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Spatial Dynamics of Contact-Activated Fibrin Clot Formation *in vitro* and *in silico* in Haemophilia B: Effects of Severity and Ahemphil B Treatment

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Abstract. Spatial dynamics of fibrin clot formation in non-stirred system activated by glass surface was studied as a function of FIX activity. Haemophilia B plasma was obtained from untreated patients with different levels of FIX deficiency and from severe haemophilia B patient treated with FIX concentrate (Ahemphil B) during its clearance with half-life $t_{1/2}$=12 hours. As reported previously (Ataullakhanov et al. Biochim Biophys Acta 1998; 1425: 453-468), clot growth in space showed two distinct phases: activation and propagation. The activation phase is characterized by the time required to start clot growth from the activator, while the characteristic parameter of the propagation phase is the clot elongation rate. This rate reaches steady state in approximately ten minutes after the beginning of growth. In haemophilia B plasma, clot formation is substantially impaired: clot starts to grow from the activating surface later than in healthy donor plasma, and its propagation rate is considerably lower. The most significant abnormalities in clot growth kinetics are observed at FIX activity below 10% of normal. Simulation of these experiments was performed theoretically using a detailed biochemical model (Panteleev et al. Biophys J 2006; 90: 1489-1500) adapted for experimental conditions used. Suitability of the assumptions used to describe triggering contact activation was verified.

Key words: blood coagulation, intrinsic pathway, haemophilia B, factor IX concentrate, mathematical modelling, autowave process
AMS classification: 92C45, 35K57, 92C50

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1. Introduction

Blood clotting system is a network of biochemical reactions designed to terminate bleeding after blood vessel wall injury. As a result of clotting system activation, polymeric fibrin clot is formed at the place of the vessel damage. Abnormalities of clotting system functioning are lethal: insufficient coagulation leads to bleeding and excessive one results in thrombosis. Complex structure of this system makes prediction of the changes of its behaviour under such abnormalities very difficult.

Clotting system is organized as a proteolytic enzymatic cascade: on each level a catalyst (active factor, see the caption to fig. 1 for nomenclature) of the next level becomes activated and then undergoes rapid inhibition. There are two pathways of clotting activation. Activation by the extrinsic pathway [1] begins when blood comes in contact with tissue factor (TF): this protein is expressed by the majority of cells except those normally being in contact with blood. TF binds with FVIIa, which circulates in tiny amounts (1% of total FVII), making FVIIa able to cleave FIX and FX to their activated forms. Intrinsic activation pathway [2] is initiated by the contact of blood with any 'foreign' material (e.g., splinter, laboratory glassware, inner surface of extracorporeal device). Upon adsorption on this surface, FXII becomes activated due to conformational changes and then stimulates its own formation both autocatalytically and by activating prekallikrein to kallikrein: kallikrein activates its cofactor high molecular weight kininogen (HK) and FXII. Generated FXIIa activates FXI, FXIa activates FIX, and FIXa activates FX. It is thought [2] that FXIIa, kallikrein and FXIa activity is localized on the glass surface possibly because of their relative protection in surface-bound complexes with HK and HKa from inhibition.

