Review Article

Hemostasis and thrombosis beyond biochemistry: roles of geometry, flow and diffusion

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Abstract

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An important trend in the modern concept of blood coagulation is the growing agreement that, in order to understand regulation of coagulation in vivo and disorders of its function, it is essential to take into account its spatial heterogeneity, diffusion, and flow. In a way, this suggests that the idea of the "coagulation cascade" itself becomes increasingly misleading because there is no such place in an organism where reactions of this cascade really co-exist: activation, propagation and termination of coagulation are regulated by different subsets of chemical reactions that have different spatial localization and depend on cofactors expressed by different cell types in different tissues, so that only diffusion and flow can link these distinct "compartments" together into the one functional system. Here we review the last two decades of evidence obtained from in vitro, in vivo and computational systems biology approaches. When combined, the data comprise into an adequately comprehensive picture of the spatial regulation and organization of blood coagulation. In addition to the basic insights into the regulatory mechanisms, these approaches provided interesting results in the fields of coagulation diagnostics and other applications. Finally, the remaining unresolved and conflicting issues in the spatiotemporal regulation of coagulation are discussed.

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1. Introduction: Is there Anything in Blood Coagulation beyond Biochemistry?

It is traditional to think about blood coagulation as a cascade (Fig. 1A), or more accurately network of proteolytic reactions [1].
Although new reactions and components are still sometimes added to this scheme [2,3], and some of the mechanisms are subject of discussion [4], its main elements have remained unchanged since early 1990-ies.

According to the cascade concept [5], coagulation is initiated by a contact of plasma with a transmembrane protein tissue factor (TF) that is normally present only in the extravascular cells, so such a contact can occur only as a result of either mechanical vascular damage or other injury, e.g., inflammation. A low active serine protease factor VIIa binds TF to become a fully functional enzyme that activates factors IX and X via limited proteolysis, factor IXa then additionally activates factor X, and factor Xa activates prothrombin into thrombin. Thrombin produces fibrin that spontaneously polymerizes resulting in gelation of plasma at the site of injury. Gelled plasma stops bleeding thus fulfilling the ultimate purpose of the hemostatic system.

In addition, thrombin strongly amplifies its own production through activation of cofactor proteins factors V and VIII. Factors Va and VIIla greatly accelerate action of factors Xa and IXa, respectively, when assembled in factor Va:factor Xa (prothrombinase) and factor VIIla:factor IXa (intrinsic tenase) complexes on the negatively charged lipid surfaces, such as those found on activated platelet membranes. Thrombin also activates platelets (not shown in Fig. 1A) and factor XI; the latter then provides an additional pathway of factor IX activation. Factor XI can also be activated by factor XIIa in a so-called contact pathway of coagulation triggered by the contact of foreign surface with blood or plasma. Contact pathway is not shown on Fig. 1A because factor XII deficiency is not associated with bleeding phenotype and therefore it probably plays a minor role in normal hemostasis [6].

A number of anticoagulation pathways are known to regulate the procoagulant cascade: some are based on a straightforward inhibition of serine proteases by plasma inhibitors (antithrombin III), others include multi-domain inhibitors with a complex mechanism of action (TFPI), and cofactors that are inactivated via either spontaneous dissociation (factor VIIla) and cleavage by activated protein C. The activation of the protein C pathway [7] is also triggered by thrombin and is greatly promoted by two transmembrane endothelial cofactor proteins, thrombomodulin and endothelial protein C receptor that bring the enzyme and the substrate together.

This cascade concept is a beautiful and logical design, beginning with the TF exposure at the site of damage and the assembly of extrinsic tenase (the factor VIIla:TF complex), leading on to the activation of zymogens one after another into active proteases and ending with the fibrin formation, with amplifying positive feedbacks of factors’ V and VIII stimulation to form complexes on the membranes of activated platelets, and negative feedbacks of the TFPI and protein C pathways to down-regulate and contain the procoagulant response. Although this cascade concept may invite philosophical questions like why all
This complexity? Why make amplifier cascades and self-amplifying feedbacks and not to make the reactions rapid from the beginning? Why do we need contact pathway of coagulation? What is the role of factor XI? for the most part any concerns about the validity of the presented cascade concept seem too abstract or too specific. The cascade approach works well, particularly when one considers clotting in a test tube induced by uniform mixing of the sample with an activator.

On the other hand, in the organism, real thrombi and hemostatic clots come in a number of flavours: large fibrin clots in the wounds (Fig. 1B), red venous thrombi (Fig. 1C), fibrin networks in arteriolar thrombi that not only consolidate the thrombi but also penetrate into the tissue as “roots” to attach them to the vessel wall (Fig. 1D). When one tries to relate in vitro biochemistry of the coagulation cascade with these real-life processes developing in space and time, the task turns out to be not so simple. The coherent picture of coagulation cascade shatters and breaks. When one looks at a thrombus, it is easy to realize that there is no such place in the vasculature, where all of the sequential cascade reactions co-exist. Some of the reactions, e.g., TF-dependent initiation, mostly occur on the damaged subendothelium; afterwards, activated coagulation factors diffuse into plasma to form complexes of tenase and prothrombinase on the activated platelet membrane (and it is intriguing that there is even a microscale segregation: procoagulant reactions occur on the membrane surface, while plasma is strongly anti-coagulant); finally, the reactions of the protein C pathway actually occur only on the undamaged endothelium due to their dramatic acceleration by thrombomodulin and binding of protein C by endothelial protein C receptor.

