LECTURE

ΔΙΑΛΕΞΗ

The clotting mechanism and how to determine its phenotype

A brief review of the mechanism of thrombin formation, according to current data, is presented. The enzymatic cascade, the bounding surfaces of the enzymes and of the complexes (tissue factor-VIIa, Xa-Va-PL, IXa-PL and APC-PS-PL), as well as the feedback and inhibition mechanisms are described. A functional test for the coagulation mechanism, the thrombogram, is proposed, and information is given about its significance, the technique of its performance and the results obtained in cases of various hemostatic disorders. Given the importance of thrombin for hemostasis and thrombosis, the significance of a laboratory test for assessing thrombin generation is emphasised.

ARCHIVES OF HELLENIC MEDICINE
2000, 17(Supplement A):13–18
ΑΡΧΕΙΑ ΕΛΛΗΝΙΚΗΣ ΙΑΤΡΙΚΗΣ
2000, 17(Συμπληρωματικό τεύχος Α):13–18

H.C. Hemker,
S. Beguin

Cardiovascular Research Institute, Maastricht, The Netherlands

Ο μηχανισμός της πήξης και τρόπος καθορισμού του φαινοτύπου

Περίληψη στο τέλος του άρθρου

Key words

Coagulation
Haemostasis
Phenotype
Thrombin

1. INTRODUCTION

It is the quintessence of thrombosis and hemostasis that thrombin generates at the site of a wound – and there and then only. Dozens of factors, cellular and humoral, cooperate to bring this about. Thrombin in its turn has dozens of actions on proteins and cell receptors, that together bring about hemostasis. The mechanism of thrombin formation is extremely complicated. This is to be expected of a physiological function that is to be so precisely controlled as to act promptly and explosively in the case of a wound, but that should remain strictly confined to the wounded region. Haemorrhage follows if not enough thrombin is formed, but thrombosis results from an excess.

In order for an individual –and hence for a species– to survive, it is in the first place required not to bleed to death, e.g. during childbirth. This is probably the reason that the hemostatic system has evoluated to a state of extreme efficiency. In this way it has become definitely too active for people living over the age of fifty in industrialized countries. The risk of lethal thrombosis, in the form of coronary infarction, stroke or otherwise, in this population is about one in two, infinitely greater than the risk of bleeding to death. The changes in human living conditions are on a time scale that evolution cannot follow. So we have to adjust to modern life by the use of our brains, as we did when first building shelter and growing crop.

Adjusting our overactive hemostatic thrombotic system to modern conditions means interfering with it pharmacologically. That again presupposes that we can measure hemostatic and thrombotic (H&T) function adequately. The mechanism of hemostasis and thrombosis is known in great detail, any known clotting factor can be estimated with precision and many of the genes involved can be screened. Nevertheless, in spite of this detailed knowledge we are not yet able to measure over-all H&T function. Clotting times are inadequate in reflecting this function, as everybody knows. Indeed, a simple adequate function test, something like blood pressure or the electrocardiogram, does not exist in this domain. Consequently, we cannot know who is at risk for thrombosis. Neither is there a simple common test for measuring the common antithrombotic effect of such diverse drugs as vitamin K antagonists, heparins or aspirin. Pharma-

cological research is greatly hampered by the fact that there is no test that measures the effect of experimental drugs on the isolated organ: blood. Therefore, this type of search has to pass directly from biochemistry (e.g. the Ki of the candidate drug for factor "so much") to animal thrombosis models and from there to clinical trials.

We present here our suggestion for measuring the function of the H&T system: the thrombogram, i.e. the course of the thrombin concentration as it develops in clotting blood or plasma. In fact this is not a very new idea. Thrombin generation has been measured for over a century already. What is new however is the development of new, automated way to determine this function in large series, without having to resource to laborious subsampling methods.

The first part of this presentation will give an overview of the mechanism of thrombin generation as we understand it today. This will show that the mechanism is altogether too complicated to allow prediction of its function from knowledge of its constituent parts. The second part will explain how we developed ways to measure thrombin generation automatically.