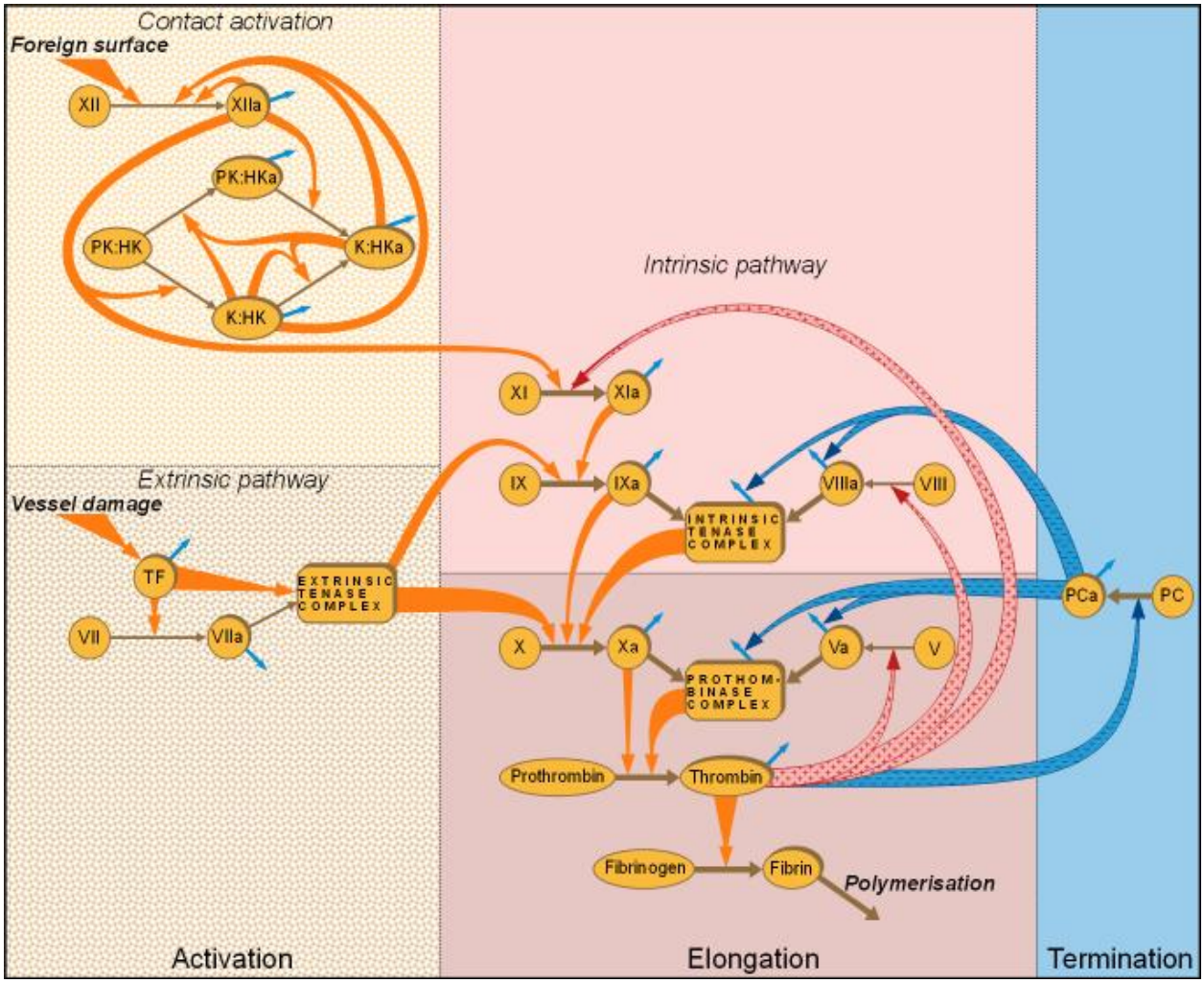

Figure 1. Kinetic scheme of blood clotting system. Roman numerals denote non-activated clotting proteins traditionally called "coagulation factors", roman numerals with "a" index denote activated factors. Tenase and

prothrombinase complexes are denoted by rectangles. For prothrombin, thrombin, fibrinogen and fibrin their commonly used names are given instead of II, IIa, I and Ia, respectively. TF is tissue factor. In contact activation system, PK is prekallikrein, K is kallikrein, HK is high molecular weight kininogen. Straight brown arrows indicate enzymatic reactions, for which catalyst is indicated by thick red arrow. Catalytic positive feedback loops are "+"-indicated, negative feedback loop is "-"-indicated. Plasma stoichiometric inhibitors' action is indicated by short blue arrows.

Both pathways unite at the activation of FX to FXa (fig. 1). FXa cleaves prothrombin to thrombin, the major protein of the system. In addition to cleavage of fibrinogen to fibrin, thrombin controls at least three positive feedback loops via activation of cascade components (FV, FVIII and FXI) located above it in the cascade. Two of these loops lead to the activation of cofactors FVa and FVIIIa thereby increasing FXa and FIXa activities by 10^4-10^5 times via prothrombinase and extrinsic tenase complexes formation. Therefore, upon the initial activation, thrombin concentration increases in a dramatically non-linear manner and, in short time, the whole plasma becomes rapidly triggered from liquid to "solid" (clotted) state due to total conversion of fibrinogen to rapidly polymerizing fibrin (fig. 2A). This scenario takes place in standard laboratory tests, when the activator is distributed throughout the domain (stirred system).