In other words, within the concept called coagulation cascade we have several distinct processes that occur at different places with little overlapping, both at microscopic and macroscopic scales, and are connected together only by such transport processes as diffusion and flow. When viewed from the locality angle, it becomes clear that coagulation cannot function without these transport processes at all, that it is diffusion of coagulation factors that is critically required for the formation of a three-dimensional fibrin clot (and not thin fibrin film on the surface of the TF-expressing cells), and that an attempt to investigate blood coagulation biochemistry without taking into account its spatiotemporal aspects and transport processes is, in a way, similar to investigating biochemistry of neural excitation without thinking about neural impulse propagation along the axon: not completely senseless, but a very limited approach.

There were several ideas that inspired different groups of researchers in 1990-ies to turn from pure “homogeneous” biochemistry of coagulation to the study of the spatial regulation, diffusion and flow. One line of thought that produced an important and influential “cell-based model of hemostasis” was realization of clear segregation of blood coagulation reactions between different cell types so that there is actually no special location for blood coagulation “cascade” to function as a whole [8]. Several groups working with animals and flow chambers began in-depth examination of diffusion and transport in thrombi [9-11]. For our group, it was similarity of the positive feedback biochemistry of coagulation to many travelling wave phenomena and importance of spatial propagation to form three-dimensional thrombi that made us propose a “reaction–diffusion” experimental model [12,13] later giving rise to the thrombdynamics assay [14]. Others became interested in clot growth from thrombogenic surfaces [15] or other problems [16]. Two factors were important in these developments, solidification of the biochemical knowledge of coagulation by that moment and increasing use of computational models for the simulation of clotting dynamics [17].

It is probably because of such different background, divergent viewpoints and the overall complexity of the subject there are few analytical or educational reviews that are available. Several cornerstone ones were published early in 2000-ies on the cell-based concept of coagulation [8,18,19]. An important methodological analysis on dealing with spatially non-uniform networks using blood coagulation as an example was provided by the Ismagilov group in [20,21]. In one of our previous studies we also suggested a systemic analysis of the coagulation network and methods of its modular decomposition [22]. Several important aspects (such as the role of flow or roles of different reactions depending on space and time) are discussed in the reviews of Mann and coworkers [23,24]. The subject of flow in general was discussed in several review papers beginning from 1990 [25-29]. Some of the spatial aspects of coagulation were recently reviewed in [30]; the authors believe that all processes of spatial propagation of blood coagulation are explained by a travelling-wave-like motion of thrombin impulse from the site of damage, while, as discussed below, this is just one possibility that is currently supported by limited in vitro evidence.

The purpose of this review is to overcome the existing shortcomings and provide a full and realistic view of the problem. We attempt to combine together and analyze the data produced by these groups in order to propose a reasonably comprehensive picture of the spatial regulation of blood coagulation (not forgetting about unresolved issues along the way), to discuss how this spatial approach can change our basic views on how coagulation works, and how this might affect diagnostics, patient treatment and drug development.

2. Stages of Spatial Clot Formation

A general framework is needed in order to speak about spatial organization of blood coagulation. Let us segregate the process of coagulation into three stages: initiation, spatial propagation, and termination (Table 1). Not all of them always need to be present in vivo, and there may be some overlapping between them, but still it is much more convenient to use them to better sort out experimental data and theoretical concepts.

It is important to clearly define terminology here, because people working with homogeneous systems and those working with spatial ones can use the same term to describe different phenomena. For example, for a homogeneous system, termination means that chemical processes are being stopped within the sample as a whole [31]; in the spatial case, one speaks about termination when a process stops to spread in space [32]. For the purposes of this review, we shall follow the “spatial” terminology. This means that initiation of coagulation is the stage that takes place near the activating surface (and not necessarily during only the first minutes, and not necessarily means that only small quantities of thrombin are formed there). Spatial propagation is the stage of spreading of the coagulation process in space from the TF-expressing surface in order to create a three-dimensional clot (not the amplification of thrombin/fibrin production itself). Finally, termination is the stage where this propagation stops, i.e. some limit for the region of clot formation is set.

Table 1 suggests the organization of these three stages that will be discussed in detail in the rest of the paper. Each of these stages has its specific (patho)physiological role, occurs at its specific location, e.g. on the membranes of different cells and is controlled by different biochemical and physical processes.

The first one is initiation. Any type of vessel wall damage (either physical disruption or inflammation) results in the contact of blood with TF of either subendothelial origin or expressed by inflamed cells of blood or vasculature. This leads to the factor VIIa-TF complex formation and factor X activation thus initiating the blood clotting cascade. This is the first step, and no large clot formation occurs yet.

