dimer[34] to assess the risk of recurrence and to make decision on the optimal duration of secondary prophylaxis.

Monitoring Patients on Antithrombotic Drugs

Parenteral or oral antithrombotic drugs (e.g., heparins, vitamin K antagonists, or direct oral anticoagulants) share the same principle. Whatever their mechanism of action, they ultimately act by downregulating TG. It is therefore obvious that TG procedures are suitable to assess their antithrombotic capacity.[35] It should however be recognized that before TG can be used in this context, it should be determined whether it is superior to the other laboratory tools (e.g., the international normalized ratio, the APTT, or the individual measurement of the direct oral anticoagulants) which are currently used to monitor antithrombotic drugs.

Investigation of the Risk of Thrombosis in the General Population

Thrombosis is cumulatively considered as one of the most important health burdens, especially in western countries. Therefore, the quest for laboratory tools able to identify those apparently healthy individuals, who will later develop thrombotic events, has attracted over the years the attention of epidemiologists, clinicians, and laboratory operators. In this context, PT and APTT play a minor role, although shortened APTT proved to be associated with the risk of VTE in retrospective case–control studies.[36] TG has been investigated as a global laboratory tool as a predictor of the risk of arterial thrombosis in studies of healthy subjects.[37,38] In particular, the results of the Gutenberg Health Study[38] that investigated 5,000 healthy subjects, who were followed up to 9 years, showed that the hypercoagulability detected by TG is associated with overall mortality. This is a promising result, although it does not appear applicable to assess the risk in individual patients.

Concluding Remarks

Thrombogenesis as well as the mechanisms of action of the many antithrombotic drugs operates through the enhancement or reduction of TG in plasma. It is therefore not surprising that the TG procedures described by the pioneer work of MacFarlane and Biggs and later by Hemker and coworkers attracted the attention of physicians, epidemiologists, and laboratory operators. Presently, TG procedures play a central role in the laboratory investigation of the mechanisms of thrombogenesis in many clinical conditions, which were poorly understood until recently. Whether they can also be used in the daily management of patients affected by hemorrhage or thrombosis requires further investigations.

Table 1 Main parameters of thrombin generation and their significance

Parameter	Significance
Lag time	Short or long lag time, suggest hyper- or hypocoagulability
Peak	High or low peak, suggest hyper- or hypocoagulability
Time to peak	Short or long time to peak, suggest hyper- or hypocoagulability
Area under the curve (ETP)	High or low ETP, suggest hyper- or hypocoagulability

Abbreviation: ETP, endogenous thrombin potential.

Table 2 Potential applications of thrombin generation (see the text for more information)

Application	Examples
Investigation of mechanisms of thrombogenesis	Liver cirrhosis, diabetes, obesity, Cushing syndrome, fatty liver, inflammatory bowel disease, and others
Investigation of hemorrhagic conditions	Hemophilia and rare hemorrhagic coagulopathies
Monitoring procoagulant agents	Hemophilia with and without inhibitors (especially treatment with bypassing agents)
Venous thromboembolism	Evaluation of the risk recurrence of venous thromboembolism
Monitoring antithrombotic drugs	Vitamin K antagonists, heparins, direct (oral or parenteral) anticoagulants
Arterial thromboembolism	Investigation of the risk of thrombosis in the general population

Conflict of Interest

None declared.

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