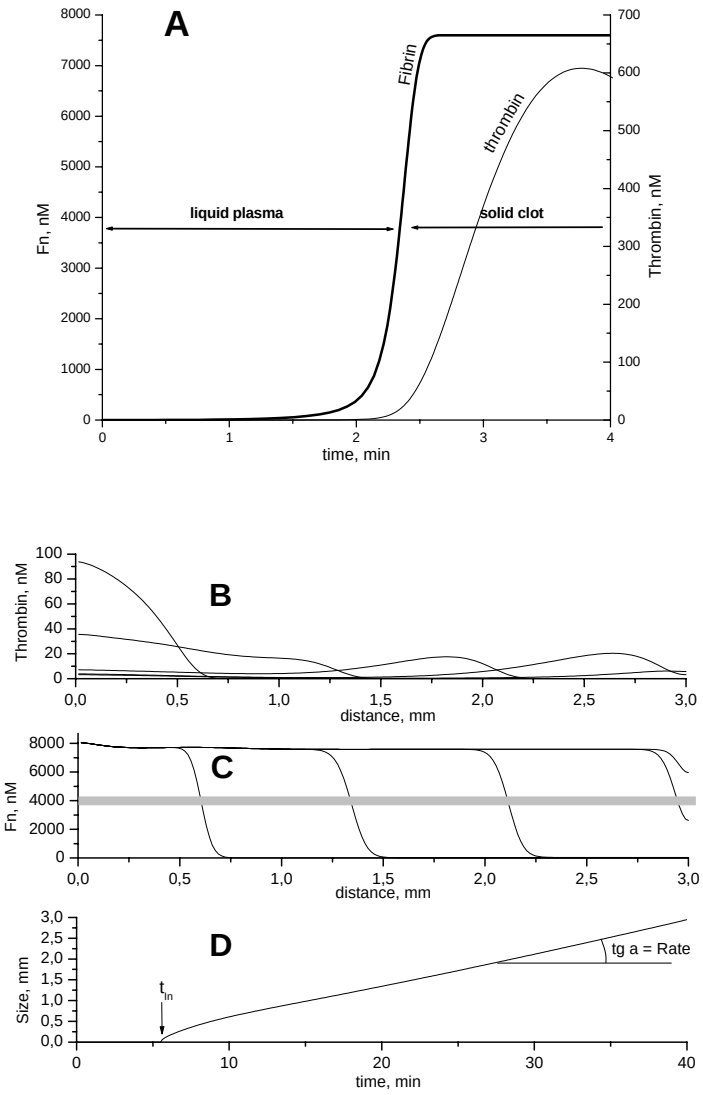

Figure 2. Simulated clotting kinetics in stirred (A) and non-stirred (B-D) normal plasma. (A): thrombin(t) and Fn(t); triggering to clotted state occur upon about 50% Fg conversion to Fn. Non-stirred system: (B) thrombin(x,t) and (C)

Fn(x,t) concentration profiles at times (from left to right) 10, 20, 30, 40 and 50 min; on (C), grey bar corresponding to the half of initial fibrinogen concentration is shown. (D): size(t) curve calculated as the bar length from zero to Fn(x, t) curve (C) at a given time. Simulation (see model description for details) instead of experimental results is presented to illustrate thrombin wave propagation in space. For experiment, see figures 3-6.

In vivo, the clot starts to grow from the site of the vessel wall injury expanding from it into the vascular lumen. Activator (the extrinsic tenase complex composed of transmembrane protein TF and FVIIa) is localized on the damaged vessel wall and it cannot be transported into the bulk, while FIXa and Xa (produced by the extrinsic tenase) can diffuse from the wall. Far from the wall, thrombin activated by FXa cleaves FXI to FXIa thereby starting clotting cascade in this local region via the intrinsic pathway. Thus, the auto-activation of clotting that exists in the bulk of plasma allows clotting to propagate in space in a self-sustaining manner. Fig. 2B and C demonstrate simulated thrombin and fibrin concentration profiles obtained with the complex mathematical model [3] describing the above mentioned reactions.

Our recent studies in recalcified and non-recalcified plasma [4,5] indicated that the spatial aspects of coagulation should be analyzed in spatially non-uniform experimental systems (i.e., without stirring). It is shown that clotting under these experimental conditions is sensitive to congenital deficiencies of FVIII, FIX [6,7] and FXI [3,8], that are associated with abnormal clotting commonly referred as haemophilias A, B and C, respectively. Extremely low concentrations of FVIII, FIX and, to a lesser extent, FXI have been shown to disrupt clot propagation in space.

In this study we examined spatial aspects of blood clotting in haemophilia B both experimentally and theoretically. Experimental investigation was performed in plasma of healthy donors, patients with different FIX clotting activity and patients with severe FIX deficiency ([FIX]<1%) during the infused FIX concentrate clearance and before treatment. For simulations, a detailed quantitative mathematical model of coagulation [3] adapted for the experimental setting was used.

2. Materials and methods of experimental investigation

Preparation of plasma. Healthy donor blood collected into 3.8% sodium citrate (pH 7.4) was centrifuged first at 2400 *g* for 15 min and then at 10000 *g* for 5 min to sediment blood cells. The obtained platelet-free plasma was lactate-treated to stabilize its pH at 7.2–7.6 as described [9]. Subsequently, plasma was supplemented with 10 μM sodium azide and kept at 37°C for 16–18 h to inhibit the coagulation factors activated during blood collection. Immediately before the assay, plasma was recalcified with 20 μl of 1 M $CaCl_2$ per 1 ml. Pooled plasma samples, each prepared from plasmas of three healthy donors, were used in experiments as normal controls. The same procedure was used to isolate and process patient plasma. A total of 12 patients were studied. Factor IX activity was determined by one-stage activated partial thromboplastin time (APTT) assay using a KC4 instrument (Amelung, Germany) and APTT kits from either Renam (Moscow, Russia) or Sigma (USA). Factor IX-deficient plasma (Sigma) was used as a negative standard. The FIX level was less than 1% in eight patients, equal to 1.8% in one patient, 2.8% in two patients, and 5.5% in one patient.

Recording of the spatial clot growth. The spatial dynamics of clot formation was studied in a special polystyrene chamber as described in details elsewhere [5-7]. One wall of the chamber was made from glass serving as activator of coagulation. The chamber was thermoregulated at 37°C and illuminated from below with red-light emitting diodes. Clot growth was monitored by capturing pictures of the light scattering from a 9 × 6.5-mm area with a digital CCD camera (Electrim Corp., USA). The first frame recorded immediately after filling the chamber with plasma was considered to be a background signal, which was later subtracted from all other frames.