As will be discussed later, even this initial stage is a subject of intricate regulation, and some of the complexity of blood coagulation cascade (specifically, feedback activation of factor VII by factor Xa and factor V activation by thrombin (other important feedbacks like factor VIII activation by thrombin [33] seem to be involved at later stages [22]) serves to form an all-or-none response of the coagulation network, also called activation threshold, and provide explosive fibrin formation [22,34]. Transport processes are less critical for this stage, but might be important (e.g., flow of blood can prevent onset of coagulation [35,
while diffusion is important in determining the onset of clotting as it depends on the size and TF density of the damaged region \[37,38\]). There is experimental and computational evidence that clotting is triggered by a transient expression of TF, and the activator does not contribute at later stages \[39,40\], which is an additional argument to consider this stage separately from the others. The next step, which we believe to be a critically important one, is spatial propagation. Obviously, without diffusion, there would be no fibrin clot, because TF is located on the cell surface. To make a solid three-dimensional plug, it is important for coagulation to propagate in space, to transfer the activating signal from the damaged surface into plasma. The data of several groups \[8,41\] suggested that the second stage that is physically separated from the initiation.

Finally, there is termination of the spatial propagation. It is obvious that blood clot cannot be allowed to propagate indefinitely, because otherwise it would turn all blood in the organism into a gel. This is the least understood stage, though certain mechanisms can be proposed. A trivial mechanism of termination assumes that the activating factors produced at the site of damage have a limited diffusion distance \[32\] that would determine the size of the fibrin clot e.g., due to adsorption of coagulation factors by mebranes of activated platelets inside the thrombus \[9\]. Then, one can imagine a situation where no biochemical controls leading to self-sustained propagation may indeed be desirable, e.g., in a large wound that needs to be completely sealed. The wave should, however, be prevented from spreading to healthy vasculature, and there are data showing that thrombomodulin can play a role in stopping the clot propagation \[32,42\]. Finally, rapid flow can prevent fibrin formation spreading beyond the platelet thrombus \[35,36\]. Still, there are many unresolved issues here.

In the next sections we will review the three stages of spatial clot formation in detail.

### 3. Clotting Initiation: Activation Threshold and Local Explosion of Thrombin Formation

The first task of the blood clotting system is to decide: to clot or not to clot? Is local activator concentration/density/size sufficient to begin mobilizing resources and proceed to clot formation? A need for such decision is not immediately obvious: one could easily imagine a blood coagulation network with a proportional response to stimulation, i.e., fibrin formation proportional to tissue factor concentration. The Nature, however, decided otherwise: blood coagulation response is vastly disproportional. Moreover, it seems to function in an all-or-none regime, also known as bistability switch in systems biology terminology \[43\]. Many biological signaling and defense systems are known to possess bistability quality. In coagulation, all-or-none switch might serve to prevent clot disruption by flow and embolization. In other words, it might be safer to have either a fully-functional clot, or no clotting at all, but not a half-hearted attempt to make one.

Bistability in a biological system is usually associated with two consequences. First, transition from one state to another is characterized by a threshold: this transition (e.g. to clot or not to clot) occurs as a trigger in an all-or-none fashion. Threshold behavior of a system is usually a fundamental property that reveals itself not only in its response to stimulation, but also in response to many parameters. The reason for this is that a threshold arises from an interplay between self-amplifying positive feedbacks and inhibition, and their balance can be shifted by changing different parameters or concentrations. Blood coagulation, in particular, responds with a threshold (depending on the experimental conditions) to perturbations in the activator concentration, density and size of the TF-covered region, blood flow velocity, or even calcium concentration. Second, transitions between the states are rapid because the functional benefit of bistability is to avoid spending time in the intermediate states. In coagulation, this means that kinetics of the thrombin and fibrin formation are also strongly non-linear, practically, explosive in nature, the fact well known for this system for a long time (for a typical curve see Fig. 2A, B versus Fig. 2C, D).

Precise determination of the threshold level and even simply establishment of its existence were not, however, technically simple. It is indicative that initial thoughts about mechanism of such phenomenon were not discovered experimentally but rather proposed in an influential 1989 theoretical paper by Khanin and Semenov \[34\], who related it to the presence of thrombin-dependent factor V activation feedback in the coagulation network. Khanin and Semenov demonstrated that coagulation in such system occurs in a threshold manner upon increase of the activating signal. Several years later, this line of study was continued in another theoretical series of papers by Beltrami and Jesty, who have also shown existence of thresholds in proteolytic cascades with positive feedbacks of different design \[44\] and were the first to publish their findings in a clinical journal \[45\]. Interestingly, it was their belief (in contrast to that of Khanin) that factor V activation is the least important compared with factor VI, factor VIII, and factor XI activation. Although Beltrami and Jesty were able to demonstrate experimentally that thresholds indeed occur in enzyme systems with feedback and inhibition \[46\], existence of a threshold in a real blood clotting remained an unresolved experimental issue. One paper (once again, a theoretical one) even suggested that there is no threshold in coagulation in the absence of blood flow \[47\]. An experimental effort by our group in 1995 demonstrated calcium concentration threshold \[48\], but not the...
activation threshold. The only evidence of an experimentally observed threshold in the coagulation system was provided by the Mann group that reported strongly non-linear response of thrombin generation in a reconstituted system of coagulation proteins titrated by TF\textsuperscript{49,50}; there was no fibrin in those experiments.

