Sensitivity and Robustness of Spatially Dependent Thrombin Generation and Fibrin Clot Propagation

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ABSTRACT Blood coagulation is a delicately regulated space- and time-dependent process that leads to the formation of fibrin clots preventing blood loss upon vascular injury. The sensitivity of the coagulation network was previously investigated without accounting for transport processes. To investigate its sensitivity to coagulation factor deficiencies in a spatial reaction-diffusion system, we combined an in vitro experimental design with a computational systems biology model. Clot formation in platelet-free plasma supplemented with phospholipids was activated with identical amounts of tissue factor (TF) either homogeneously distributed (concentration 5 pM, homogeneous model) or immobilized on the surface (surface density 100 pmole/m², spatially heterogeneous model). Fibrin clot growth and thrombin concentration dynamic in space were observed using video microscopy in plasma of healthy donors or patients with deficiencies in factors (F) II, FV, FVII, FVIII, FIX, FX, or FXI. In the spatially heterogeneous model, near-activator thrombin generation was decreased in FV-, FVII-, and FX-deficient plasma. In the homogeneous model, clotting was not registered in these samples. The simulation and experiment data showed that the coagulation threshold depended on the TF concentration. Our data indicate that the velocity of spatial clot propagation correlates linearly with the concentration of thrombin at the clot wave front but not with the overall thrombin wave amplitude. Spatial clot growth in normal plasma at early stages was neither reaction nor diffusion limited but became diffusion limited later. In contrast, clot growth was always diffusion limited in FV-, FVII-, and FX-deficient plasma and reaction limited in FVIII-, FIX-, and FXI-deficient plasma. We conclude that robustness of the spatially heterogeneous coagulation system was achieved because of the combination of 1) a local high TF surface density that overcomes activation thresholds, 2) diffusion control being shared between different active factors, and 3) an early saturated stimulus-response dependence of fibrin clot formation by thrombin.

INTRODUCTION

The blood coagulation system is a network of proteins that activate each other in an intricate cascade of proteolytic reactions. The main function of this system is the formation of an insoluble fibrin gel upon vascular damage to stop bleeding. Dysregulation of coagulation can lead to dangerous hemorrhagic or thrombotic disorders (1).

Coagulation is a spatially distributed process that is triggered by the contact of blood with transmembrane protein tissue factor (TF). TF is expressed by cells that are normally not exposed to flowing blood, such as subendothelial cells and cells surrounding blood vessels; endothelial cells do not express TF except when they are exposed to inflamma-
tory molecules (2) (Fig. 1). TF is a cofactor for coagulation factor VIIa (FVIIa), which circulates in blood at picomolar concentrations. These factors form the extrinsic tenase complex, which activates coagulation factors FX and FIX. These factors diffuse from the damaged area, initiating clotting propagation. FXa and FIXa form complexes with their cofactors, FVa and FVIIIa, to convert prothrombin (FII) to thrombin (via the FXa:FVa complex) and FX to FXa (via the FIXa:FVIIIa complex), respectively. Thrombin converts soluble fibrinogen to fibrin, which polymerizes and causes plasma gelation.

Thrombin is the main regulatory protein of the blood coagulation system. In addition to converting fibrinogen to fibrin, thrombin activates FXI and FVII and cofactors FV and FVIII as well as anticoagulant inhibitor protein C (PC), thereby forming positive and negative feedback loops regulating the whole coagulation network.
The complex structure of the coagulation network was suggested to endow it with functional properties (3). There is theoretical and experimental evidence that positive feedback of FV activation in combination with TFPI (tissue factor pathway inhibitor) and antithrombin III inhibition forms an activation concentration threshold (4). The positive feedback loop of FVII activation was suggested to create a threshold in response to blood flow rate (5). The threshold behavior of the system in response to the TF distribution (5) and the size (6) and shape (7) of the activating area was revealed when studied in heterogeneous conditions that simulated coagulation in vivo with TF localized on vessel walls. The long-range positive feedback of FVIII and FXI activation by thrombin are critical for the transfer of coagulation signals from TF-expressing cells to platelets (8,9). Thus, coagulation in space is a self-sustained excitation-wave-like propagation of clot (10). The PC pathway acts as a clot-size-limiting factor in these experiments and simulations (9,10). Several experimental and computational studies attempted to identify the sensitivity of homogeneous thrombin generation to coagulation factor deficiencies, the variability of parameters, and the “fragile points” of the coagulation cascade (11–14).

However, analysis of sensitivity of blood coagulation and the studies on the spatial propagation remain disconnected. Specifically, it remains unclear which reactions, diffusion processes, or their parameters control the velocity of the spatial clot propagation (and to which it is not sensitive).

Previously, we developed a technique and created a device for the measurement of thrombin in an experimental in vitro spatial reaction-diffusion model in which clotting was activated by a surface with immobilized TF (10). Here, we apply this approach in combination with computer modeling to investigate the sensitivity of the fibrin front and thrombin wave propagation to coagulation factor deficiencies and to reveal the rate-limiting processes.

**MATERIALS AND METHODS**

### Materials

The following reagents were used: 7-aminooctadecylcoumarin (AMC) and low-gelling-temperature agarose (Sigma Aldrich, St. Louis, MO); phospholipid-TGT (thrombin generation test) containing a mixture of highly purified phosphatidylycholine (42%), phosphatidylserine (28%), and sphingomyelin (30%) (Rossix, Malmö, Sweden); Alexa Fluor 647-conjugated annexin V (Biolegend, San Diego, CA); relipidated recombinant TF (Instrumentation Laboratory, Bedford, MA); and Thrombodynamics-4D kits consisting of corn trypsin inhibitor, fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland), calcium acetate, and a plastic insert with immobilized relipidated recombinant TF from Instrumentation Laboratory (HemaCore Labs, Moscow, Russia).