Characterizing of the spatial clot growth kinetics. To quantitatively characterize the spatial clotting process we calculated the size of the clot as a function of time from the light scattering profiles. We assumed that the clot size at a given time, Size(t), was equal to the distance from the activator to the point where fibrin concentration (proportional to the light scattering intensity [8]) declined to 50% of the plateau value (fig. 2 C-D). Due to the initial absence of fibrin in the system, Size(t) graph always has a lag-phase where clot size equals to zero (which is approximately the time required for the half-conversion of fibrinogen to fibrin near the activator) and a propagation phase where the clot size monotonically increases with time (fig. 2D). The duration of the lag-phase is the time of clotting initiation (t_{ln}), the slope of Size(t) curve is the rate of clot propagation. In all experiments conducted, this rate became constant in some ten minutes after the clot size exceeded zero (fig. 8 A, B). We calculated this steady, activator-distanced, rate on the last ten minutes (t=50..60 min) linear segment of Size(t) curve.

Changes in the spatial dynamics of clot growth after factor IX concentrate treatment. Effects of a single factor IX concentrate infusion on the FIX coagulation activity in plasma and the spatial dynamics of clot growth were studied over a period of 30 h in five patients with severe haemophilia B ([FIX] < 1%). The factor IX concentrate was a domestic product Ahemphil B [10] developed by the National Center for Hematology (Russian Academy of Medical Sciences). Ahemphil B has passed clinical trials and was approved for routine use in management of haemophilia B. Patients were administered with Ahemphil B at a dose of 200 IU/kg in the absence of bleeding episodes and no earlier than 7–10 days after the previous administration (against an undistorted background). Blood samples were withdrawn before and in 15 min, 30 min, 1 h, 3, 6, 9, 12, 24, and 30 h after Ahemphil B administration.

3. Mathematical simulation of blood clotting

The mathematical model used in this study was a modification of a previously published one [3], which described clotting activated by both extrinsic and intrinsic pathways in homogeneous and spatial-distributed conditions and was verified by extensive comparison of the simulation results with experiments. It consisted (in the homogeneous case) of 27 ordinary differential equations (ODEs)

$$\frac{dc_i}{dt} = W_i(\bar{c}) \tag{3.1},$$

where $\bar{c}$ was a vector of all clotting factors, their inhibitors and platelets concentrations, $\overline{W}$ was a vector of their total conversion rates. In the spatial case, diffusion terms were added to 24 equations in the bulk of plasma:

$$\frac{\partial c_i}{\partial t} = D_i \frac{\partial^2 c_i}{\partial x^2} + W_i(\bar{c}) \tag{3.2},$$

where $\overline{D}$ was a vector of diffusion coefficients. The rest 3 ODEs described reactions on the activating via the extrinsic pathway surface:

$$\frac{d\sigma_i}{dt} = w_i(\bar{\sigma}, \bar{c}_{|x=0}) \tag{3.3}$$

where $\bar{\sigma}$ was a vector of TF, TF-FVIIa and TF-FVII complexes surface densities, w_i was a total rate of σ_i conversion. Coupling of bulk PDEs (3.2) with surface ODEs (3.3) was introduced by means of 8 non-zero Neumann boundary conditions conveyed the equality of the surface reaction rate $\tilde{w}_i$ and the corresponded flux of bulk reagent or product through this surface:

$$-D_i \frac{\partial c_i}{\partial x}|_{x=0} = \tilde{w}_i(\bar{\sigma}, \bar{c}_{|x=0}) \tag{3.4}$$

The full set of equations is available online in the supplement materials for the paper [3] at *http://www.biophysj.org/cgi/content/full/biophysj.105.069062/DC1*

Activation solely from glass in our experimental system denotes switched-off extrinsic pathway. Our experimental set-up had two distinct sources of intrinsic pathway activation: one

from glass activator surface and another one from the polystyrene dish surface. The latter was described in [3] assuming constant surface-bounded FXIa density throughout the domain, which gave

$$W_{IX} = -k_{9,V} \cdot [IX] - \ldots \quad (3.5a)$$

$$W_{IXa} = k_{9,V} \cdot [IX] + \ldots \quad (3.5b)$$

where $k_{9,V}$ was a first-order kinetic constant with dimensionality of 1/min. Dots in (3.5) are used to denote non written out terms describing activation of FIX to FIXa by not-bounded to the surface FXIa and inhibition of FIXa.

To describe activation from glass, we replaced the block of extrinsic pathway reactions (3.3),(3.4) by 2 boundary conditions

$$-D_{IX} \frac{\partial}{\partial x}[IX]_{|x=0} = -k_{9,S} \cdot [IX]_{|x=0} \quad (3.6a)$$

$$-D_{IXa} \frac{\partial}{\partial x}[IXa]_{|x=0} = k_{9,S} \cdot [IX]_{|x=0} \quad (3.6b)$$

In (3.6) $k_{9,S}$ is a first-order surface kinetic constant with dimensionality of mm/min. Potency of the contact activation is unique for every surface, and thus we had to estimate kinetic constants both for glass ($k_{9,S}$) and polystyrene ($k_{9,V}$) surfaces. All other constants in the model [3] were from experimental reports, and no adjustment was done.