2. THE PHYSIOLOGICAL MECHANISM OF THROMBIN GENERATION

Thrombin in its free form in plasma is extraordinary dangerous. Some snake poisons owe their lethal power to the fact that they convert circulating prothrombin quickly and massively into thrombin. Circulating thrombin in plasma is inactivated by the plasmatic antithrombins with a half life time of between 10–20 sec, just long enough to allow the enzyme to do its work in the area of a wound. There is some low grade thrombin generation going on in the body, as can be judged from the non-zero levels in normal plasma of activation peptides of prothrombin and of thrombin-antithrombin complexes. Only in a wound the velocity with which thrombin forms is such that appreciable concentrations arise because the antithrombotic forces lag behind the prothrombotic ones. If thrombin formation is triggered, its concentration first rises and then falls. This concentration-time curve contains all the relevant information on the thrombin generating and inactivating system. That is why we think that the "Thrombogram" is the reflection of the function of the H&T system of the blood.

The thrombin inactivation system is relatively simple. About 66% of thrombin is bound to antithrombin (AT), about 25% to α_2-macroglobulin and the remaining thrombin is bound by various other inhibitors. The rate of disappearance of thrombin is proportional to the concentration of thrombin and to the concentration of inhibitor. In contrast to thrombin disappearance, prothrombin activation is a very complicated process. Those that in the year 2000 still speak of the clotting system as a cascade are at least 25 years behind time. Cascade-wise proenzyme-enzyme sequences do remain the skeleton of the mechanism. Two more principles however govern thrombin generation, surface bound enzyme complexes and feedback activation and inhibition. Furthermore, the role of blood platelets is a subject that seems to have been almost forgotten for several decades, whereas from our recent work it appears to be primordial.

2.1. The enzyme cascade

Factor VII (a) binds to tissue factor (TF), a membrane protein of perivascular cells. The surface bound complex thus formed activates factor-X and activated factor X (Xa) splits prothrombin so as to generate active thrombin. There is a reinforcement loop, the Josso loop: VIIa-TF also activates factor-IX and factor-IXa in its turn can activate factor-X. This mechanism starts to be important if a little TF is available, so that a little Xa is formed directly. So the original cascade is simplified to VII>X>II. At low TF it is complicated by the coexistence of the pathway VII>IX>X>II. At still lower availability of TF the fact that factor-XI can be activated by thrombin begins to count, and the pathway XI>IX >X>II also plays its role.

2.2. Surface bound enzymes

In 1963 Pappahadjopoulos and Hanahan observed that prothrombin converting activity could not be obtained as a single clotting factor. The minimal requirements were factors Xa, V(a) and phospholipid (PL). Later, we showed that the prothrombin converting moiety consists of a reversible complex of these three components, in which factor-Xa carries the enzyme activity and the other two serve to augment its activity. Rosing showed that factor-Va serves to augment the catalytic power of Xa about one thousand fold and that the phospholipid causes this enzyme to be saturated with prothrombin at normal, plasmatic concentrations. The phospholipids that are capable to sustain the coagulation reactions need to contain a certain amount of the negatively charged aminophospholipids: phosphatidyl serine (PS) and phosphatidylethanolamine (PE). These phospholipids are as a rule not to be found in the outer face of the cell membrane double layer, but they are present in the inside. Therefore, the intact cell is not capable to sustain the

coagulation reactions. In a wound, broken cells will expose the inner face of their membranes and activated platelets exert their procoagulant action by making procoagulant surfaces available (see below).

There are four complexes of this type operative in coagulation. The tissue factor-VIIa complex is slightly aberrant in that TF is normally anchored to the outside face of an extravasal cell membrane and factor-VIIa binds to it when blood comes in contact with perivascular tissue. The other three complexes are the Xa-Va-PL that activates prothrombin, the IXa-VIIIa-PL that activates factor-X and the APC-protein S-PL that inactivates factors-Va and-VIIIa.

The kinetic constants of these complexes are dependent upon the phospholipid composition of the surface. This composition changes in time with the degree of activation of the platelets. This makes that the rate of thrombin formation is subject to changes dependent upon the activation state of the platelets.

