Thrombin generation assays

Thrombin generation assays: accruing clinical relevance

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Purpose of review

After decades of near oblivion, thrombin generation is being revived as an overall function test of the plasmatic coagulation system in platelet-poor plasma (PPP). In platelet-rich plasma (PRP) it assesses platelet procoagulant functions as well.

Recent findings

The recently developed use of special fluorogenic thrombin substrates allows monitoring of thrombin concentration in clotting PPP and PRP on line in up to 24 parallel samples. Studies in model systems stress the importance of cell-bound thrombin generation such as measured in PRP.

Summary

The method can be profitably applied to various hitherto unyielding problems such as the control of (low-molecular-weight) heparin therapy, the detection of lupus anticoagulant, and various forms of thrombomodulin and activated protein C resistance (including the use of oral contraceptives) as well as monitoring the treatment of hemophiliacs by factor VIII bypassing therapy. In PRP it reflects the abnormalities encountered in von Willebrand disease and Glanzmann and Bernard-Soulier thrombopathy as well as the action of antiplatelet drugs.

Keywords

thrombin generation, platelet-poor and platelet-rich plasma, monitoring antithrombotics, lupus anticoagulant, hemophilia and von Willebrand disease

Curr Opin Hematol 11:170–175. © 2004 Lippincott Williams & Wilkins.

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Current Opinion in Hematology 2004, 11:170–175

Abbreviations

APC	activated protein C
aPTT	activated partial prothrombin time
CAT	calibrated automated thrombogram
ETP	endogenous thrombin potential
α_2M	α_2-macroglobulin
PAR	protease activatable receptor
PPP	platelet-poor plasma
PPR	platelet-rich plasma
TF	tissue factor
TG	thrombin generation
TM	thrombomodulin
vWF	von Willebrand factor

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Introduction

Because of its numerous positive and negative feedback controls, the hemostatic-thrombotic system is so complicated that it is practically impossible to judge the overall hemostatic function of the blood from the concentration or structure of its components. Information on details of the system therefore is not an alternative for an overall function test. Clotting times (prothrombin time, activated partial prothrombin time [aPTT], activated whole blood clotting time) do not indicate hypercoagulability and are insensitive to mild bleeding disorders. For over a century (*eg*, Hayem [1]), the generation of thrombin in clotting blood or plasma has been used to assess the coagulation system, but only recently have technical developments brought it into reach of the nonspecialized laboratory.

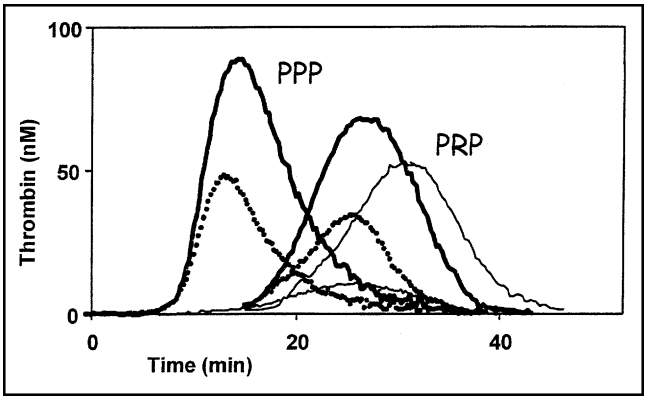
Ex vivo thrombin generation (TG) should be distinguished from *in vivo* TG, revealed by products of an ongoing clotting process in the body (prothrombin fragment 1-2, D-dimer etc.). Increased *in vivo* TG indicates an ongoing pathologic process. Increased or decreased *ex vivo* TG means that the function of the coagulation process is abnormal (*eg*, hyperprothrombinemia [2], hemophilia [3••], use of anticoagulants [4]). It does not necessarily mean ongoing pathology but indicates an increased risk of thrombosis or bleeding [5]. *In vivo* TG is a smoke detector signaling ongoing evil; *ex vivo* TG is like the smell of gasoline indicating an increased risk.

