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Introduction

One of the most interesting properties of thrombosis is that it seems to be caused by the hobby of each particular author. You may thus have seen thrombosis attributed to psychosociological behaviour, feeding habits, unbalanced prostanoid metabolism, lack of certain plasma proteins, excess of other plasma proteins, platelet properties, physical activity and what not . . . It will not come as a surprise, therefore, that we defend the position that thrombosis is caused by thrombin generation. Before doing so, it might be safe to explain that we do not think that any of the so-called causes of thrombosis has the exclusive right of way. The majority of them are not mutually exclusive but rather mark sequential or parallel steps in a still poorly understood sequence of events. The point that we should like to make here is that all the problems around thrombosis finally pivot on one single hinge and that this hinge is thrombin generation.

Thrombin

If thrombin generation is not the unique cause of thrombosis, it is *the final common path* of all causes of thrombosis. This becomes quite obvious if one uses a yardstick that must be recognized by basic scientists, theoreticians and clinicians alike; that is therapeutic success. They would probably agree that, if we list the successful therapies against thrombosis and if we can then find a common denominator, this common denominator is likely to point to a key mechanism in thrombosis.

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Anti-thrombotic Agents

At this moment there are only two drugs that are universally recognized as efficient antithrombotics: vitamin K antagonists and heparins.

Anti-vitamin K therapy

Let us consider oral anticoagulants first. Their use in preventing venous thrombosis is unchallenged at the present time. In arterial thrombosis it has taken longer for the item to be settled. After the trial published in 1980 by the Leyden group I feel that we can accept that oral anticoagulation can prevent coronary reinfarction and it does not take us too far to extend this conclusion to all arterial thrombosis (de Vries *et al.*, 1980). The more so, as from the same study a positive effect of anti-vitamin K treatment on cerebral arterial thrombosis can be concluded. These results are no longer annihilated by the negative results of other trials, because Loeliger was able to show that unsuccessful trials are unsuccessful *always* and *only* if the level of anticoagulation that is obtained is not continuous and of a suitable low level. We therefore at this moment can safely accept that prolonged treatment with a high enough dose of a vitamin K antagonist has an antithrombotic effect (Loeliger, 1984).

Now what does a vitamin K antagonist do to a patient? Recent research, notably from the group of Vermeer in our laboratory, has shown that there are many vitamin K dependent proteins in the body. Kidney, bone, testis, vessel wall and liver all produce such proteins (Vermeer and de Boer van den Berg, 1985). The clotting factors II, VII, IX and X and the anti-clotting factors C and S are only a small subgroup among them. Yet patients that have been subjected to an oral anticoagulant treatment for many years have never been reported to show effects other than a tendency to bleed and, strictly in parallel, a tendency not to develop thrombosis. This makes it unlikely that the main effect of these drugs should be anything other than on the haemostatic mechanism in blood or on the arterial wall.

Heparin

Let us now turn our attention to heparin. Here the situation looks hopelessly complicated because heparin is an uncanny mixture of a large number of different substances while each of them may have several different actions (Hemker *et al.*, 1986a). As you know, heparin can interact with antithrombin III (AT III) to form a complex that can inactivate all proteases of the coagulation cascade. Heparin also interacts directly with thrombin and so can inhibit several important feedback activations, independently from AT III. This means that, in coagulation only, at least seven different reactions exist that are possible targets for heparin action. On the other hand, it has been found that lipid-bound coagulation enzymes, especially when accompanied by their protein cofactor, are less sensitive to the

inhibiting action of AT III–heparin than are the free enzymes (Josso and Béguin, 1981; Marciniak and Tsakamura, 1972; Ofosu *et al.*, 1984; Teitel and Rosenberg, 1983). The question of where exactly heparin therapy inhibits coagulation thus remains entirely open. This problem cannot be solved by the study of isolated subsystems of coagulation, because we are not interested in knowing what reactions are possible but we want to recognize those reactions that set the scene *in vivo*. We have therefore decided to return to the study of thrombin generation in whole plasma.