2.3. Feedback activation and inhibition

Factor-Va is formed from plasma factor-V by thrombin. So the first traces of thrombin cause a tremendous explosion of further prothrombin conversion. Thrombin, however, also binds to thrombomodulin, a membrane protein of the endothelial surface. The resulting complex activates protein C (APC), the enzyme that degrades factor-Va (also VIIIa) and hence stops further thrombin generation. Because the activation of factor-V by thrombin is direct, whereas the inactivation is mediated via TM and proteins C and S, a time slot is created in which thrombin generation is particularly efficient. Because TM occurs on intact endothelium, the inactivation is more important in an intact vessel than in a wound.

Oral contraceptives influence the thrombin generation mechanism because they inhibit the APC-dependent inactivation of factor-V, as do congenital malformations of factor-V (factor-V-LEIDEN) and deficiencies of proteins S and C. Completely comparable to the role of factor-V in prothrombinase is that of factor-VIII that helps factor-IX in activating factor-X. Also here thrombin activates VIII whereas APC kills it.

Another negative feedback mechanism arises when factor-Xa binds to tissue factor pathway inhibitor (TFPI). The resulting couple binds to the VIIa-TF complex and blocks its activity. If only few TF is available then not enough active Xa may survive to ensure adequate thrombin generation. At that moment it is useful that factor-IXa is present so as to create an independent source of factor-X activator. At still lower TF concentrations still another positive feedback loop comes into play: thrombin, the end product of the activation sequence can activate factor XI, that in its turn activates IX and thus keeps thrombin generation going. The activation of platelets by thrombin and fibrin (see below) are other examples of feedback activation. All in all, we thus recognize at least five positive and three negative regulatory feedback mechanisms.

2.4. The role of platelets

Platelets that adhere to collagen or to fibrin and that are exposed to traces of thrombin undergo a membrane alteration that makes that procoagulant phospholipids (PS & PE) turn from the inside leaflet of their membrane to the outside. After this "flip-flop" reaction they support massive thrombin generation. Platelets adhere to each other via GPIIb/IIIa and activation via this receptor also induces flip-flop – drugs that block this receptor inhibit thrombin formation. When fibrin is present, von Willebrand factor adsorbs to the fibrin and platelets adhere to the altered vWf via GPIb. This interaction again makes them procoagulant.

Our research on thrombin generation in platelet rich plasma very much stress that platelets and the plasmatic clotting factors operate in concert. Thinking of them as two separate systems is a laboratory artifact. Von Willebrand factor is a clotting factor, because it is required for normal thrombin production. Fibrin, the presumed inert end-product of blood coagulation is an active component that, via platelet activation, fosters thrombus growth. Also the adherent platelet plug acts as a sponge, in the interstices of which the formed thrombin is not washed away by the flowing blood. That is why the primary hemostatic plug and the arterial thrombus consist mainly of platelets.

In fact, the sharp distinction between primary hemostasis and arterial thrombosis on the one hand, that are recognized as platelet dependent mechanisms, whereas secondary hemostasis and venous thrombosis are seen as blood coagulation dependent should, in our opinion, be revised. This paradigm is easy to maintain as long as platelets and clotting are conceived as distinct physiological entities, which in fact they are not. Until recently platelets were usually studied in experimental setups, in which thrombin could not form and clotting was studied in platelet free plasma. This is not the setups in which the interconnections that exist *in vivo* are easily recognized. Since we started to study these interactions we are more impressed by the similarities

than by the differences. We are ready to defend the position that primary hemostasis and arterial thrombosis is essentially the same mechanism as secondary hemostasis and venous thrombosis, except for changes in the flow conditions. At high flow, as in a wound or an artery, generated thrombin is easily washed away and its effects are limited to the area where no flow is possible, i.e. the interstices of the platelet aggregate and a very thin surface around. In venous thrombosis thrombin is not washed away, so that large clots form around the primary, platelet rich head of the thrombus. Defective secondary hemostasis in our opinion is due to fibrinolysis-induced re-bleeding. Thrombin is required for the formation of TAFI (thrombin-inducible fibrinolysis inhibitor) and fibrinolysis is not sufficiently inhibited if not a sufficient amount of thrombin has been formed.