4. Results

4.1. Clot shoots up with constant rate in healthy donor plasma. Contact activation of plasma by glass led to the formation of a dense fibrin clot that scattered more light than the liquid plasma [4]. An example of a sequence of images of a growing clot is shown on fig. 3. The light scattering intensity profiles were obtained along the straight line marked as (A) in the last image. Figure 4A shows the corresponding family of light scattering profiles at shorter (2 minutes) time intervals.

In all normal plasma samples, fibrin reached substantial concentration near the activator in 5-10 minutes after the plasma had been brought in contact with the activator. After this, the clot began to expand farther from the activator into the bulk of plasma. The shape of the growing clot resembled a "step" function: a plateau (maximal intensity of light scattering) in the area adjacent to the activator and an abrupt drop in the scattering at the clot–liquid plasma interface (fig 4A). This shape and rate of clot propagation remained constant throughout the experiment if no spontaneous clots formed in the bulk of plasma.

Formation of spontaneous clots is a typical feature for healthy donor plasma [4]. Their formation did not depend on the presence of the activator. They arose randomly, at any point of the chamber, in some 15-25 min after the activator-induced clot growth started. On fig. 3 clot near the activator can be seen starting with the third image, and spontaneous clot – starting with the sixth image. If a spontaneous clot formed on the way of the clot growing from the activator, the two clots fused (last 3 images on fig. 3). Light scattering profiles for this case are shown on fig. 4B.