The first law of hemostasis and thrombosis

"The more thrombin the less bleeding but the more thrombosis, the less thrombin the less thrombosis but the more bleeding" may be called the "first law of hemostasis and thrombosis." The converse is not true. The cause of thrombosis or bleeding can be in the vessel wall, with the hemostatic function of the blood being perfectly normal. The circumstantial evidence for the first law is overwhelming; we know of no refutation. Bleeding may be caused by a lack of any known clotting factor or by an excess of antithrombin activity (antithrombin Baltimore, heparin); thrombosis by an excess of clotting factor (*eg*, prothrombin); or a shortage of coagulation delimiters (antithrombin, proteins C and S).

The time course of TG (the "thrombogram") is shown in Figure 1. After a lag time, a burst of thrombin is observed. Clotting occurs at the end of the lag time, when more than 95% of all thrombin is still to be formed. We

Figure 1. Thrombin generation curves as obtained with the calibrated automated thrombogram (CAT) technique



Platelet-poor plasma (PPP) was triggered with low (5 p*M*) tissue factor (TF); platelet-rich plasma (PRP) with traces (0.5 p*M*) of TF to mask variations in endogenous TF. Bold lines: no addition; dotted lines: 10 n*M* thrombomodulin (TM) added; thin lines: 6 n*M* APC added (lower curve: PPP; upper curve PRP).

may well ask what the purpose is of all the thrombin that is formed after clotting [6]. We see two main functions: *In vivo* thrombin diffuses out, around the primary focus of its formation (*eg*, wound, ruptured plaque). Above a certain threshold concentration, it will autocatalytically promote more thrombin formation and thus thrombus growth. Under that threshold it will be washed away or neutralized. The amount of thrombin formed in a focus will thus determine the extent of a thrombus/hemostatic plug. Thrombin in a clot also prevents subsequent lysis via the activation of thrombin-activatable fibrinolysis inhibitor [7,8]. This explains why hemophiliac bleeding often develops after a bleeding-free interval, as if a formed hemostatic plug is precociously dissolved (*eg*, Verstraete [9]). Thrombin also acts on a number of different cells in the neighborhood of a focus and has a function in tissue repair and proliferation of surrounding cells [10,11].

Models of *ex vivo* thrombin generation

The course of thrombin concentration in a hemostatic plug or thrombus is technically impossible to measure. Measuring thrombin (-products) in samples from the blood in a wound comes close [12,13], as does subsampling from clotting blood [14,15]. Both require heavy experimentation. Two essential different types of model are of more practical use: reconstituted systems and plasma.

Reconstituted systems (for reviews see Monroe *et al.* [16] and Mann [17•]) use purified clotting factors to represent the physiologic situation. Reaction conditions are under tight control and can be varied at will. To investigate TG at cell surfaces, notably platelets with or without cells bearing tissue factor (TF) or thrombomodulin (TM) (monocytes, endothelial cells) they proved very useful [18]. However, purified factors will not necessarily retain their native activity (*eg*, Hemker [19]). Reconstituted systems are as realistic as our insight into the clotting mechanism allows; extrapolation to physiology should therefore be regarded with due suspicion. Minor players (*eg*, β1glycoprotein Ib, $\alpha_2$macroglobulin [α_2M]) and *a fortiori* unrecognized proteins/functions escape notice; fibrin(-ogen) and von Willebrand factor (vWF) are often absent but do play a role in rendering platelets procoagulant [20]; see below).

Thrombin generation in plasma (in platelet-poor or platelet-rich plasma, PPP or PRP [21,22]) represents the function of a relevant slice of the *in vivo* system with all the plasma proteins present, unmodified, near their physiologic concentrations and independent of *a priori* hypotheses. It represents a function test of the "isolated organ" PPP or PRP. The vessel wall is lacking, however. To simulate its presence, the two most important known elements, TF and TM, may be added to the plasma (Fig.1).

Cell-bound thrombin generation: the role of platelets

The arm-to-tongue circulation time of the blood (~30 seconds) is short compared with a whole blood clotting time, so thrombin formed in flowing blood *in vivo* is rapidly diluted and inactivated before clotting can occur. Thrombin will only build up in unstirred boundary layers at cell surfaces and in the unstirred plasma caught in a clot or an aggregate. Transport by diffusion will therefore tend to govern reaction rates. According to our interpretation [23] diffusion limitation, for instance, explains the kinetics observed in a cell-bound model of TG by Allen *et al.* [24•].