3. THE THROMBOGRAM, A FUNCTION TEST OF THE CLOTTING MECHANISM

The diagnostics of H&T disease, the control of antithrombotic and substitution therapy and the development of new antithrombotics are all severely handicapped by the fact that no adequate function test exists of the H&T system. The sheer complicatedness of the reaction mechanism, as explained above, makes that it is not only practically but also theoretically impossible to predict the course of thrombin formation; even when one should know the concentration of all the relevant components of the system and the reaction constants of their interactions. Biochemistry, in the strict sense, i.e. the lore of the interaction of biomolecules, is able to predict the behavior of very complicated reaction mechanisms only up to a certain limit. Beyond a certain degree of complication a mechanism becomes non-linear– it might be called chaotic, in the sense that small changes of one reactant may have unforeseen large consequences, whereas big changes in another may be damped and go unnoticed. The clotting mechanism is certainly complicated enough to be beyond the limit of complete predictability.

If we recognize, thus, the limits of understanding the complete mechanism, then it becomes immediately clear that it is impossible to calculate the course of thrombin formation, even from an almost complete knowledge of all the concentrations of all the interacting clotting factors. In fact the problem that we face is not a biochemical problem, it is a problem in chemical physiology: how to assess the function of the "isolated organ" plasma, knowing that this function is the generation of thrombin.

The classical answer is the measurement of the clotting time. But blood clots already when only 1% of all the prothrombin present has been converted into thrombin. The large majority of thrombin is formed after coagulation. That this thrombin determines the extent of the H&T reaction becomes evident when one realizes that an antithrombotic like heparin does not influence the clotting time when thromboplastin is present –i.e. in close to *in vivo* situation– but significantly reduces the amount of free thrombin that appears.

If we want to know about thrombin generation, there is no solution but to measure thrombin generation. The classical approach is to subsample from clotting blood or plasma and to measure the concentration of thrombin in these samples. This, of old, was done by adding the sample to fibrinogen and determining the clotting time. Later, thrombin activity was measured by the conversion of chromogenic substrates. This induced an extra complication. Of all free thrombin 20–25% will bind to ß-2-macroglobulin, which extinguishes all biological activity but not the amidolytic action on small (chromogenic) substrates. The amidolytic activity curve therefore is not the same as the curve of thrombin activity. The course of free thrombin can be derived from that of the amidolytic activity by a simple mathematical procedure, that we shall not further elaborate upon here.

The thrombogram is an extremely useful reflection of the H&T function. We used it to investigate the action of low molecular weight and unfractionated heparins, as a precise indicator of the activity of the protein C system (which led to the recognition of the essential difference between third and second generation oral contraceptives), the recognition of different interactions between blood platelets and the clotting system, such as the procoagulant effect resulting from the interaction of fibrin and platelets, etc.

These results suggest that it would be worthwile to investigate the thrombogram in large series of patients and to assess the effect of drugs. In an attempt to make continuous measurement possible, one of us (SB), in the eighties, added a chromogenic thrombin substrate to clotting plasma and measured the development of colored product, which reflects the appearance and disappearance of thrombin. The "normal" thrombin substrates, however, were completely consumed before thrombin generation was over. We therefore developed substrates that were converted only slowly, in fact so slowly that no more than ~10% of it was consumed, so that, during the whole course of thrombin generation the velocity of color production remained proportional to the amount of enzyme. In this way the first derivative of color production reflects the thrombin concentration.

Because the method depends upon the measurement of optical density, it cannot be carried out in a mixture that becomes turbid because of a clot. The plasma therefore has to be defibrinated first, with a snake venom enzyme that does not influence the coagulation factors except for clotting fibrinogen. Later we applied the same principle using fluorogenic substrates. This allowed to measure in the presence of fibrin(-ogen) and in the presence of platelets. In order to approach the *in vivo* system still more closely, we are now developing a system that measures thrombin generation in whole blood and under flow conditions.

The thrombogram in general has the form as depicted in figure 1: there may or there may not be a lag phase, then there is a quick rise to peak and a slower decay to zero. Clotting invariably occurs in the beginning of the rapid thrombin generation, when a level of ~10 nM has been reached. The lag time therefore is for all practical purposes equal to the clotting time (more precisely: the clotting time equals the lag time plus the time required to reach the ~10 nM level, which is usually between 10–20 s). The peak represents the thrombin concentration at which thrombin generation velocity equals thrombin breakdown. Thrombin breakdown is proportional to thrombin concentration, so at the peak its velocity is maximal. The peak-height therefore gives a good indication of maximal prothrombin conversion velocity. Probably, the most important parameter is the area under the curve. This area, that we called the endogenous thrombin potential (ETP) indicates the number of "man-hours" of thrombin, i.e. the amount of enzymatic work that can be done by thrombin during its lifetime in clotting plasma.