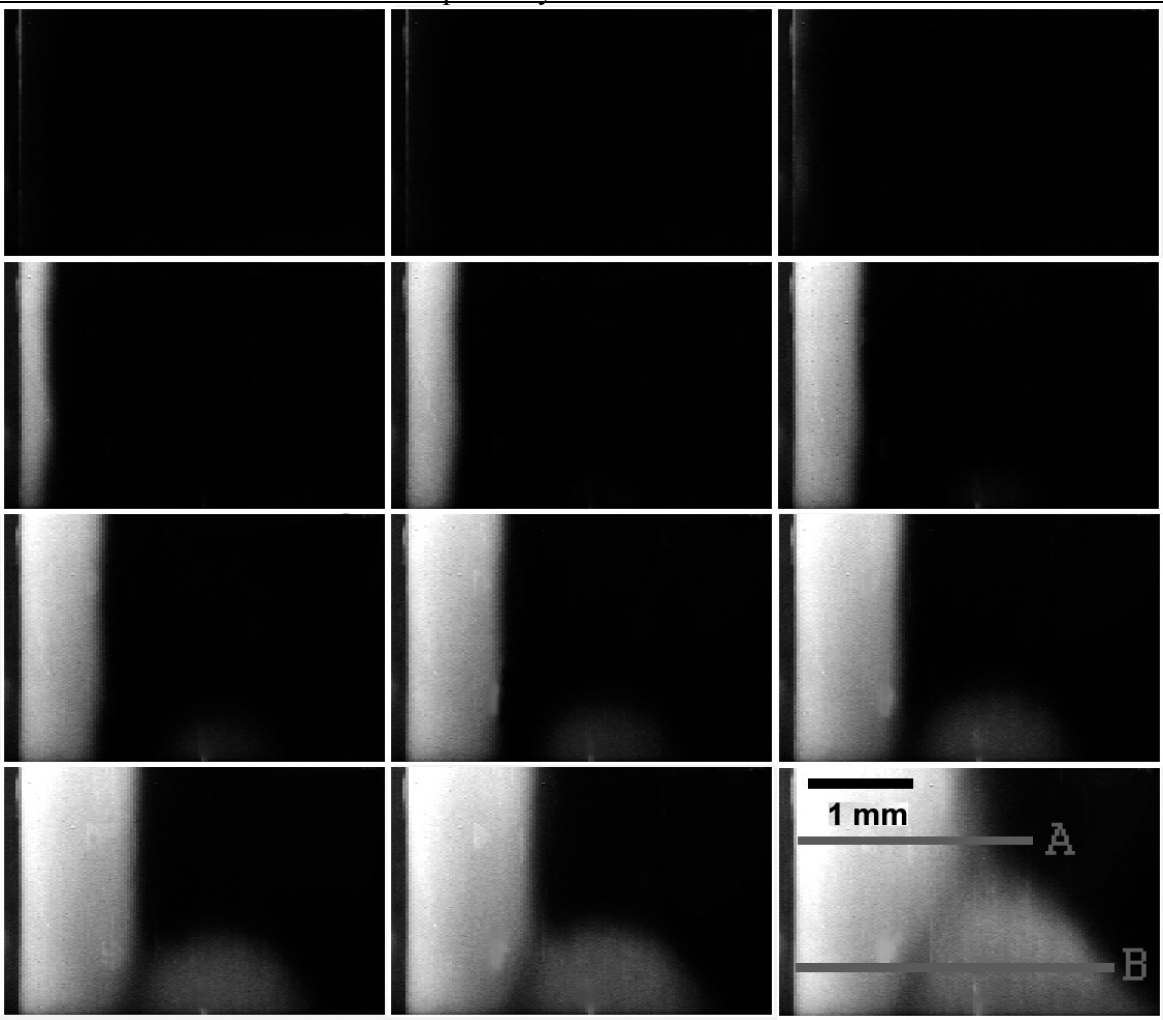

Figure 3. Spatial dynamics of clot growth in healthy donor plasma activated by contact with the glass surface (leftmost vertical line in each frame). In images, clot is light, and liquid plasma is dark. The first frame was taken 2 min after the recalcification, the subsequent at 4 min intervals. The last frame corresponds to 46 min after recalcification. The light scattering intensity profiles on fig.4 A and B were obtained along the straight lines indicated on the last frame.

4.2. Clot formation is substantially reduced in haemophilia B plasma. In the plasma of patients with severe haemophilia B (FIX activity < 1%) spatial clot formation was heavily impaired (fig. 5A). The clot started to grow from the activating surface later than in healthy donor plasma and its propagation rate was considerably lower (empty markers in fig. 8A and B). The light scattering profiles, in contrast to normal plasma, rapidly declined without a plateau with the increase of distance from the activator. Throughout the experiment (1 h), the patient plasma remained free of spontaneous clots.

The effect of FIX activity can be studied by comparing clotting in plasma of patients with haemophilia B having different severity of FIX deficiency (fig. 5 A-D) and/or clotting in plasma of patient treated with FIX concentrate before administration and during its two-day clearance from blood (fig. 6 A-D). As figure 5 shows, the more FIX clotting activity was, the more light scattering profiles (fig. 5) resembled those in normal plasma (fig. 4A). The corresponding kinetics of clot growth also tended to become normal with the increase of FIX activity (fig. 8A). Similarly, profiles distorted by severe haemophilia B (fig. 6A) were nearly normal just after Ahemphil B administration (compare fig. 6B with fig. 4A). During subsequent clearance of Ahemphil B (this pharmacokinetics is shown on fig. 7) spatial clot growth became more and more distorted (fig 6. B-D). Corresponding spatial clot growth kinetics finally returned to its original (before treatment) form (fig. 8B).

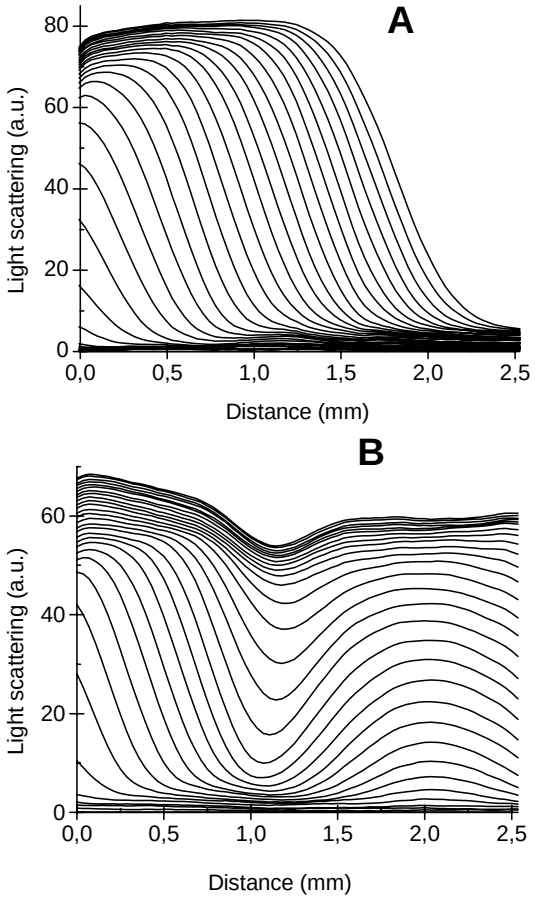

Figure 4. Clot grows away from the glass surface in a healthy donor plasma. Curves are light scattering profiles at 2-minutes time intervals. A: no spontaneous foci of activation on the way of the clot; B: interaction with a clot spontaneously formed at a 2-mm distance from the activator.

The parameters of clot growth kinetics in plasmas with different levels of FIX activity from fig. 8 A and B along with their experimental errors are summarized in figure 9 (points), where it can be seen how the dynamics of clot growth depends on the FIX activity. The smaller the activity, the longer the time to the onset of clot growth (t_{In}) and the smaller the propagation rate and final (at time = 1 hour) clot size.