### Patients and plasma preparation

Plasma of healthy volunteers and patients with FII, FV, FVII, FVIII, FIX, FX, and FXI deficiencies were commercially available (George King Biomedical, Overland Park, KS) or collected at the Hematology Department of the Christian Medical Hospital (Vellore, India). A total of 34 plasma samples of patients with severe coagulation factor deficiencies (<1% of clotting factor normal concentrations, except for prothrombin deficiency, which had a cutoff of 2% of normal FII levels) were used in this study (11 from George King Biomedical and 23 from patients of Christian Medical Hospital). Factor activities were assessed using a one-stage method.

The patients who participated in this study gave their written informed consent. The study was approved by the Ethics Committee of the Christian Medical Hospital. Blood was drawn into 5 or 9 mL plastic vacuum tubes with 106 mM sodium citrate buffer at a 9:1 blood/anticoagulant volume ratio. Blood was centrifuged for 15 min at 1600 × g. Plasma was aliquoted and stored at −80 °C.

Frozen samples of plasma were thawed in a water bath at 37 °C for 15 min and then incubated for 1 h at room temperature. Then, plasma was centrifuged for 5 min at 10,000 × g and used for the experiments.

### Experimental design

First, 120 μL of plasma was added to dried corn trypsin inhibitor (final concentration 0.2 mg/mL), dried substrate (final concentration 400 μM), and 5 μL of phospholipids (prepared according to manufacturer’s instructions, aliquoted, and stored at −80 °C, final concentration 2 μM). We used frozen lipids because this allowed us to have a better standardization. Freezing does not affect thrombin generation and clot growth; the effect of freezing did not exceed 5%, which is comparable with the measurement errors.

After that, the sample was incubated for 15 min at 37 °C. Subsequently, plasma was supplemented with dried calcium acetate (final concentration 20 mM), and the sample (100 μL) was placed in a plastic cuvette.

Clotting was activated using 5 μL of TF diluted in distilled water (stored at −80 °C, final concentration 5 pM) poured directly into plasma or using a plastic plate with a 1 × 5 mm² TF-coated surface (TF density 100 pmole/m²). We used the designation of a “homogeneous model” and “spatially heterogeneous model” for these experimental designs, correspondingly. TF was covalently attached on a plastic plate with a cross-linking agent as described in (15). For making gel activators, we used a liquefied 5% agarose solution in distilled water, one part of which we mixed with four parts of diluted TF; so the final TF concentration was...
5 or 2000 pM. The resulting solution was poured on the tips of a plastic insert without immobilized TF and left to gelate. The solidified gel was cut into a rectangular shape with dimensions 5 × 1 × 3 mm (width, thickness, and length) attached to the tips of the plastic insert.

We used an ACTICHOME TF activity assay (BioMedica Diagnostics, Stamford, CT) for concentration of TF measurement as described in (5). The manufacturer (HemaCore) provided the information about density of the TF in the Thrombodynamics kits; ACTICHOME TF activity assay was used.

To demonstrate the presence of phospholipid vesicles in our activators, we made microscopic photographs of a plastic activator with TF immobilized on the surface and gel with TF. The plastic surface with immobilized TF was placed on the cover glass and covered with 100 μL of buffer (20 mM HEPES, 150 mM NaCl, 2 mM CaCl₂, 5 mg/mL bovine serum albumin) containing 12.5 mM annexin V conjugated with Alexa Fluor 647. The same buffer with Alexa 647-Annexin V and TF (100 pM) in 1% agarose gel (to prevent movement of lipid vesicles) was placed on the microscopy slide and covered with the cover glass. Images were acquired using an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) with long-distance Plan-Neofluar 20×/0.4 objective. Optical filter 647 + nm (Semrock, Rochester, NY) was used. The excitation wavelength was 635 nm. Analysis of obtained images was carried out with Imaged software.

Experiments were performed using a video microscopy system (10) that allows the observation of the spatial dynamics of fibrin clot growth and thrombin generation (Thrombodynamics-4D Analyzer; HemaCore Labs). Fluorogenic substrate added to the plasma was cleaved by thrombin and produced a fluorescent AMC molecule. Its generation rate was proportional to the thrombin concentration. The plasma sample was illuminated in turns by red (625 nm) and ultraviolet (365 nm) light-emitting diodes. The fibrin clot formation was detected by red light scattering, and the fluorescence of AMC was excited by ultraviolet emission (440 nm). Scattered light and fluorescence passed through a multiband filter and were detected by a digital camera every 30 s for 90 min.

**Data processing and calculating coagulation parameters**

Parameters of fibrin clot growth and thrombin generation were calculated from a series of images (Fig. 2A). Data processing was done using the program T2-CALC (HemaCore) as described previously (10). A detailed description of the procedure is given in Supporting Materials and Methods, Appendix A.

The light scattering of fibrin clots and thrombin concentrations were plotted as functions of time, and the following parameters were calculated (Fig. 2, B and C): clotting time (the time it takes to reach half of maximal light scattering, \( T_{1/2} \)); the amplitude of the thrombin peak (\( A_{max} \)); time to peak (\( T_{max} \)), and endogenous thrombin potential (ETP, the area under the curve). We compared thrombin generation in a homogenous system measured with a Thrombodynamics-4D assay and measured with a fluorescent plate reader (Fig. S3). Thrombin generation parameters from the Thrombodynamics-4D assay were consistent with those from the plate reader (the same protocol was used for comparison).