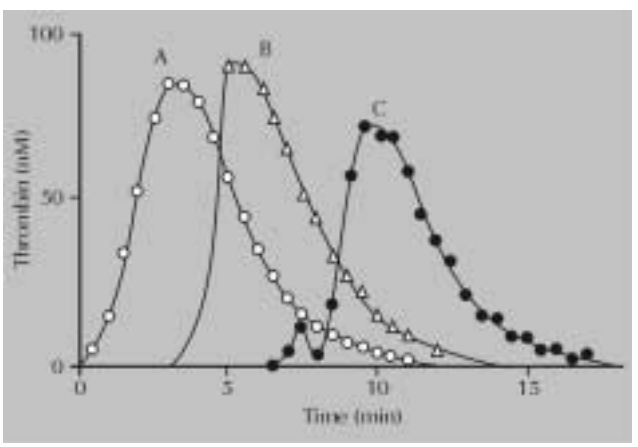

Figure 1. The thrombogram. Thrombograms as obtained with the classical subsampling method. From left to right: platelet poor plasma, extrinsically triggered; platelet poor plasma, intrinsically triggered; platelet rich plasma.

4. RESULTS OBTAINED WITH THE THROMBOGRAM

The results obtained with the thrombogram until now suggest that indeed we have in hand a method that reflects the over all function of the hemostatic and thrombotic system.

– In congenital deficiencies of the clotting factors II, V, VII, VIII, IX, X and XI the lagtime is prolonged and the ETP is lowered. It is interesting to see that the ETP becomes significantly lower in the range of clotting factor concentrations that are known to cause clinical problems. In the hemophilias, for example serious lowering of the ETP is observed only at factor VIII or IX concentrations of <10% and at >30% the ETP is practically normal.

– Oral anticoagulation and heparin treatment, and in fact any anticoagulant drug that we tested, diminishes the ETP. As a rule, the therapeutic dosage of a drug causes a reduction of the ETP to 40–20% of its normal level. We have reasons to believe however that any reduction of the ETP translates in an antithrombotic effect. In this respect it is interesting to note that aspirin, that causes a ~10% decrease of thrombin generation has recently been shown to have a modest beneficial effect in venous thrombosis.

– Deficiency of antithrombin or congenital increase of the prothrombin level brings about a proportional increase of the ETP.

– Disturbances of the APC system, such as deficiency of proteins S and C, factor-V Leiden, but also acquired dysfunction induced by oral contraceptives or pregnancy, augments the ETP by about 10%. This increase becomes much more evident when the APC system is activated by addition of e.g. thrombomodulin.

– In 41 patients who suffered from a thrombotic stroke at an age <50 years, about one third had a high thrombogram in platelet poor plasma and one third a high thrombogram in platelet rich plasma only. This suggests strongly that there exists a kind of thrombophilia that is determined by the platelet.

– In Glanzmann's and von Willebrand's disease thrombin generation is strongly impaired.

– Platelet inhibitors such as GPIIb/IIIa blockers, but also others, notably aspirin and clopidogrel, diminish thrombin generation in PRP.

– Pure fibrin, obtained from purified fibrinogen by a specific snake venom (that per se does not activate platelets, provokes platelet procoagulant activity in a von Willebrand factor dependent reaction. Thus natural fibrin, because it contains adsorbed thrombin, has a double platelet activating action. This is possibly one of

the most important mechanisms that are responsible for thrombus growth.

The use of the thrombogram in platelet rich plasma thus stresses the fact that plasma and platelets are not two separate systems but operate in concert in the body, despite the fact that they are usually studied in different setups in different laboratories.