The principle of our approach is simple. In plasma, thrombin is generated from prothrombin by prothrombinase. We cannot measure prothrombinase activity directly but we can measure the resulting thrombin. At any moment the observed velocity of change in thrombin concentration is the sum of prothrombin conversion and thrombin neutralization. The latter occurs in two ways: (a) by α_2-macroglobulin, which leaves a complex that is still active towards chromogenic substrates and (b) by AT III that makes thrombin activity disappear completely. The reaction constants of thrombin decay caused by α_2-macroglobulin and by AT III can be obtained from independent experiments. If we now determine a thrombin generation curve with sufficient precision, it is possible to obtain the *prothrombinase curve* from the thrombin generation curve by a calculation based upon the differential equations that describe the chemical system. This calculation can be carried out with the aid of a computer. This instrument thus acts as a kind of enzyme kinetic microscope that shows us details in our experimental material that are not immediately apparent to the naked eye (Hemker *et al.*, 1986b).

We measured thrombin generation in the presence and in the absence of heparin, and of course reaffirmed the much smaller thrombin generation in the presence of heparin. When we then calculated the velocity of prothrombin conversion, we expected to find it had also decreased due to the factor Xa and/or factor IXa inactivation induced by the heparin. To our great surprise, however, the prothrombinase activity as induced by diluted thromboplastin appeared not to be inhibited. The intrinsic coagulation pathway on the other hand was inhibited, but this inhibition vanished as soon as factor VIII was preactivated before coagulation was started. This indicated that again inhibition of thrombin forms the basis of the action of heparin, i.e. that in the intrinsic system it expresses itself via the non-activation of factor VIII.

We must conclude that in clotting plasma all three of the factors VIIa, IXa and Xa are completely insensitive to inactivation by AT III–heparin and that classical unfractionated heparin acts mainly on thrombin (Béguin and Hemker, 1987). The common denominator of the effect of oral anticoagulation and of heparin in platelet-poor plasma seems to be thrombin and nothing but thrombin.

Of course, platelet-poor plasma is only a small sub-system of the whole organism. We would not like to claim that our results exclude the possibility of

an action of anticoagulant therapy on the vessel wall, the platelet or elsewhere. We feel, however, that we may exclude from the possible list of target reactions those that have been shown not to be inhibited in the system that we studied, i.e. all those leading to thrombin generation in platelet-poor plasma. It might also be possible that vitamin K antagonists act by some mechanism not involving thrombin, and heparins by another. For the moment, we feel this to be highly unlikely and therefore we claim a pivotal role for thrombin in thrombosis formation.

The next thing to do will be to extend our studies to the next larger sub-system, i.e. platelet-rich plasma. We will meet some rather formidable technical problems but they do not seem insurmountable.

At this moment one may recall Popper and say that, if we could produce an example of an effective antithrombotic therapy that does not affect thrombin generation, our thesis would be invalidated. There are two possible candidates: first antiplatelet therapy and secondly low dose heparin therapy.

Zwaal (this volume) describes how small amounts of thrombin will transform platelets that are sticking to collagen, into very powerful foci of thrombin generation. It is thus quite possible that platelet inhibition will prevent thrombin formation at the site of a wound. Conversely, because thrombin is a very potent platelet activator, it is also possible that inhibition of thrombin generation is the best possible way to inhibit platelets. Platelets and thrombin generation are anyhow linked so closely that an antithrombotic effect of an antiplatelet drug cannot be taken as counter evidence for the role of thrombin in thrombosis.

The case of low dose subcutaneous heparin may seem more challenging. Up to this moment no significant antithrombin action of such therapy has been demonstrated, whereas its antithrombotic properties appear from clinical trials (Kakkar *et al.*, 1982). However, we were recently able to show that subcutaneous heparin administration does have an influence on thrombin generation. Basing our approach on experiments from Professor Josso's laboratory we investigated the activation of factor VIII in the blood emerging from a finger prick (Josso *et al.*, 1976). It has been demonstrated that this activation is the best indicator of trace amounts of thrombin. Consequently inhibition of this activation of factor VIII indicates inhibition of thrombin generation in the wound. If we follow factor VIII activation from a capillary puncture after administration of 1,000 units of classical heparin subcutaneously, we see that heparin activity can only be demonstrated with difficulty by the APTT or other common laboratory tests. The activation of factor VIII, however, is inhibited between 2 and 4 h after the injection. Therefore this therapy inhibits thrombin formation (J. Fiolet and D. P. Devilée, personal communication) and its clinical success does not invalidate our conclusion.