For each light scattering profile in the spatially heterogeneous system (Fig. 2D), clot size was determined as the coordinate of the half-maximal light scattering intensity (Fig. 2E). The clot size as a function of time was used to calculate the following parameters: lag time (\( T_{lag} \), time when the clot starts to grow) and clot growth velocity (\( V \), slope of the tangent line that approximates clot size as a function of time in the time period from 15 to 25 min after the start of clot growth). The linear least squares regression was used for \( V \) calculation. Using AMC and thrombin profiles (Fig. 2G), we calculated the amplitude of the thrombin peak after 60 min (\( A_{peak} \)).

We used algorithms for smoothing (moving average) to eliminate noise, which resulted in an inability to calculate parameters for the zone near the activator (250 μm). In addition, the optical problems associated with image defocusing in the 1 mm layer also did not allow calculation at the boundary with the activator plate. For a more detailed examination of the initiation phase of clot formation, we calculated the thrombin curve in the near-activator (na) area (Fig. 2F). High local concentration of TF on the activating surface causes rapid changes of thrombin concentration in the area close to the site of activation. To describe the process of thrombin generation in the area close to the activator, we averaged the fluorescence in the whole area and used it to calculate thrombin as if it were distributed evenly within the area. The AMC concentration was averaged in the area 0.05–0.2 mm from the site of activation. To describe the process of thrombin generation in the area close to the activator, we averaged the fluorescence in the whole area and used it to calculate thrombin as if it were distributed evenly within the area. The AMC concentration was averaged in the area 0.05–0.2 mm from the site of activation.

![Figure 2](https://example.com/figure2.png)

**FIGURE 2** Fibrin clot formation and thrombin generation in homogenous and spatially heterogeneous experimental systems in normal plasma. (A) A scheme of clot activation (left column) and pictures of the fibrin clot at 0, 5, 20, and 40 min after the clotting activation by TF in homogenous (5 pM TF) and spatially heterogeneous (100 pmole/m² TF was immobilized on the surface that appears as a black horizontal line at the top) systems. The rectangle shows the averaging region (all data inside the area were averaged) for light-scattering signal calculation in the homogeneous system; the arrow shows the averaging region (the averaging was performed within horizontal lines) for light-scattering signal calculation in the spatially heterogeneous system. (B) Light scattering of a fibrin clot and (C) thrombin concentration as functions of time in the homogeneous system. (D) Light scattering of a fibrin clot, as a function of time and space, and (E) clot size as a function of time in the spatially heterogeneous system. (F) na thrombin concentration in the spatially heterogeneous system as a function of time and (G) thrombin concentration as a function of time and space in the spatially heterogeneous system. The fibrin clot size is indicated as a point on the thrombin profile for each time point. Typical experiments from n = 3 replicates are shown.
the activator and was then transformed into a thrombin generation curve. After that, we compared the na thrombin curve and the thrombin curve in the homogeneous model, in which the same amount of TF was distributed throughout the whole sample volume. We calculated the same set of parameters as for the homogeneous thrombin generation test: $A_{\text{max, na}}, T_{\text{max, na}},$ and ETP$_{\text{na}}$.

**Computational model of the spatially heterogeneous system**

A computational systems biology model of blood clot formation and dissolution was designed to reproduce the experimental design of Fig. 2, D-G. Simulations with Comsol 5.2 (Comsol, Burlington, MA) were performed in a one-dimensional region that was 6 mm long (Fig. S1). Clot growth was initiated by TF located at $x = 0$. The model equations were reaction-diffusion equations based on the laws of Michaelis kinetics and of mass action. A detailed description of the model equations is presented in Supporting Materials and Methods, Appendix B, Tables S1–S3.

We used a one-dimensional computer model to describe our experimental spatially heterogeneous setup (which is formally three-dimensional) because this in vitro system can be considered as one-dimensional: the surface with TF is flat, so the signal is the same along it (the signal was averaged along the $x$ axis during processing) and across it (the integral scattering/fluorescence signal from the entire thickness of the 1 mm thin experimental cuvette was registered).

The computational model of one-dimensional spatial clot growth was based on a model previously developed and validated by our group (16). Briefly, this model included several distinct modules: 1) binding/dissociation of FVIIa/FVII to TF and feedback activation of FVII; 2) activation of FX and FIX by FVIIa-TF; 3) activation of FII by FXa; 4) feedback stimulation of FVIIa/FVII to TF and feedback activation of FVII; 2) activation of FX and FIX by FVIIa-TF; 3) activation of FII by FXa; 4) feedback stimulation of FVIIa/FVII to TF and feedback activation of FVII; 2) activation of FX and FIX by FVIIa-TF; 3) activation of FII by FXa; 4) feedback stimulation of FVIIa/FVII to TF and feedback activation of FVII; 2) activation of FX and FIX by FVIIa-TF; 3) activation of FII by FXa; 4) feedback stimulation of FVIIa/FVII to TF and feedback activation of FVII.

Briefly, this model included several distinct modules: 1) binding/dissociation of FVIIa/FVII to TF and feedback activation of FVII; 2) activation of FX and FIX by FVIIa-TF; 3) activation of FII by FXa; 4) feedback stimulation of FVIIa/FVII to TF and feedback activation of FVII; 2) activation of FX and FIX by FVIIa-TF; 3) activation of FII by FXa; 4) feedback stimulation of FVIIa/FVII to TF and feedback activation of FVII.