This began to change by the end of 2000s. The first paper to directly demonstrate activation threshold (though not a threshold in the sense of activator concentrations) was an elegant study of the Ismagilov group\textsuperscript{51} who demonstrated a threshold size of TF-covered patch required to initiate clotting in plasma at a given TF density (Fig. 2E). For a TF density of 0.5 pM/m\textsuperscript{2}, this critical size was of the order of 100 μm in diameter. This study was expanded later for stimulation of coagulation by clusters of bacteria\textsuperscript{52}. A year later, the next step was made in the laboratory of Scott Diamond who used flow perfusion chambers with whole blood and printed TF microarrays of approximately 175 microns in diameter of different density to look for the activation threshold in a more classical sense. They found that a critical threshold TF density required to trigger coagulation is 4 to 10 molecules of TF per micron\textsuperscript{2} depending on shear rate\textsuperscript{53}. Finally, our team used carefully prepared plasma, where contribution of contact activation was prevented by corn trypsin inhibitor (CTI), to measure a homogeneous threshold TF concentration required to turn on thrombin and fibrin formation\textsuperscript{22}. The threshold turned out to be on the order of 0.01 pM of TF. We also demonstrated theoretically and experimentally that both threshold formation and explosive dynamics of fibrin formation strongly depend on factor V activation (see disappearance of the explosive response in factor V-deficient plasma in Fig. 2C, D) and do not depend on factor VIII or factor XI thus confirming the groundbreaking Khanin theory. Later we expanded this study to plasma stimulated with patterned TF and have shown importance of TF concentration in local spots of high density\textsuperscript{37}; once again, this turned out to be strongly dependent on the positive feedbacks of coagulation network. To summarize, there is extensive in vitro and in silico evidence that blood coagulation should and indeed does possess threshold properties.

Fig. 2. Activation threshold in blood coagulation and its regulation. (A–D) Dynamics of TF-initiated fibrin formation in normal (A, B) and factor V-deficient (C, D) plasma: computer simulations (A, C) and experiments (B, D). Reproduced from [22]. (E) Critical role of the activation region size in the initiation of clotting process. Reproduced from [51].
in a wide sense of the word: its activation depend in a bi-stable manner on activator concentration, density and patch size/shape in various experimental systems: with plasma and whole blood, with and without flow, for TF-induced and contact activation. Furthermore, there is substantial evidence that threshold behavior is due to the positive feedbacks of the coagulation network, in particular, to factor V and, to some extent, factor VII feedback activation. That is why we included threshold as part of the coagulation network displayed in Table 1, i.e. the module responsible for triggering regulation. One important reservation, however, is that we are not aware of any in vivo studies able to decisively confirm or disprove these threshold concepts (though it is understandable, as deficient function of too many of the required factors is not compatible with survival).

It is interesting that the activation stage, although originally designated as "purely biochemical", is not completely pure after all. In particular, the role of flow in determining the threshold TF density, existence of the activating patch threshold, importance of spatial distribution of TF on the activator strongly suggest importance of diffusion and flow in the modulation of clotting biochemistry even at the first stage.


A number of biological systems is known where a chemical signal develops not only in time but also in space, including neural impulse propagation, morphogenesis, spreading of gene in a population, and complement system activation. These systems cannot be understood unless we take into account the following interplay of reactions and diffusion: while biochemical reactions transform the components of this system, the diffusion physically moves them in space. The simplest example where the reaction and diffusion processes can be clearly separated is the attack on the pathogenic microbe by the complement system: biochemical reactions on the surface of the microbe generate C3a and C5a peptides that diffuse into surrounding plasma and allow chemotaxis of immune cells [54]. In more complex cases, reaction and diffusion processes are tightly intertwined. According to Fick’s law, velocity of diffusion is determined by a concentration gradient. If reactions can locally produce a steep gradient this propagates the signal in space further with a good speed.

For blood coagulation, the research focus was originally on elucidation of the biochemical details with little attention paid to the fact that this process has somehow to propagate in space. In a homogeneous system, generation of factor Xa during the activation phase gives an impulse for further thrombin generation. From the biochemical point of view, the coagulation cascade represents a system designed for amplification with the tiny amounts of thrombin, factor V and platelets that are activated in the initial phase. Paradoxically, fibrinogen is already converted into fibrin by that moment [55]. Further development of coagulation in a homogeneous case is characterized by a burst of thrombin generation. About 95% of all thrombin is produced during this second stage, although there is no more fibrinogen to cleave. This “thrombin paradox” raises the question "why do we need this burst?" [31]. The burst of thrombin is dependent on the intrinsic pathway reactions, but the sheer volume of post-clot thrombin, 95%, seems to be excessive with respect to clot formation. It might look like the intrinsic tenase is not required for clot formation. Alas, clinical evidence of bleeding in hemophilia suggests that intrinsic tenase is absolutely essential. One possible explanation is the rapid inactivation of the extrinsic tenase [56], which does not seem very convincing from the evolutionary stand point: why make a rapidly inhibited extrinsic tenase in the first place?