Cell-bound TG is dependent on TF-bearing cells (monocytes, perivascular cells) and platelets [18,16]. The role of the platelet in physiologic thrombin generation is twofold. By adhesion and aggregation it forms a maze in which plasma can clot without the thrombin being washed away; conversely, activated platelets provide the surface on which TG can take place. Upon activation of the platelet [25], procoagulant phospholipids appear at its outside. Thrombin (PAR 1) and collagen (GPVI) bring about this process, especially in combination. GPIIb/IIIa plays a role as well and GPIIb/IIIa antagonists inhibit [26]. vWF adsorbs onto polymerizing fibrin and this probably brings about a molecular change (like ristocetin), which makes it interact with GPIb-V. This enhances the formation of a procoagulant surface [27,28•].

Techniques of thrombin generation measurement

The thrombogram can be obtained through subsampling [29] or through monitoring the conversion of a suitable substrate directly added to the clotting plasma [30]. The former method is straightforward and time consuming;

the latter allows automatic continuous measurement of many samples in parallel. Via subsampling, the thrombin (~30% of total) that adsorbs onto the formed clot [31•,32,33] escapes notice. Such thrombin can activate factors V, VIII, and XI or platelets and thus probably is essential in thrombin growth [34].

Added thrombin substrate occupies part of the thrombin formed. Enough free thrombin should remain to allow for natural feedback reactions and for adequate removal of thrombin by antithrombins; therefore binding should be relatively loose (low Km). Suitable substrates should also be converted slowly (low k_{cat}) so as not to be consumed during the experiment [35].

The fluorescent signal has the drawback of not being linear with product concentration. To compensate for this and for the effects of substrate consumption, the calibrated automated thrombogram (CAT) method has been developed that continuously compares the signal from the experimental sample to that of a fixed known thrombin activity [36•]. This method allows visualizing the thrombin concentration in clotting PPP or PRP in 24 parallel experiments.

The thrombogram

Typical thrombograms as obtained with the CAT are shown in Figure 1. The three most important parameters are the lag time, the peak value, and the area under the curve or endogenous thrombin potential (ETP), which quantifies the enzymatic "work" that thrombin can do during its lifetime ("person-hours" of thrombin) [37]. Plasma clots at the end of the lag phase so the clotting time can be read from the thrombogram. During the lag phase the reaction mechanisms are essentially different from those during the thrombin burst [15]); this is one of the reasons that the clotting time does not represent TG. The normal values and coefficients of variation as obtained with the CAT-method are given in Table 1.

Partial techniques

Both the lag time and the ETP can be obtained by alternative techniques without monitoring the complete thrombogram. The clotting time represents the lag time. The ETP can be assessed by measuring the product from any natural or added substrate that is not exhausted during the clotting process. One natural substrate is α_2M, which, in defibrinated plasma, will bind ~30% of the thrombin formed (~5% with fibrinogen). The final concentration of the (amidolytically active) α_2M-thrombin complex is proportional to the ETP [30]. Rosing *et al.* [38] used this approach to demonstrate acquired activated protein C (APC) resistance through the use of oral contraceptives. The ETP can also be assessed by measuring the end level of conversion of a slow-reacting artificial substrate, provided that it does not react with α_2M-thrombin.

Surrogate techniques of thrombin generation

Several techniques have been published that depend on fibrinogen polymerization. Apart from the clotting time, they give little information on thrombin generation because fibrinogen is exhausted before 5% of all thrombin is formed. In so far as the properties of the clot are determined by the velocity or the amount of thrombin formed, some information can be retained, however. Indeed tensile strength [39•], clot retraction [40•], turbidity [41], and fibrinolysis [42•] are derived variables that to some extent are determined by the amount or velocity of thrombin formation. They recently have been (re-)used for the assessment of overall hemostatic function. Some of these methods have the advantage of using full blood. A disadvantage is that the indicating substance (fibrinogen) may increase when TG decreases, *eg*, in active thrombosis under anticoagulant treatment. It may be extremely confusing that curves are obtained that resemble real TG curves but are not.

Applications of thrombin generation measurement

Thrombin generation measurement has been shown to be a useful tool in several different domains.