The effect of TF spatial distribution on coagulation in healthy donor plasma

To investigate clotting regulation, we characterized spatial and homogeneous experimental models side by side (Fig. 2). Numerical values of the clotting parameters are given in Table S4. Clotting in the homogenous experimental model was activated by 5 pM homogeneously distributed TF (Fig. 2 A, top row). Light scattering of a fibrin clot as a function of time in normal plasma had a sigmoidal form and reached half of the maximal light scattering in 2.2 ± 0.2 min after activation (Fig. 2 B). Thrombin concentration as a function of time had an asymmetric peak; the thrombin was maximal in 9.3 ± 0.2 min, and the peak concentration was 188 ± 19 nM (Fig. 2 C).

In the spatially heterogeneous model, the total TF was the same as in the homogeneous model (Supporting Materials and Methods, Appendix C), but this TF was localized on a 5 × 1 mm surface with a high (compared with the homogeneous model value) local density of 100 pmol/m² (Fig. 2 A, bottom row). During clot formation in the spatially heterogeneous system, we could distinguish the initiation phase near the activator, where the diffusion processes were very intense because of high concentrations of active factors; even a 100-fold decrease in diffusion rate did not affect clot formation, and the propagation phase did not depend on reactions with TF (9). Fibrin clotting started near the activator in this case and then propagated as a front (Fig. 2 D). Clot size as a function of time is shown on Fig. 2 E. In normal plasma, clotting started 0.7 ± 0.2 min after activation, which was three times faster than in a homogeneous assay activated by 5 pM TF. The clot growth velocity reached 37 ± 3 µm/min. The na thrombin curve is shown in Fig. 2 F. This curve was similar to the curve in Fig. 2 C except for the numerical characteristics: the peak was registered at 1.8 ± 0.2 min and amounted to 225 ± 10 nM. The spatiotemporal thrombin distribution is shown on Fig. 2 G. Thrombin propagated from the activator as a traveling wave; its amplitude at the steady stage was 40 ± 6 nM, which was ~5-fold lower than the peak amplitude in the na thrombin and homogeneous thrombin.

To see how thrombin and fibrin correlate with each other, we combine the information on how the clot boundary and thrombin profiles correlate in one figure (Fig. 2 G). The thrombin concentration at the clot boundary is additionally marked with dots on the thrombin generation curves. The fibrin clot boundary corresponded to the outer (right) slope of the thrombin wave. The concentration of thrombin on the boundary of the clot is not the same during the clot growth but decreases asymptotically to a certain basal level, which is ~10 nM of thrombin (Fig. 2 G).

It is important to emphasize that our homogeneous and spatially heterogeneous models are equivalent in terms of ongoing chemical reactions (some of them occur on lipid surfaces, some in volume) and differ only in the geometry of the location of the TF. Thus, the reactions of FIX and FX activation by extrinsic tenase take place on the lipid vesicles of the TF reagent (Fig. S4). As an additional control, to check whether the observed effects are due to spatial distribution of TF or to some other factor like its changes upon immobilization, we performed a series of experiments in which the clotting activator was a low-temperature agarose gel with TF (Fig. S5; Video S1). Such a gel is impermeable to TF (i.e., TF and lipid microvesicles are localized inside the gel). We used gels with two TF concentrations: 5 and 2000 pM. First, we compared the thrombin generation near the gel boundary and the homogeneous thrombin generation. The na peak of thrombin for a “gel” model with 5 pM of TF corresponds to the peak of thrombin in a homogeneous model with 5 pM of TF activation (Fig. S5 B).
These curves are substantially lower than the curve of the na generation of thrombin in a heterogeneous model with immobilized TF. Moreover, the na thrombin curve for a “gel” model with 2000 pM TF slightly differs from the curve of the na thrombin generation in a heterogeneous model with immobilized TF (Fig. S5 C). Distribution of the clot from the activator in heterogeneous models with gel or immobilized TF was independent of the amount of TF (Fig. S5, D–G; Table S5): the clot growth velocity and amplitude of the thrombin wave are not sensitive to the activation (which corresponds to the definition of the autowave and agrees with our previous results (10)). The fact that in the gel model we receive the very same results as in the spatially heterogeneous model with covalently bound vesicles confirms that the major observed effects are due to the different localization of the activator rather than due to some difference caused by chemical immobilization.

In our spatially heterogeneous model, TF-bearing phospholipid vesicles were immobilized on the surface, unlike in the homogeneous model in which vesicles were free to diffuse. It could possibly change the activation rate (because it can change the rate of complex formation). However, according to (17–19), the immobilization of vesicles larger than 50 nm changes the complex formation rate less than by 10%, and we neglected this difference (Supporting Materials and Methods, Appendix D).

Thus, the localization of TF led to an accelerated formation of thrombin in larger quantities near the activator. In addition, we can observe the qualitatively different form of clotting in this case—clot growth and thrombin propagation (in the form of a traveling wave) in a plasma volume that did not contain an activator (TF).

**The sensitivity of coagulation to factor deficiency as a function of TF distribution**

Further, we characterized the sensitivity of spatial and homogeneous blood clotting and thrombin generation to clinically relevant coagulation factor deficiencies (Fig. 3; Table S4).

In the extrinsic-factor (FVII)-deficient plasma, a very small amount of thrombin (20% of normal, Fig. 3, A and B) was generated near the activator, and the time until the thrombin peak as well as the lag time were prolonged (Fig. 3 B). The propagation phase was normal: a thrombin wave with the normal amplitude propagated from the activation surface. Clot growth velocity stayed within the normal range. In the homogeneous system, neither a thrombin nor a fibrin clotting signal was detected.