The intrinsic tenase paradox (or thrombin paradox) together with many others can be resolved if one leaves a homogeneous system for a spatially heterogeneous one. Several groups of researchers began to study coagulation from a reaction–diffusion point of view in 1990-ies, and the above-described lack of clear understanding of the role for intrinsic pathway in TF-induced coagulation became one of the starting points. The elegant experimental studies of M. Hoffman, D. Monroe and their co-workers considered thrombin generation in a reconstituted system of proteins and blood cells, with one critically important difference not seen in experiment published previously: Hoffman and Monroe did not mix repleted tissue factor (or TF-expressing cells) uniformly with the sample, but instead used a monolayer of monocytes on the bottom of a multiwell plate, with the synthetic plasma sample put on top. This simple approximation of a real-life situation led to the revision of the cascade concept, because TF-activated coagulation factors had to diffuse from the monocytes to platelets; as a result, factors Xa and IXa played distinct roles [57,58]. Factor Xa activation was crucial for initial thrombin formation and rapid activation of platelets, while subsequent thrombin generation on the surface of activated platelets strongly depended on factor IXa that is inhibited slowly in plasma and thus can diffuse further from the monocyte surface. These data showed different functions of extrinsic and intrinsic pathways and underlined the formulation of the cell-based model of hemostasis [8] stating that initiation and propagation phases of coagulation occur on the surface of different cells and determined by different reactions in the coagulation cascade.

To study the effect of spatial heterogeneity of coagulation process one needs new experimental systems that explicitly consider diffusion process. Such kind of systems was developed by several research groups and included models in non-stirred plasma with immobilized activator [13,15,59]. In the experimental system that was developed by our group, coagulation was initiated either by TF-bearing cells, glass edge [13], or TF immobilized to a plastic surface [60]. After activation on the surface, coagulation propagated freely into the bulk of non-stirred plasma, and fibrin clot growth was observed by light scattering. In a more advanced version, a “spatial fibrin & thrombin generation” approach shown in Fig. 3A, B, spatial dynamics of fibrin and thrombin can be monitored simultaneously [42]. One might notice that initial explosion of thrombin generation at the site of activation is followed by the propagation of thrombin wave to the right, into the bulk of plasma; the wave that strongly resembles travelling pulses of neural propagation and that leaves fibrin clot formed in its wake. What are the mechanisms of this propagation?

A major part of the response was the action of the intrinsic pathway. Experiments performed in early 2000s revealed that when intrinsic tenase could not assemble because of either factor IX or factor VIII deficiency, the clot growth was dramatically impaired, while initiation was not affected (Fig. 3C) [13,61]. In both hemophilia A and B the rate of clot growth was significantly decreased, showing that propagation in space cannot occur without intrinsic tenase. These observations are in line with the results of the “cell-based model” and highlight the different functions of the intrinsic and extrinsic pathways; important difference is explicit addition of spatial dimension and the role of feedbacks.

Indeed, a lot of thrombin is generated on the activating surface, together with other coagulation factors, and can (theoretically) diffuse into the bulk of blood and spread the clotting process there. However, this does not occur: both thrombin and factor Xa are rapidly (with a half-life time of less than 1 min) inhibited by antithrombin and other inhibitors [32], so their activity rapidly decreases with distance from the activating cells. Fig. 3D shows spatiotemporal distribution of factor Xa produced by extrinsic tenase obtained from computational simulations: it is produced in relatively large quantities but cannot get far from the activator. However, if we include positive feedback reactions into this model, amplification of coagulation can occur in every point where diffusion of active factors can reach. New active factors are produced at larger distances from the initial activating cells. Therefore, self-sustained production of thrombin due to the feedback reaction dramatically changes the distribution of active factors in space [32]. Fig. 3E displays factor Xa produced by intrinsic tenase by a combination of diffusing factor IXa and thrombin-activated factor VIIIa: it has no role near the activator but forms a pulse propagating from the activator.
The basic idea behind this is the same one as in other spatially heterogeneous systems with positive feedbacks. Because of these feedbacks, each point in space within the reach of the front of coagulation becomes in its turn a source of new activated factors. As mentioned above, the rate of propagation is determined by a concentration gradient. Without positive feedbacks, the rate of propagation rapidly decreases with distance from the activator. In the case of the self-sustained reaction, the gradient value can be sustained, and the rate of propagation remains high.