Low molecular weight (LMW) heparins and heparin-like substances offer the next challenge. Let us take pentosan polysulphate (PPS, Hemoclar®) as an example. This drug has been shown to be an effective antithrombotic agent even if AT III is absent. Recently Wagenvoord *et al.* (1986) from our laboratory, determined

that, in the concentrations used therapeutically in humans, PPS has only two actions on blood coagulation; a low, heparin-like, AT III enhancing capacity and, more important, a specific action at the level of factor VIII (Wagenvoord *et al.*, 1986). Because factor VIII has a key function in the reinforcement loop of extrinsic coagulation, PPS will inhibit thrombin formation caused by small amounts of thromboplastin as well as thrombin formation via the intrinsic pathway, in a way comparable to the inhibition seen in haemophiliacs. This in essence is again an action on thrombin formation at the site of vascular damage. In a similar way, LMW heparins that have only an anti-factor Xa activity may be good anti-thrombotic drugs because they will inhibit the generation of thrombin.

Perspectives

If thrombin indeed is the pivotal enzyme in thrombosis generation it nevertheless is also a critical factor in maintaining haemostasis. We therefore are confronted with the question of how it will ever be possible to make drugs that prevent thrombosis without causing a haemorrhagic diathesis. Yet the preliminary experience obtained with LMW heparins forcefully suggests that it may be possible to obtain such drugs (Barrowcliffe *et al.*, 1984; Cade *et al.*, 1984; Holmer *et al.*, 1982). How can the enzymology of thrombin formation explain this and how can it help to design drugs? This question can only be approached if we take into account the form of the thrombin generation curve (Fig. 1).

Such curves when obtained in patients with a condition that leads to a thrombotic tendency, such as AT III deficiency, tail behind and above the normal one. Those from patients with a haemorrhagic diathesis like haemophilia or over-anticoagulation show very low and slowly rising thrombin concentrations. It nevertheless is possible to markedly diminish thrombin generation in a way that does not cause an important haemorrhagic diathesis but does reduce the risk of thrombosis. In fact one gets the impression that, if a fairly steep rise in thrombin generation at the beginning of the reaction can be assured, haemostasis is not affected but thrombosis is prevented. This leaves us with the idea that it is not (only) the amount of thrombin formed that determines whether thrombosis (or haemostasis) can develop, but also the form of the thrombin-time curve. We stress that at this moment we do not have enough information to determine what disease is linked to precisely what form of curve. We only present examples that make it clear that the form of the thrombin time curve may indicate whether haemostasis or thrombosis is affected. Now the new heparin fractions, such as CY 216 or PK10169, have the interesting property of inhibiting more or less specifically certain steps of the thrombin generation and breakdown process. More specific inhibitors, like Choay's pentasaccharide (Choay *et al.*, 1983), are no longer science fiction. This means that in the near future we will be able to shape the thrombin generation curve to our liking. After that we can compare the thrombin generation curves obtained with different drugs, and compare them to the clinical antithrombin

