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LETTER IN FOCUS

During coagulation, thrombin generation shifts from chemical to diffusional control

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Hemostasis and thrombosis hinge on the formation of thrombin. The underlying reaction mechanism is a web of proenzyme–enzyme conversions, controlled by positive and negative feedback reactions [1]. Current paradigm has it that thrombin formation is the results of chemical interactions between the components of this web and therefore is uniquely determined by the initial concentrations and reaction constants of its components [2,3].

Essential steps of the thrombin forming process occur at membranes that contain negatively charged phospholipids [1]. In an *in vitro* model system such procoagulant phospholipids are normally added in the form of relatively small vesicles that remain in suspension, so that the binding sites for the clotting factors are available in free solution. *In vivo* the binding takes place on membrane surfaces that present in a wound or ruptured plaque [4]. Factor VII(a) binds to tissue factor incorporated in cell membranes and factors II, X(a), V(a), IX(a) and VII(a) on adhering and aggregating activated platelets and on membrane remnants. Experiments in the presence of platelets and tissue factor bearing cells suggest that, as soon as such structures are present, physical transport through diffusion might play a role in determining thrombin generation velocities [5,6,7]. In coagulation experiments in plasma *in vitro,* procoagulant phospholipids are added to platelet-poor plasma (PPP) in the form of vesicles; in platelet-rich plasma (PRP), they are provided by activated platelets [8].

At the moment that a clot forms, ~98% of thrombin is still to be generated [9,10]. The total amount of thrombin formed is an important determinant of the quality of hemostasis or the extent of thrombosis [11] and this thrombin is almost all formed within the fibrin mesh of the clot. In that mesh, the plasma is stagnant and the procoagulant surfaces of blood platelets and membrane fragments stick to the fibrin fibers [12]. Prothrombin conversion and other activation reactions thus are likely to be located on the insoluble fibrin mesh rather than in free and homogeneous solution and it is conceivable that not only chemical reaction rates but also diffusional transport to these surfaces determines the rate of thrombin formation.

Whether diffusion or chemical interaction is rate limiting can be decided on the basis of temperature dependence. Diffusion velocity is proportional to absolute temperature, i.e. it will show a ~14% increase between 10 and 50 °C, whereas biochemical reactions will roughly double their pace if the temperature is raised by 10 °C (see e.g. 13). Here, we show that the temperature dependency of thrombin generation in clotting plasma is such that we must assume that, when a clot has formed, diffusion starts to co-determine the rate of thrombin formation.

Thrombin inactivation, on the contrary, is caused by stoichiometric reaction with specific plasma proteins, the most important of which is antithrombin. In contrast to the activation reactions, inactivation takes place in free solution and therefore is likely to be less diffusion dependent.

$$\text{Prothrombin} \xrightarrow[\text{at Membrane}]{\text{Prothrombinase}} \text{Thrombin}$$

$$\text{Thrombin} \xrightarrow[\text{in Solution}]{\text{Antithrombins}} \text{Inactivated Thrombin}$$

If the velocities of both activation and inactivation are determined by chemical reaction rates, a rise in temperature will enhance both reactions to roughly the same extent and the change in thrombin activity with temperature will be small. If diffusional transport would govern activation but thrombin inactivation would be a chemical reaction, then activation would increase ~3% by raising the temperature from 25 to 35 °C, but inactivation would double so that an almost 50% decrease of the free thrombin formed would result. If diffusion plays a role that is more important in the activation reaction than in the inactivation, a limited decrease of thrombin activity would be seen with rising temperatures.

We tested thrombin generation in plasma at four temperatures: 25, 30, 35 and 40 °C under three conditions. A: in defibrinated plasma with added procoagulant phospholipids;

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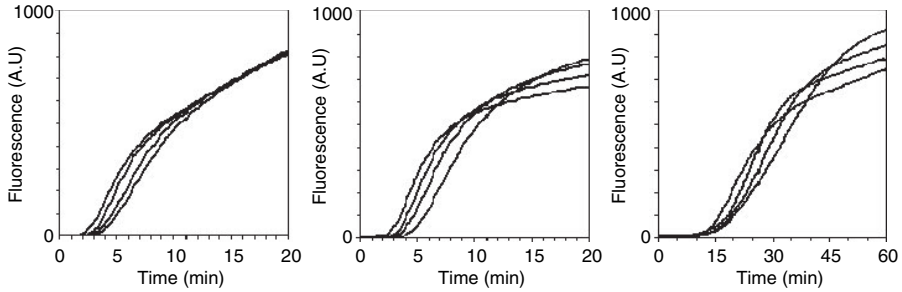

Fig. 1. Fluorescence development from a thrombin-specific substrate in plasma. Left frame: defibrinated PPP with added phospholipids; middle frame: non-defibrinated PPP with added phospholipids; right frame: PRP. In all experiments, the fluorescence was measured in 120 μL of citrated plasma to which (final concentrations) 5 pM TF, 16.7 mM of $CaCl^2$ and 0.42 mM of ZGGR-AMC were added at zero-time. In each frame, from left to right the temperatures were: 40, 35, 30 and 25 °C.

B: in normal plasma with the same amount of added phospholipids and C: in PRP (Fig. 1 from left to right). Defibrination with Arvin is known to precipitate fibrin but not otherwise to affect the clotting system [14].

To 80 μL of citrated plasma, we added 20 μL of a mixture of 30 pM recombinant tissue factor and 24 μM phospholipid vesicles (20% of dioleoyl phosphatidyl serine, 20% of dioleoyl phosphatidyl ethanolamine and 52% of dioleoyl phosphatidyl choline and 8% of cholesterol). Thrombin generation was started by adding 20μL of $CaCl_2$ containing 2.5 mM of the thrombin substrate Z-Gly-Gly-Arg-aminomethylcoumarine (AMC). The fluorescent product AMC was detected by its emission at 460 nM upon excitation at 390 nM [15]. When fibrinogen is present, the clot forms at the moment that also the fluorogenic thrombin substrate starts to be converted, the bulk of thrombin being formed within the clot.

In Fig. 1, it is seen that, with increasing temperature, the lag-time of thrombin formation shortens under all circumstances. This is to be expected because during the lag-time all reactions take place in free solution and will follow chemical rate laws, they i.e. are accelerated significantly by a rise in temperature.

In Fig. 1, middle and right frames, it is seen that as soon as fibrin is present less thrombin is formed at higher temperatures than at lower ones. This indicates that thrombin forming reactions are less accelerated by a rise in temperature than thrombin inactivating reactions are, which can be explained by assuming that the role of diffusion in the generation of thrombin is more important than in its decay.

This effect of diffusion starts to play a rate determining role in the generation of thrombin around the moment of clot formation. So, whereas the initiation phase is a purely chemical process, the contribution of physical restraints to thrombin generation during the propagation phase is not to be neglected. In mixtures without fibrinogen or in mathematical simulation, thrombin generation velocity is governed by chemical conversion rates only and diffusion is not taken into account. In models where the prothrombin converting enzyme is adsorbed at a macroscopic surface, thrombin generation is diffusion limited at all but extreme concentrations of the reactants [16]. Because of the transition of chemical to diffusional limited reaction velocity around the moment of clotting, neither of these approaches can be quantitatively extrapolated to the *in vivo* situation. It follows that thrombin generation in the body is best modeled by measuring it directly in a sample of isolated organ i.e. in clotting PRP with addition of the relevant components of the vessel wall (tissue factor, thrombomodulin) and/or white cells.

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