5. Spatial Propagation Continued: A Special Role for Factor XI in the Presence of Sufficient Phospholipid

Positive feedbacks of factor VIII and factor V activation have one essential limitation: these proteins are cofactors and are unable to act by themselves; therefore, their effect is limited by the distance of factor IXa diffusion, without it they do not work. In contrast, feedback activation of factor XI by thrombin [62] can generate new factor IXa and this allows to complete the circle of thrombin activation without contribution of TF. Based on this, we proposed a hypothesis that blood can act as an active media and support autowave-like self-sustaining thrombin propagation [12,13].

When autowave hypothesis was tested in experiments using the above-described reaction–diffusion model in platelet-free plasma and detailed mechanism-driven mathematical models, the concept seemed to be confirmed to an extent [32]. Fig. 4A shows the clot size as a function of time in normal and factor XI deficient (also known as hemophilia C) plasma obtained using model simulations; it can be seen that contribution of factor XI is not great and is observed only during the late stage of clot formation. The same was seen in experiments. In contrast, when factor IXa diffusion was “turned off”, this produced a defect in clot propagation similar to that observed in the deficiency of factors VIII or IX (hemophilias A and B, respectively).

However, in the spatial thrombin generation model in phospholipid-supplemented plasma the effect of factor XI was much more pronounced [42]. In normal plasma thrombin generation demonstrated two distinct phases: generation of thrombin peak near the activator that was dependent on the density of TF coating and the stationary

Fig. 3. Spatial propagation of blood coagulation from the site of damage in the reaction–diffusion model. (A, B) Fibrin (A) and thrombin (B) are displayed as functions of space (distance from the activator) and time. Clotting was induced by bringing recalcified phospholipid-supplemented plasma in contact with immobilized tissue factor (on the left). Reproduced from [42]. (C) Defective spatial propagation of coagulation in hemophilia A plasma. Time is indicated in min, scale bar is 2 mm. Reproduced with permission from Ovanesov et al. [61]. (D, E) Contribution of extrinsic (D) and intrinsic (E) tenase to factor Xa formation in the reaction–diffusion model. Computer simulations from [32].
phase when peak of thrombin concentration moved from the activator with a constant speed (Fig. 4B). Height, shape and speed of this propagating peak did not depend on the amount of TF on the activator (compare Fig. 4B and C). Still, this phenomenon occurred only in the presence of phospholipid surface, otherwise clot growth was not truly stationary and depended on TF density [37] (Fig. 4D), once again both in experiments and computer simulations.

This difference is easily explained by the key role in feedback amplification of thrombin generation played by the enzymatic complexes intrinsic tenase and prothrombinase that assemble on the phospholipid membrane surfaces. When lipids are available, the output of the tenase and prothrombinase reactions helps to sustain high gradient of active factors and propagate coagulation in space in much the same way as neural impulse. When not, there is no sufficient amplifying power in the feedbacks for moving impulse formation. The main source of procoagulant lipids in blood is believed to be the surface of activated platelets. Actually, any surface with proper lipid composition can promote coagulation complex assembly, including circulating microparticles or other cell types.

The effect of factor XI activation increased with distance from the activator where the effect of TF diminishes (Figs. 3B and 4B-D). In homogeneous experiments, the difference between normal and factor XI-deficient plasma became significant only when TF concentration was decreased up to zero. All these data show that this feedback loop plays a major role when TF is not available [63,64]. It seems to be logical to suggest that TF has progressively lesser effect on thrombin generation as the site of fibrin formation moves further away from the site of activation, i.e. as the clot grows.

This description of the propagation phase is to a certain extent idealized but it was observed in the experimental in vitro model setup. In what situations can propagation phase occur in vivo? There is no direct experimental data on whether such travelling excitation wave propagation of coagulation really occurs during hemostasis or thrombosis. Still, some intravital microscopy and morphological data can give indications of possible situations.

Judging from the in vitro data, travelling wave is more likely to form in the absence of blood flow and in presence of lipid surface, and its characteristic length scale is millimeters (though it can be much smaller in a platelet plug where diffusion is slow [9]). The first possible situation is a hemostatic plug in a large wound. In this case, there is no flow after initial platelet plug formation, and the large volume of blood should be coagulated. Self-sustained thrombin propagation can be the fastest way to coagulate blood. Morphology studies showed that normally fibrin formation and platelet activation occur inside a hemostatic plug [65]. But in the case of hemophilia, fibrin is concentrated on a site of TF and collagen exposure and the platelets in the middle of the plug are not fully activated. These facts suggest that propagation of thrombin generation in space is of great importance for normal hemostasis. Secondly, propagation of thrombin activation can occur inside the growing platelet thrombus.

But the self-sustained mechanism of propagation is not always required. Other mechanisms can also work during hemostasis process. One of the examples is formation of fibrin along the damaged tissue [11]. It also can be named as propagation of coagulation, but in this case TF is present on all the cells and only reactions of extrinsic pathway really work. Intrinsic pathway and feedback activation of factors VIII and XI is probably not required to produce such fibrin mesh.