4.3. Mathematical model agrees well with experimental results. The experiments conducted were simulated using the complete biochemical model of clotting in non-stirred plasma. Due to specific surface activity of every laboratory glassware kinetic constants of contact blood activation by glass $k_{9,S}$ and by polystyrene $k_{9,V}$ were unknown. Estimation $k_{9,S}=5\cdot10^{-4}$ mm/min was obtained by fitting the model t_{In} to the experimental value (about 5 min) in normal plasma (filled squares on fig. 8). Similarly, $k_{9,V}=1.5\cdot10^{-5}$ min^{-1} was estimated by fitting the model's spontaneous clotting time far from the activator to mean experimental spontaneous clotting time in normal plasma (about 40-50 minutes, fig 8). This activator-distanced spontaneous clotting was alternatively modeled in homogeneous approach, i.e. system with full stirring, the same $k_{9,V}$ and $k_{9,S}=0$, giving similar clotting time.

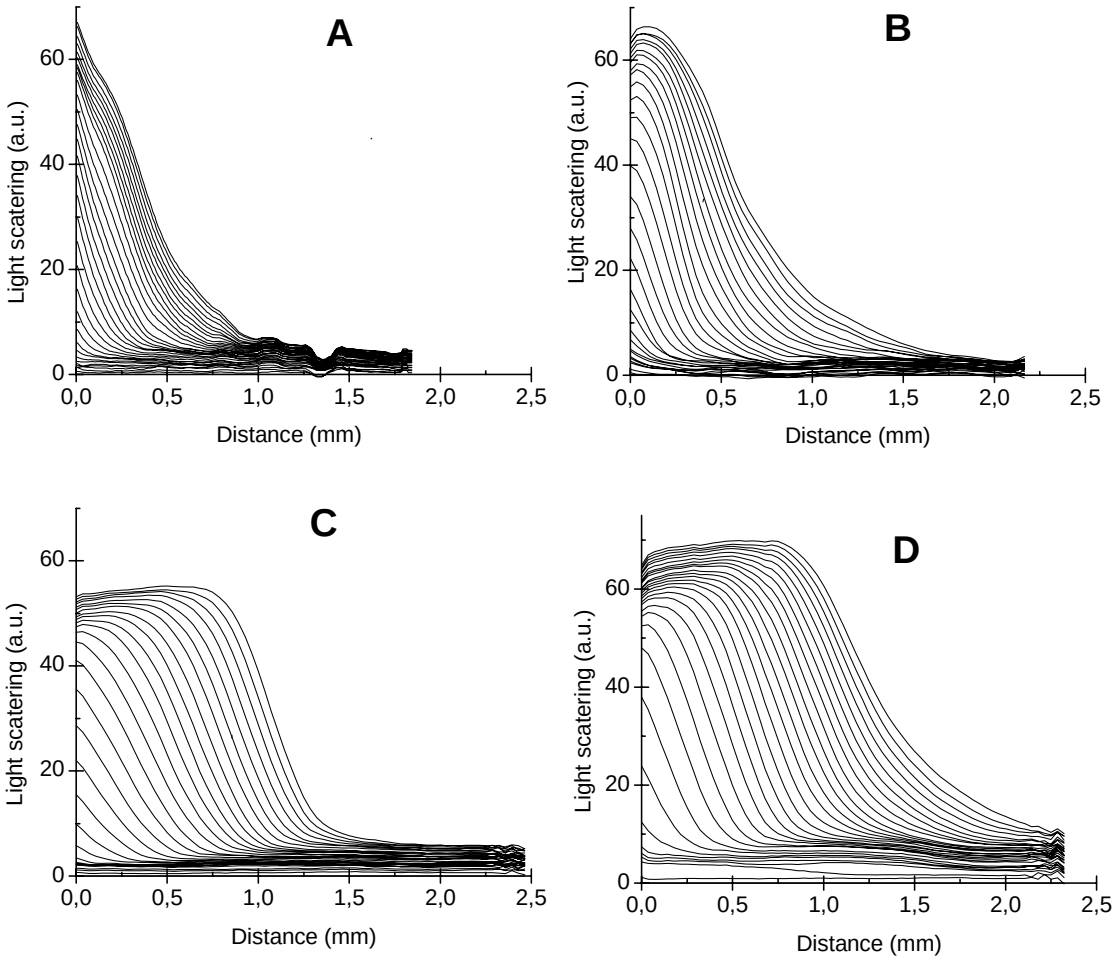

Figure 5. Clot growth from the glass surface in plasma of patients with (A) less than 1%, (B) 1.8%, (C) 2.8%, and (D) 5.5% of normal FIX clotting activity. The first curve was recorded in less than 1 minute after the start of the experiment; all other curves were recorded at a 2-minute intervals.

The entire range of FIX activity from 100% down to 1% was studied. Figure 8C shows calculated clot growth kinetics in normal and haemophilia B plasmas. The parameters of these curves (t_{ln}, Size at t=1h, Rate at t=50..60min) were calculated just as experimental ones were (figure 9, solid lines). A remarkable agreement with the experimental data was obtained. The one exception was a slightly overestimated growth rate at t=50..60 min in the model compared to the experiment at [FIX]<3%. This was caused by faster initial, at $t \geq t_{ln}$, clot growth at these FIX concentrations in the model (two rightmost curves in fig. 8C). Therefore, activator-distanced rate of clot growth was determined at increasing times (dash and dot lines on fig. 9C). Steady rates of clot growth in the model and in the experiment were in good agreement.