We are convinced that the thrombogram may be a powerful tool in the search after newer and better antithrombotics. In studying "the isolated organ" it bridges the gap between biochemistry (finding an inhibitor of factor "so much") and experimental thrombosis. Moreover it allows searching for effects in the relevant species, i.e. the human. Our experience until this moment has shown no exception to the rule that a drug that diminishes thrombin generation in platelet rich plasma has an antithrombotic effect. We surmise that any systematic inhibition of the thrombogram will cause an antithrombotic effect, proportional to the decrease brought about. We think that this is independent of the mode of action of the drug, and that the pharmacokinetic aspects (how stable is the effect, how easy is it to administer the drug) are more important than the pharmacodynamic ones (how is the effect brought about). As far as we can see now, a decrease of the ETP of around 40% will prevent most venous thrombosis and will reduce the occurrence of arterial thrombosis by about 50%. Bleeding risk is increased when the ETP drops to below 20%.

We cannot summarize our findings more concisely than by formulating the first main law of hemostasis and thrombosis: the more thrombin, the more thrombosis - the less thrombin, the more bleeding. And by admitting that every known test for thrombin is essentially a thrombogram.

ΠΕΡΙΛΗΨΗ

Ο μηχανισμός της πήξης και τρόπος καθορισμού του φαινοτύπου

H.C. HEMKER, S. BEGUIN

Cardiovascular Research Institute Maastricht, The Netherlands

Αρχεία Ελληνικής Ιατρικής 2000, 17(Συμπληρωματικό τεύχος Α):13–18

Συνοπτική ανασκόπηση του μηχανισμού σχηματισμού θρομβίνης, σύμφωνα με τα σημερινά δεδομένα. Περιγράφονται ο ενζυμικός καταρράκτης, οι επιφάνειες πρόσδεσης των ενζύμων και των συμπλεγμάτων (ιστικού παράγοντα-VIIa, Xa-Va-PL, IXa-PL και APC-PS-PL), καθώς και οι μηχανισμοί ανάδρασης και αναστολής. Ο ρόλος των αιμοπεταλίων περιγράφεται επίσης. Προτείνεται μια λειτουργική δοκιμασία του μηχανισμού πήξης, το θρομβογράφημα και δίνονται στοιχεία για τη σημασία του, τον τρόπο εκτέλεσής του και τα αποτελέσματα που λαμβάνονται σε περιπτώσεις διαφόρων διαταραχών της αιμόστασης. Δεδομένης της σημασίας της θρομβίνης, για την αιμόσταση και θρόμβωση, επισημαίνεται η εργαστηριακή δυνατότητα ελέγχου του σχηματισμού της.

Λέξεις ευρετηρίου: Αιμόσταση, Θρομβίνη, Πήξη, Φαινότυπος

References

1. HEMKER HC, WIELDERS S, KESSELS H, BEGUIN S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. *Thromb Haemost* 1993, 70:617–624
2. WIELDERS S, MUKHERJEE M, MICHIELS J, RIJKERS DT, CAMBUS JP, KNEBEL RW, KAKKAR V, HEMKER HC, BEGUIN S. The routine determination of the endogenous thrombin potential, first results in different forms of hyper-and hypocoagulability. *Thromb Haemost* 1997, 77:629–636
3. BEGUIN S, KUMAR R, KEULARTS I, SELIGSOHN U, COLLER BS, HEMKER HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. *Blood* 1999, 93:564–570
4. HEMKER HC, GIESEN PL, RAMJEE M, WAGENVOORD R, BEGUIN S. The thrombogram: monitoring thrombin generation in platelet rich plasma. *Thromb Haemost* 2000, 83:589–591
5. BEGUIN S, KUMAR R. Thrombin, fibrin and platelets: a resonance loop in which von Willebrand factor is a necessary link. *Thromb Haemost* 1997, 78:590–594
6. HEMKER HC, BEGUIN S. Thrombin generation in plasma: Its assessment via the endogenous thrombin potential. *Thromb Haemost* 1995, 74:134–138
7. KUMAR R, BEGUIN S, HEMKER HC. The influence of fibrinogen and fibrin on thrombin generation-Evidence for feedback activation of the clotting system by clot bound thrombin. *Thromb Haemost* 1994, 72:713–721

Corresponding author:

H.C. Hemker, Cardiovascular Research Institute, Maastricht, The Netherlands