Another possible mechanism is activation of coagulation cascade on the surface of procoagulant vesicles. There are experimental evidences that TF-bearing microparticles can accumulate inside growing thrombus [66] and therefore clot propagation can be achieved by TF accumulation from flowing blood. Also, the surface of activated platelets and platelet-derived microparticles can activate contact pathway of coagulation [67,68] and therefore thrombin formation can propagate in space due to activation of factor XI on a surface of platelets inside the plug. Further experiments in vivo are required to elucidate this.
6. Multiple Possible Ways of Termination of Fibrin Clot Propagation

Clot formation should be strictly localized to allow not to turn all blood into gel and not to spread clotting into healthy tissues. However, the exact mechanism behind localization is a matter of dispute and plenty of unresolved issues remain.

The possible mechanisms can be roughly divided into three groups: biochemical, diffusion-dependent and flow-dependent. Apparently, none of the known mechanisms can work exclusively and on its own. Rather, all of them can work in a range of conditions independently and together.

As mentioned above, special stopping mechanism is not always required. When hemostatic plug forms inside a wound, a whole surface of damage becomes covered with a plug and all blood that flow out can be turned into clot (Fig. 5A) [65,69]. In this case localization is achieved by a surface of wound.

From the biochemical point of view, coagulation system has a good candidate for the dynamic inhibition, protein C (PC) pathway. When thrombin binds to its cofactor thrombomodulin (TM), it switches its activity from activation factors VIII and V toward activation PC. Activated PC inactivates factors Va and VIIIa, therefore shutting down the amplification of coagulation cascade. Indeed, activation of the PC-pathway by artificial means of TM addition to plasma can dose-dependently stop clot growth in reaction–diffusion experimental system (Fig. 5B,C) [32, 42]. At the same time, added TM does not affect the initiation phase.

However, in reality, TM is a membrane protein which is expressed almost exclusively by healthy endothelium. Therefore, all of the PC-pathway reactions should occur on the endothelial cells, i.e., the range of conditions where PC pathway can work is narrow. For example, it was shown that TM-dependent mechanism of clotting termination may play significant role but only in small capillaries with high surface to volume ratio (Fig. 5D) [59]. Thus, thrombomodulin can prevent
propagation of coagulation to healthy tissues in narrow wounds, but cannot limit size of 3D plug inside the broad vessels.

Another mechanism of clot growth termination, diffusion-dependent mechanism, is based on the data showing that activating factors have limited diffusion distance. Therefore, slow diffusion rate can limit the size of fibrin clot under the conditions where clot growth is exclusively driven by factor diffusion. But, as we discussed earlier in Propagation, this diffusion limitation can be overwhelmed if there is any source of factor activation that does not rely on the activating TF-bearing surface, such as a feedback activation of factor XI by thrombin or accumulation of TF-bearing microparticles from flowing blood.

The most sophisticated but nevertheless possible mechanism is associated with blood flow. The flow rates can vary over a huge range of values in vessels of different size, e.g., from capillaries to large arteries. As flow can remove active factors from the site of production, dilution by flow can act as a strong inhibitor of coagulation. It was shown experimentally that shear rate of flow results in another threshold for coagulation cascade [35, 70]. When the flow of plasma is faster than the threshold limit, removal of active factors becomes sufficient to prevent clotting initiation and propagation is space. Model studies showed that the onset of coagulation is controlled by flow via removal of factor Xa. Positive feedback of activation of factor VII by factor Xa can maintain significant factor Xa concentration for further cascade activation, but only until the shear rate is under the threshold value (Fig. 5E) [35].

In another series of studies employing flow chambers, termination of spatial coagulation propagation was shown to be dependent on flow alone [20, 70]. In-vivo studies showed that thrombin generation is localized in the central part of the clot, where platelet plug is rather dense (Fig. 5F) [11]. In the area of loose packing of platelets, which is easily permeable by flow, coagulation cannot propagate further as active factors are washed out.

In summary, there are several possible mechanisms of clotting termination, one of them is implemented as a negative feedback loop into the coagulation cascade, others are outside of the coagulation system but may have a strong effect on the cascade reactions. Also, in some cases, termination of coagulation is not required (wounds). Therefore, distinct mechanisms work together to provide strict localization of a clot on a site of injury.

7. Effects of Flow beyond the Possible Clot Growth Termination

In general, blood flow influences coagulation via several mechanisms. The most important of them are supplying the clot with new zymogens and pro-cofactors, removing active factors, and physically affecting the fibrin network. There are indications that flow can radically change the roles and the mechanisms of many reactions in the coagulation network: prothrombin activation [71], action of therapeutic anticoagulants [72], membrane binding [73]. The overall effect of flow on coagulation is discussed by several important reviews [25–29].

8. Hierarchy of Experimental Models of Hemostasis

As outlined in previous sections, a picture of spatial regulation of blood coagulation emerges, a picture that is still in development but that, in many of its elements, is confirmed by experiments performed by different groups using a variety of experimental models. This very variety makes it important to discuss differences in the experimental design, which becomes particularly important because different experimental systems sometimes agree surprisingly well while can give drastically different results in others.