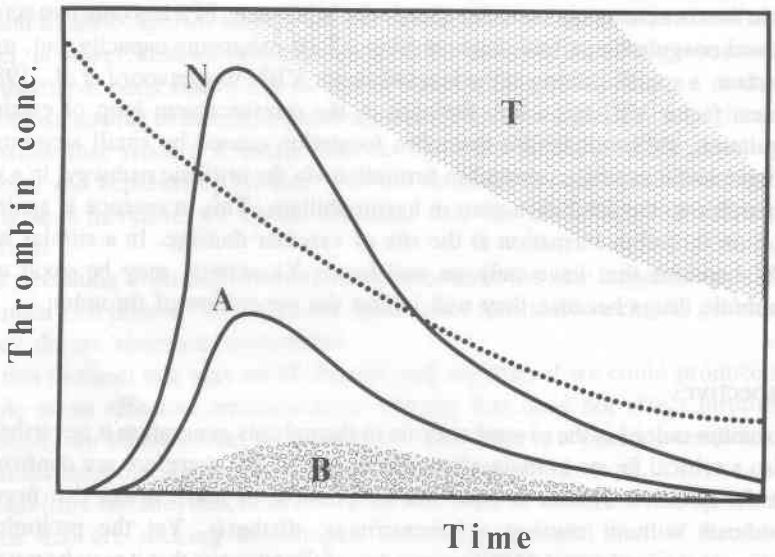

FIG. 1 N shows a normal thrombin generation curve, i.e. the development of thrombin in time after triggering of coagulation. As soon as it extends into area T, the thrombosis risk zone, even people with healthy vessels tend to thrombosis (e.g. antithrombin III deficiency). With the increase of age (and other risk factors) the boundary of the thrombosis danger zone shifts downwards (dotted line). Anticoagulant therapy can move the thrombin generation curve out of the danger zone (curve A). Anticoagulation should not lead, however, to curves entering the bleeding risk zone (B).

potency of these substances. This will instruct us about the relation of the thrombin generation curve and bleeding or thrombosis. In this way the coagulation laboratory together with the medical research profession and pharmaceutical industry may hope to define the ideal anticoagulant.

Summary

All efficient antithrombotic therapeutic agents cause a decrease in thrombin generation in the blood of the patient. Thrombin therefore is very probably crucial to the final common path in the pathogenesis of thrombosis. On the other hand, it is also the key enzyme of haemostasis. Yet the time course of thrombin generation leading to haemostasis and to thrombosis is different. It must therefore be possible to obtain a non-haemorrhagic antithrombotic drug by specific inhibition of certain steps in the reactions that determine this time course.

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Discussion

Dr Coller: Could Dr Hemker comment on the perplexing role of factor XI?

Dr Hemker: I can comment on it, but I cannot say anything sensible about it. In 100 patients lacking factor XI there may be five who indeed have a serious bleeding tendency. I cannot explain why they have that, or why the other 95 patients do not.

Dr Zwaal: Dr Hemker will of course know that if a small amount of thrombin is added to an animal, that does not lead to a thrombotic state but rather to a bleeding state. This has recently been explained by the fact that thrombin is carried away by thrombomodulin. Is it known whether there is any thrombomodulin on the venous endothelial cell?

Dr Hemker: I think there is — but if thrombin is injected, there is a situation that is much more akin to disseminated intravascular coagulation than to local haemostasis. This is because there is immediate mixing of the thrombin with the bulk of the blood. Of course, it is well known that in disseminated intravascular coagulation the end effect is an anticoagulation.

Dr Zwaal: Is factor VIII activation a sensitive parameter for the amount of thrombin?

Dr Hemker: That is what is seen in disseminated intravascular coagulation. Factor VIII is activated, but this factor is highly unstable in the circulation, so its level drops in these cases as do the factor V and the platelet count.

Dr Sultan: With regard to the activation of factor VIII in the capillaries, Dr Hemker showed that it can be increased by 25%?

Dr Hemker: No, that is not correct. We cannot convert the clotting time to the amount of factor VIII, because after its activation, it becomes another molecular species; that is the reason why I express roughly its activation by taking the difference between the long time that is found in the first drop and the short time in the second drop divided by the time in the first drop. It is completely arbitrary, but I know of no other way to express it.

Dr Sultan: So the reduction is from 25% to 5% when a low dose of heparin is given?

Dr Hemker: Yes, something like that, between 2 and 4 hours after injection.

Dr Caen: When some 20 years ago, we studied a patient with congenital hypofibrinogenaemia with developed thrombosis, we looked at the thrombin generation test. As expected, not only was it normal, but more than normal. At that time, we used a small dose of heparin to cure that kind of thrombosis in congenital hypofibrinogenaemia. I would consider that fibrinogen has no importance in that regard, but that thrombin generation is quite important, as Dr Hemker stressed.

Dr Nurden: I think that Dr Hemker has made an important contribution to the meeting because he has made us look away from the platelets towards the whole system that may be responsible for thrombus formation.