In the intrinsic-factor (FVIII, FIX, and FXI)-deficient plasma, the na thrombin curve was close to normal (Fig. 3, C–E). However, in plasma with a deficiency of either FVIII or FIX, thrombin almost did not depart from the activator area, and clot growth velocity was lower than in plasma from healthy donors. In FXI-deficient plasma, the thrombin wave decayed with increasing distance from the activator, and the clot growth velocity decreased with time. In the homogeneous system, the thrombin peak was more than two times lower in factor VIII-, IX-, and XI-deficient plasma than in healthy plasma.

In common-pathway-factor (FV and FX)-deficient plasma, a very small amount of thrombin (2% of normal) was generated near the activator, and the time to the thrombin peak and the lag time were prolonged (Fig. 3, F and G). As in the previous case, no thrombin wave was present, and the clot growth velocity was much lower than in healthy plasma. In the homogeneous system, neither a thrombin nor a fibrin clotting signal was detected.

Clotting therefore occurred in all cases in the spatially heterogeneous model. Different factor deficiencies caused significant changes in different clotting parameters, which can be divided into three groups: prolonged lag time (FVII deficiency), reduced clot growth velocity (FVIII and FIX deficiencies), and both changes at once (FV and FX deficiencies) (Fig. 4, A and B). In thrombin behavior, three different variants were observed: excitation wave propagation (FVII deficiency), wave fading (FXI deficiency) and diffuse distribution (FV, FVIII, FIX, and FX deficiencies) (Fig. 4 C). In cases of deficiency of the vital factors FV, FVII, and FX, the values of the clotting parameters in different plasma samples were widely dispersed (Fig. 4; Table S4). Coagulation might depend on the concentrations of these factors in a threshold-like manner, this threshold being lower than 1% of a factor’s normal concentration.

The mathematical model gave the same results for the spatially heterogeneous system (Fig. S2). We calculated two variants: total deficiency of factor (ideal case) and the deficient factor having 0.1% of its normal concentration (simulation of the experimental conditions; the patient plasma most often had a nonzero mutation, resulting in residual activity of the deficient factor). Here, we observed that the factors V, VII (the concentration of FVIIa was also zero), and X were necessary—with a complete deficiency of them, clotting did not occur at all. In the presence of 0.1% of the deficient factors, coagulation occurred in all cases. In addition, we had the opportunity to exclude the effect of AMC generation in the model with the thrombin-α2 macroglobulin complex, which in part distorted the signal in the experiment (see (10) for details). Calculations showed that thrombin propagated in the form of a wave in all cases (in contrast to experiments, in which we observed the exponential attenuation of the signal with the distance from the activator for FV-, FVIII-, FIX-, and FX-deficient plasma), and only the amplitude and speed of propagation significantly decreased with factor deficiencies.

**The effect of normal plasma addition on coagulation in factor-deficient plasma**

To estimate the concentration of a deficient factor necessary for a system to return to normal clotting, we conducted a
A series of experiments in which we spiked FV-, FVII-, and FX-deficient plasma with different amounts of normal plasma. The results are shown in Fig. 5.

Spiking deficient plasma with normal plasma in the homogeneous system led to the start of clotting, but the ETP did not reach its normal value even in the presence of 5% normal plasma in FVII- and FX-deficient plasma (the ETP value was 40 and 17% of its normal value, respectively; Fig. 5, A and B). ETP was normalized in the presence of 2% normal plasma in FV-deficient plasma. The clotting time was prolonged for all plasma.

In the case of the spatial setup, the thrombin parameters were normalized at 2–5% of their normal plasma levels when FV, FVII, or FX were deficient (Fig. 5 C), but the lag time was slightly prolonged even if 5% normal plasma was present (Fig. 5 D).
Clotting normalization in the propagation phase as estimated by fibrin clot growth occurred (clot growth velocity achieved a value of 70% of its mean normal value) at 0.1–0.2% normal plasma (Fig. 5E).

As the amount of normal plasma in factor-deficient plasma increased, the shape of the spatial thrombin distribution changed from diffuse to a peak. We measured the level of thrombin or the wave peak (if a peak was formed) at the

FIGURE 4 Clotting activation and growth parameters for different factor-deficient plasmas in the spatially heterogeneous experimental system. (A) Lag time, (B) clot growth velocity, and (C) the amplitude of thrombin in blood plasma of 34 patients and healthy volunteers (control). The box plots indicate the following parameters: the mean value (the dot), the 5th and 95th percentiles (the ends of the whiskers), the 25th and 75th percentiles (the bottom and top of the box, respectively), and the median (the horizontal line inside the box). * significantly differs from healthy volunteers, \( p < 0.05 \), t-test.

FIGURE 5 The effect of normal plasma on fibrin clot formation and thrombin generation in homogeneous and spatially heterogeneous experimental systems in coagulation-factor-deficient plasma. (A) Clotting time and (B) endogenous thrombin potential in the homogeneous system; (C) na thrombin endogenous thrombin potential, (D) lag time, (E) clot growth velocity, and (F) the amplitude of thrombin in the spatially heterogeneous system as functions of normal plasma concentration (0, 0.0625, 0.125, 0.25, 0.5, 1, 2, and 5%) in FV- (squares), FVII- (circles), and FX-deficient (downward-pointing triangles) plasma. Data are presented as the mean \( \pm \) SD, \( n = 3 \). Thrombin profiles (profiles from every 10 min are presented) in the spatially heterogeneous system in FV-deficient plasma spiked with (G) 0%, (H) 0.0625%, and (I) 5% normal plasma. Typical experiments from \( n = 3 \) replicates are shown.
end of the experiments in FV-, FVII-, and FX-deficient plasma spiked with normal plasma. In the case of FVII deficiency, the thrombin wave distribution was present in plasma at any FVII concentration, and its amplitude stayed within the normal range. With FV and FX deficiencies, the thrombin wave appeared at 0.1–0.2% normal plasma (Fig. 5, F–I).