There is no and there could not be a single "forever true ultimate final experimental model of coagulation" simply because coagulation proceeds differently under different conditions. From the beginning of this paper we have seen that different geometry, activator density, availability of procoagulant cell surface, availability of anticoagulant cell surfaces, flow results in different dynamics of blood coagulation process and contributions of various components of coagulation. There could be a travelling wave of thrombin propagation if there is sufficient procoagulant surface and sufficient size of the wound to clot, but then there could be conditions when even diffusion is not very important. And this agrees well with what we know in clinical experience: there could be different thrombi and different hemostatic plugs.

Conditions may define the outcome, therefore the theories and models should be considered in proper pathophysiological context, i.e., under the ranges of conditions that make biological sense.

And although there is no in vitro model that includes all possible complexities of in vivo clotting, there is an increasing tendency of developing models including more and more such complexities. Each step along this way open a new field of information for research and diagnostics (Fig. 6). From the basic assays of APTT and PT [74] to the reduced degree of activation to improve sensitivity [75], which became particularly important in the thrombin generation assay [76].

The meaning of thrombin generation curve is a subject of intense debate [31] because it is difficult to understand: why do we look at generation of hundreds nM of thrombin when 1–3 nM of thrombin is sufficient to convert all fibrinogen into fibrin? It is most likely that the answer is dual. On the one hand, there are two types of thrombin-dependent reactions: a) factors V, VIII, fibrinogen, platelet aggregation and release are stimulated by very low concentrations of thrombin (~1 nM); b) on the other hand, factors XI, XIII, platelet procoagulant activity require 10–100 nM of thrombin, so reaching these concentrations is important to produce more stable clots [77]. Another line of reasoning is the spatial one [42]: if we take into account that activated blood coagulation factors can diffuse from where they are produced then it is not a problem that more thrombin is produced if necessary, as the activation process can spread into the nearby regions (though, interestingly, it seems that it is factor Xa rather than factor Xa or thrombin, whose diffusion is the most important).

An explicit addition of diffusion and spatial propagation (of fibrin, and then of thrombin as well [42]) can be considered as the next step towards more refined and physiologically relevant model (Fig. 6). Although there were versions that added the next logical step, flow, they did not make into the clinical field yet: all existing perfusion chamber methods are mostly focused on platelet adhesion and aggregation. Aggregation became first combined with clotting with the introduction of thrombelastography and other viscosimetry devices [78] thus opening an additional new dimension compared with the assays focused on coagulation only. While thrombin/fibrin generation can potentially evaluate platelets via their procoagulant activity [79], viscosimetry methods allow direct monitoring of aggregation, and shear flow-based methods (PFA and particularly TTAS) add adhesion thus mimicking microvascular thrombosis in great detail.

9. Spatial Coagulation: Possible Applications beyond Basic Research?

As was discussed in the previous sections, the spatial methods employed by different groups produced a lot of important information, and one of the versions led to development of a clinical assay of thrombodynamics. Some of the clinical and pharmacological results obtained with it are shown in Fig. 7, and more can be found in recent reports and reviews [67, 80–86].

10. Conclusions

The progress of the last two decades employing new experimental (in vivo and in vitro) and computational models resulted in a waterfall of new data overhauling our views on the regulation of blood coagulation. The emerging picture is not yet established but it definitely seems to be different from the previous views in several important respects. One is that there is a clear agreement that "cascade thinking" has major drawbacks, and a spatial, diffusion- and cell-controlled view on the processes of fibrin formation in thrombi and wounds appear to provide useful new insights. The second consideration is the understanding that
different modules of the coagulation network carry out specific functions responsible for creating thresholds, allowing spatial propagation and overall regulation of the process depending on the geometrical considerations and flow. Role of factor V activation in the explosive response, role of factor IXa diffusion in spatial clotting propagation are likely to be established elements of this picture that has already led to some pharmacological and diagnostics developments.

And yet there is also a substantial work ahead before we can speak about a new theory of coagulation that would be complete to a reasonable degree. One important element lacking for many elements of this picture is intravital and clinical confirmation of the results obtained mostly in vitro and in silico. As of the present moment, such confirmations are few and scattered. Another line is to unify these views with those on platelet-dependent hemostasis and other physiological systems (it is not accidental that complex interactions with platelets, factor XIII, fibrinolysis, contact pathway, role of coagulation in angiogenesis and immunity remained beyond the scope of this review). Finally, even this picture is not uniformly painted: there is much more...
information, understanding and agreement between different groups with regard to initiation/threshold and propagation/spreading stages than with the termination, where a lot remains to be understood.

**Conflict-of-interest Disclosure**

M.A.P., N.M.D., and F.I.A. are employees and/or founders of HemaCore LLC and HemaCore Labs LLC (Moscow, Russia) that hold several patents and patent applications on the diagnostic use of coagulation assays in spatially distributed systems, which are currently developed under the trade name of Thrombodynamics®.

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