Platelet-plasma interactions

Thrombin generation has been instrumental in unveiling the role of platelet receptors in the production of a procoagulant surface by platelets. In short, a role of GPIIb/IIIa, of GPIb/V, and vWF [28•] and of GPI (collagen), as well as of the PAR receptors, has been demonstrated (see Hemker and Lindhout [43] for a review).

Detection and quantification of thrombotic tendency

Deficiencies of proteins S or C are readily recognized when TM is added to the plasma, factor V_{Leiden} as well [44,45]. APC resistance, either acquired (oral contracep-

Table 1. Normal values and variability

		n	Average (pop.)	StDev (pop.)	CV (pop.)	CV (ind.)	CV (exp.)
PPP	ETP (n*M*.min)	36	1879	284	15	4.5	2.5
	Peak (n*M*)		458	60	13	5.5	4.5
	Lag (min)		3.1	1.4	47	8.1	5
PRP	ETP (n*M*.min)	24	1678	371	22	7.8	3
	Peak (n*M*)		117	42	35	8.3	3.5
	Lag (min)		8.1	1.9	23	9.1	7

CV, ; ETP, endogenous thrombin potential; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

tive treatment) or congenital, can also be detected with a TG-based endpoint technique [45].

Thrombin generation also solves the long-standing enigma of the prothrombotic anticoagulant in lupus erythematodes. It has been shown that this antibody prolongs the lag phase (*ie*, is anticoagulant in clotting tests) but induces TM and APC resistance [46••].

Detection and quantification of bleeding tendency

In deficiencies of factors II, V, VII, VIII, IX, X, and XI, it has been demonstrated that TG is diminished in PPP and that clinical bleeding is observed at ETP values less than 30% [3••,47].

The thrombasthenias of Glanzmann [48] and Bernard-Soulier [28•], as well as severe thrombopenia, show a moderately diminished TG in PRP. Von Willebrand disease, unless accompanied by severe factor VIII deficiency, shows normal TG in PPP but decreased TG in PRP [20].

Control of procoagulant therapy

As expected, restoration of the factor VIII level of hemophiliac plasma restores TG, as does DDAVP treatment in mild hemophilia and vWD [49]. More interesting: inhibitor bypassing therapy with either factor VIIa, or this factor in combination with other factors (Feiba), can be monitored with TG [50•,51••].

Control of antithrombotic therapy

Under oral anticoagulation the incidence of bleeding increases as soon as the international normalized ratio drops below 3 [52], which is equivalent to ETP = 20% [36•]. Heparins, including the low-molecular-weight types, inhibit TG primarily by increasing thrombin breakdown [53•]. Twofold prolongation of the aPTT corresponds to ~80% inhibition of the ETP [54•]. TG is the only available method to quantify the combined effect of heparin and vitamin K antagonists or other anticoagulants.

Platelet "aggregation" inhibitors in general such as abciximab [48], clopidogrel [55], and aspirin [56] also inhibit TG to a certain degree. This is not to say that inhibition of aggregation as such would not have an—or even be the main—antithrombotic action. It is an interesting possibility that through decreasing the size of the platelet aggregate, the volume in which thrombin can form undisturbed by flow is also diminished.

Pharmacologic research

As yet we have not encountered any antithrombotic, either anticoagulant or antiaggregant, that did not inhibit TG [5]. We can assume that any drug that inhibits TG to ~50% of normal will show an antithrombotic effect at an acceptable bleeding risk. By introducing TG as an intermediate step between the biochemical experiments and thrombosis models in experimental animals, we can significantly diminish the latter, especially in dose-finding experiments. The test can also be used to assess the effects of a candidate molecule in volunteers.

Conclusion

To understand thrombin generation, we have to measure thrombin generation, the whole thrombin generation, and nothing but thrombin generation—under conditions as close as possible to those *in vivo*. This offers a wealth of information that is not otherwise available. Measurement in an undisturbed fibrin clot in which activated platelets are fixed, as is possible with fluorogenic substrates, probably resembles the situation in a hemostatic plug or thrombus more closely than stirred systems do. The calibrated automated technique makes it possible to obtain a graph of thrombin concentration against time in real time in up to 24 parallel experiments. Whole blood measurement is as yet technically impossible.

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