Experiments demonstrated that coagulation factor deficiency impacted clot formation and thrombin generation less in the spatially heterogeneous model than in the homogeneous model, indicating that the system’s sensitivity to factor deficiency was lower in the spatially heterogeneous system.

The sensitivity of clotting to prothrombin deficiency as a function of TF distribution

It was not possible for us to study coagulation in plasma with less than 1% FII activity because of lack of patients with such low levels of factor activity. Unlike other factors, the lowest possible activity of FII was thus 2% (we did not work with plasma immunodepleted of coagulation factors). The experimental results of clotting in plasma with 2% FII are presented in Fig. 6 A and in Table S4. In the homogeneous system, clotting occurred with only a small clotting time increase despite the small amount of thrombin generated (8% of normal). In the spatially heterogeneous system, the amount of thrombin was also low, the lag time was prolonged, and the clot growth velocity was close to normal even though the thrombin wave amplitude was very low (18% of normal).

For investigating the clotting with FII levels of 1% and below, we used the mathematical model. The mathematical model showed that the complete absence of FII led to the absence of clotting, but clotting started with even a small amount of FII (Fig. 6, B–D). After we increased the FII concentration, the thrombin distribution took the form of a fading wave (Fig. 6 C) and became a stationary wave at ~30% factor II; the amplitude of the wave was proportional to the concentration of prothrombin (Fig. 6 D).

Extrinsic tenase and diffusion differentially regulate the sensitivity of the coagulation system to factor deficiency

We assumed that the cause of the coagulation system’s low sensitivity to factor deficiency in the spatially heterogeneous model was related to the high local TF density. To test this assumption, we conducted a series of experiments in FV-, FVII-, and FX-deficient plasma in the homogeneous model with different concentrations of TF: 5, 20, 50, 100, and 200 pM (Fig. 7, A and B). Indeed, with increasing TF concentration, the clot began to form, and thrombin was generated in all factor-deficient plasma. In FV-deficient plasma, thrombin was generated monotonously over time without a distinct peak; rather, the signal was spread out over time (Fig. S6).

The role of TF concentration was also indicated by the main clot size increase during the first 10 min in all deficient plasma except FVII-deficient plasma (Fig. 3). We therefore used the mathematical model to investigate in detail the roles of different reactions and diffusion processes. As in experiments, clot formation in factor-deficient plasma was observed during the first 10 min after the clotting onset (Fig. 7 C). Decreasing the TF density by a factor of 20 led to the disappearance of detectable clot formation in
Using the clot growth velocity at 80 min after clotting onset to estimate the impacts of reactions and diffusion on the clotting propagation phase, we found that clotting was greatly slowed when diffusion was decreased and therefore heavily diffusion limited in normal plasma (Fig. 7 F); clotting also remained diffusion limited in most extrinsic-pathway-deficient plasma samples (although the velocity was already decreased with either FV or FX deficiency). Very interestingly, clot propagation velocity at later stages was generally low in FVIII-, FIX-, and XI-deficient plasma, but it was not further affected by a decrease in diffusion, suggesting that this remaining velocity was reaction limited.

In addition, we reduced the diffusion coefficient 100-fold for each active factor separately in different factor-deficient plasma samples and found that only the slowed diffusion of FIIXa significantly changed the growth of the clot. Decreasing the diffusion coefficients for all other factors together, we obtained a comparable effect (Fig. S7). Therefore, not only was FIIXa diffusion important for clot propagation, but the remaining diffusional control was shared by all active factors.

The low critical concentration of thrombin required to produce fibrin was important for clot propagation and its robustness

In the spatial model, the fibrin clot grows rather well and similarly with all types of coagulation factor deficiencies despite differences in the thrombin signal (Fig. 3). We determined the thrombin concentration on the edge of the clot, where fibrin was generated (as shown in Fig. 2 G). We found that in the propagation phase, the fibrin clot formed after the thrombin concentration reached the mean threshold level of $4.1 \pm 2.4 \text{nM}$, whereas in the initiation phase, the level of thrombin at the edge of the fibrin clot was different for plasma with different deficiencies (Fig. 8 C). At the beginning of clotting near the activator, the thrombin concentration at the edge of the clot was high for normal plasma and FVIII-, FIX-, and FXI-deficient plasma (60–100 nM) and significantly less for FII-, FVII-, and FX-deficient plasma (10–20 nM). The reason for this difference was the high rate of thrombin generation and the difficulty in determining the exact moment of clot formation (some time was needed for the polymerization, and we can detect the clot only after that). After some time, the threshold thrombin concentration necessary for clot formation decreased to 1.5–9 nM (the maximal level was for normal plasma) and stayed at this level during the experiment for all factor deficiencies. The only exception was prothrombin- and FX-deficient plasma, in which the same value of the thrombin concentration was observed from the first minutes of clot growth. This result was in line with data on adding thrombin to non-recalcified plasma (Fig. 8 A); even 1–2 nM of thrombin caused plasma clotting within 10 min.

FIGURE 7 The role of extrinsic tenase in the regulation of the coagulation system’s sensitivity to factor deficiency. (A) Clotting time and (B) endogenous thrombin potential in the homogeneous experimental system as functions of TF concentration (5, 20, 50, 100, and 200 pM) in FV- (empty squares), FVII- (empty circles), and FX-deficient (downward-pointing triangles) plasma and the plasma of healthy volunteers (filled squares) are shown. Data are presented as the mean ± SD, n = 3. Mathematical model calculations of the clot size as a function of time in the normal system (control) and a system with a factor deficiency (0.1% deficient factor) activated by (C) 50 pmole/m^2 and (D) 2.5 pmole/m^2 TF, respectively. Mathematical model calculations of clot growth velocity after (E) 10 and (F) 80 min from the beginning of the clot growth with normal diffusion velocity (gray columns) and 100-fold reduced diffusion of active factors (hatched columns).

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FV-, FVII-, and FX-deficient plasma, and the clot propagation velocity in all other cases dropped more profoundly from that in the case with the base TF concentration (Fig. 7 D).

We further compared clot growth velocity at 10 and 80 min after the clotting onset for two cases: normal behavior and 100-fold decreased diffusion of all activated coagulation factors. Under normal diffusion conditions, the initial velocity did not change greatly in the case of coagulation factor deficiency (except for FVII deficiency); it decreased only twofold when the diffusion was decreased (Fig. 7 E). However, decreased diffusion caused sufficient decreases in clot growth velocity in plasma lacking extrinsic pathway factors (FV, FII, FX, or FVII) but not in those lacking intrinsic pathway factors. Therefore, under normal conditions, clotting initiation is limited only by the FII level; a comparable level of clotting inhibition can be achieved only when extrinsic factor deficiency is coupled with hindered diffusion.

The low critical concentration of thrombin required to produce fibrin was important for clot propagation and its robustness
In contrast, computer modeling indicated a perfect linear correlation between the concentration of thrombin in the fibrin wave edge (Fig. 8, B and D) with the clot growth velocity. The insufficient sensitivity of the experimental system to low thrombin concentration did not allow this phenomenon to be measured experimentally, but there was no correlation between the thrombin wave amplitude and velocity in either experiments (Fig. 8 E) or simulations (Fig. 8 F).

The dependence of clot growth lag time and velocity on deficient factor concentration was almost the same without the addition of phospholipids, which are essential for thrombin wave formation (10), as in the presence of phospholipids (Fig. S8), which also indicates that the thrombin wave is not essential for spatial clot growth. The amplitude of the thrombin wave and even the presence of the wave thus barely affected spatial clot growth.

**DISCUSSION**

This study investigated the sensitivity and robustness to clotting factor deficiency of blood coagulation activated by a layer of immobilized TF at a physiologically relevant density (in comparison with homogeneously distributed TF) in a spatial reaction-diffusion experimental model combined with computational systems biology analysis. Our main conclusions include that 1) the spatial propagation of clotting was more robust with respect to coagulation factor deficiencies than homogeneous thrombin generation because of a local high TF surface density, which overcame activation thresholds; 2) the initial stages of clot growth in normal plasma were neither reaction nor diffusion limited, whereas later stages were reaction limited; and 3) the robustness of clot propagation was due to the need of only a low concentration of thrombin at the edge of the clot to form the clot that drove spatial propagation of clotting. We considered robustness as a fundamental feature of a complex system, which allows the system to maintain its function despite any subthreshold external or internal perturbations, in particular, clotting factors’ concentration decreasing.

All data on the sensitivity and robustness to clotting factor deficiency of the spatial clot/thrombin propagation aspect were mostly novel to the best of our knowledge. The majority of previous studies on reaction-diffusion models investigated FVIII, FIX, or FXI deficiency (8,10,20), and there was a single attempt to investigate fibrin formation with some other deficiencies but without determining a concentration dependence or thrombin generation as functions of space and time (8). The role of diffusion of a single factor has not been analyzed, either, except for a single attempt to compare the roles of FIXa and FXa diffusion in normal plasma (9). The picture arising from our study is much more comprehensive and fills numerous previous gaps. According to this study, we can assume that physiological coagulation is initiated by a potent stimulus of TF concentrated at the extravascular cells that generated excessive amounts of coagulation factors that diffused to form a clot and generated more factors far from the activator. Our, to our knowledge, novel additional answer to the ancient question “what is all that thrombin for?” (21) is “for robustness.” Because this excessive coagulation factor generation vastly exceeds what is needed for fibrin formation, blood clotting spatial propagation in a reaction-diffusion system is not sensitive to either a two-order-of-magnitude decrease in any coagulation factor concentration or a two-order-of-magnitude simultaneous decrease in all diffusion coefficients. Thus, we can speak about the following hierarchy of thrombin and its role in the system: 1) 250 nM thrombin is required near the activator for a rapid start of coagulation, 2) the propagating wave of thrombin with an amplitude of ∼50 nM is necessary for the growth of a large clot, and 3) the thrombin concentration of the edge of the fibrin clot determines the velocity of clot spatial propagation.
The relationship of the activating wave amplitude propagating in the active media and the velocity of its propagation in the general case is not established (22). Here, we showed that in blood plasma, the velocity of fibrin clot propagation (transition from liquid state to gel) depended on the concentration of activator (thrombin) on the phase border rather than on the thrombin amplitude. In classical autowaves, the peak propagates in a self-similar manner. This means that the concentrations at all points on the front change proportionally with the change in the peak. However, in coagulation, it turned out that this is not so. The frontal segment of the peak and the concentration at the front and at the maximum are not self-similar. The frontal segment of the thrombin wave (at the clot boundary) decreases more slowly than the peak. So, the peak can disappear altogether, and the clot growing remains (Fig. 3). This is an unusual property not described for other systems.

Our experiments have shown that the localization of TF (heterogeneous model) leads to the propagation of thrombin in the form of an autowave into an area where TF is absent. Regardless of whether TF was immobilized on the activation site or TF-bearing vesicles were distributed in gel, the parameters of thrombin and fibrin distribution depend on the activator properties only in a small region. Far from the activator, all processes are determined only by the properties of the plasma itself (10).

Interestingly, in FV-deficient plasma, thrombin is generated monotonously over time without a distinct peak (Fig. 3 F, Fig. S6), which confirms the role of FV in providing explosive clot growth (23).

For larger clots (at times after 30–40 min and at distances more than 1 mm from the activator), regulation is slightly different. This stage is clearly reaction limited, meaning that diffusion is much more rapid than needed. Within this stage, FIXa diffusion plays a major but not exclusive role; long-lived FIXa diffusion is responsible for ~50% of propagation efficiency, whereas the control of other 50% is somewhat distributed among the other factors. In addition, we can find the same principle of robustness: the amplitude of the thrombin wave (or simply that of thrombin because there is no wave in many of the factor-deficient plasmas) may change far from the activator, but those little quantities of thrombin are sufficient to perform the complete conversion of fibrin. Taken together, these two mechanisms (i.e., vastly excessive TF-dependent thrombin generation near the activator and excessive TF-independent thrombin generation far from the activator) allow the formation of a large solid clot that is mildly affected by deficiency or hindered diffusion of any component. Clot propagation becomes diffusion limited only in patient plasma without critical factors.

Using the mathematical model allowed us to overcome the limitations of real experiments, such as 1) the impossibility of switching off the diffusion of individual proteins and 2) the inability to measure thrombin concentrations less than 1–2 nM. Model simulations were used to identify the role of diffusion of individual components and to determine the relationship between the distribution of thrombin and the growth rate of the clot.

Although this view is novel, to our knowledge, its elements are in good agreement with previous reports. All results of this study in its homogeneous model are well in line with previous reports on the sensitivity of the clotting cascade to coagulation factor deficiency (11,24–33). The decrease in clotting sensitivity to coagulation factor deficiency with the increase in TF activation level is also well documented (34). The importance of our homogenous experiments consisted in accurately repeating the experimental conditions of the heterogeneous model for their adequate comparison.

The two limitations of our study, applicable to all conclusions, are that our factor-deficient plasmas have different (nonzero) concentrations of the remaining factors and that our experimental ability to measure edge thrombin concentrations or modify some parameters (like diffusion coefficients) is limited. As a result, although the majority of the conclusions are based on experiments, some of the missing links could be obtained only from the computer model. Although the model was mechanism based and solidly validated in our previous studies using a significant amount of experimental data (4,5,9,10,16), these aspects require additional validation using other approaches. The third limitation is that we used lipids rather than platelets, which require activation by thrombin, whereas the lipids used represent already activated phospholipid surfaces. In addition, it should be noted that our results are not applicable to every model of bleeding and model of hemostasis. Thus, in (35), it was shown that in patients with hemophilia, mainly the time of the secondary bleeding is prolonged rather than the time of the primary bleeding; this model of the study of secondary hemostasis is often used in the study of bleeding in mice (36). In our model, we cannot separate these processes.

The major issue is the applicability of the conclusions to real-life clotting in vivo. The components in our system do not flow; however, there is evidence that fibrin clot is very attenuated in the presence of even very slow flows (37,38). Clotting within platelet aggregates (where clotting is protected from flow) or in the stasis region would occur similarly as the clotting in this model system; venous thrombosis might be an exception, though some of the aspects of this study might also be applicable to this case. It is cautiously speculated that the revealed properties are fundamentally intrinsic to the clotting cascade and should be applicable to clotting under a wide range of conditions that could adjust these properties but not drastically change them. For example, the well-known hindrance of diffusion in platelet aggregates (39,40) might change the typical spatial and temporal scales wherein the described phenomena are observed. In any case, experimental validation using...
in vivo models is required to confirm and deepen our understanding of clotting network regulation.

Biological systems are fantastically robust, but the nature of this robustness is not always clear. For some of these systems (such as metabolism), the origin of the robustness was identified and proved as a theorem (41). The results of our study might also provide general insight into the surprising robustness of the clotting system and into other spatially nonuniform cascade-controlled signaling networks. The development of diagnostic assays, personalization of therapy, and creation of novel therapeutic agents require understanding such things.

SUPPORTING MATERIAL

Supporting Materials and Methods, eight figures, six tables, and one video are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(18)31230-X.

AUTHOR CONTRIBUTIONS

A.N.B., F.I.A., A.S., and M.A.P. designed the study. A.D.K. performed all experiments except for the factor activity assays, which were performed by R.V. S.C.N. and R.V. recruited the patients and collected the clinical data for the patients. A.N.B. and A.M.S. performed mathematical model calculations. A.D.K., A.N.B., F.I.A., A.S., and M.A.P. analyzed the data. A.D.K. and A.N.B. performed the statistical analysis. A.D.K. and A.N.B. wrote the manuscript. S.C.N., F.I.A., A.S., and M.A.P. edited the manuscript.

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