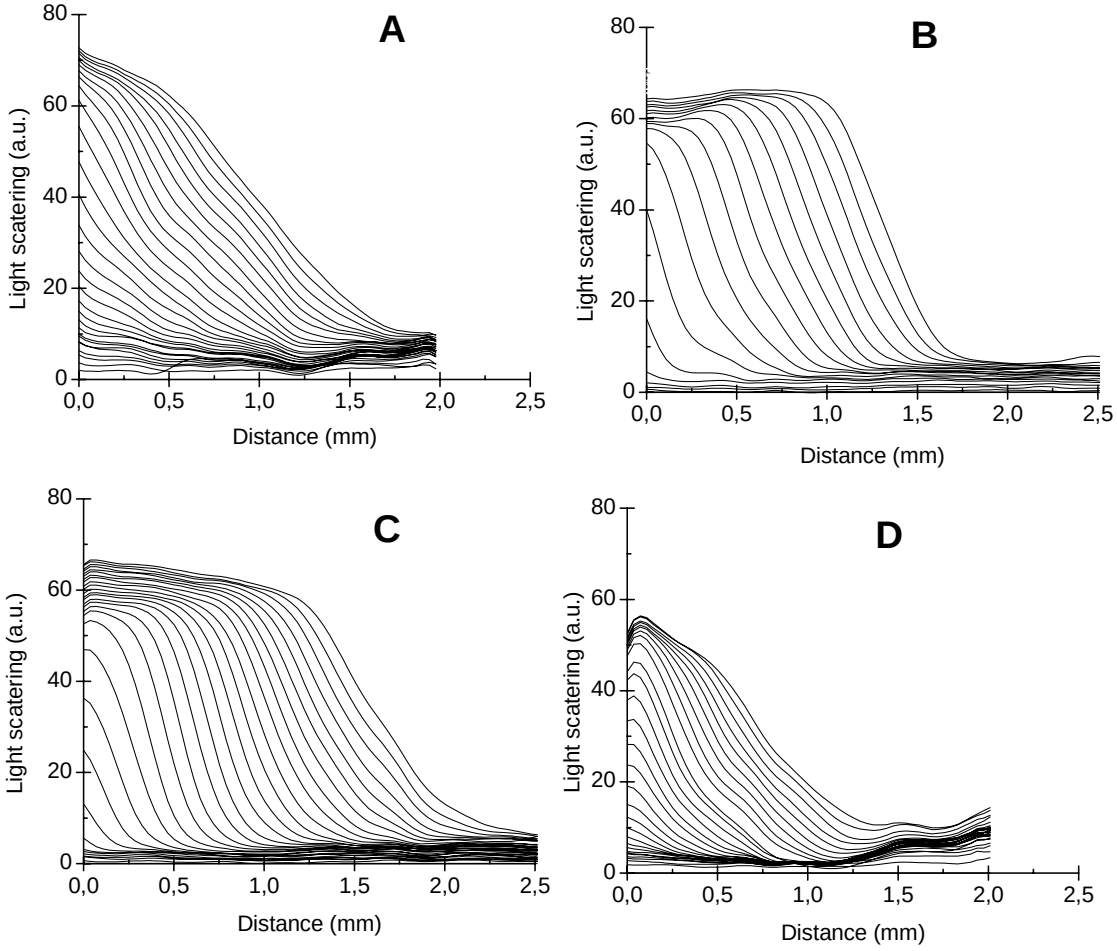

Figure 6. Clot growth in patient plasma taken (A) before and (B) 0.5, (C) 21, (D) 48 h after a single administration of Ahemphil B (200 IU/kg). On each panel, the first curve was recorded in less than 1 min after the start of the experiment; all other curves were recorded every 2 min after the first one.

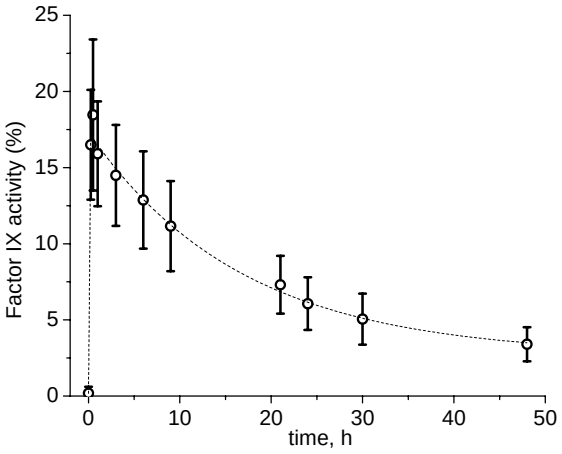

Figure 7. FIX clotting activity (percentage of the norm) before and after the single dose (200 IU/kg) of FIX concentrate Ahemphil B administration. The data are means for five patients with the basal FIX level less than 1%. Their FIX level increased to 18.5 ± 5% in 30 min after administration and then exponentially decreased ($t_{1/2}$ =12h) to 3.5 ± 1.1% over the subsequent 48 h.

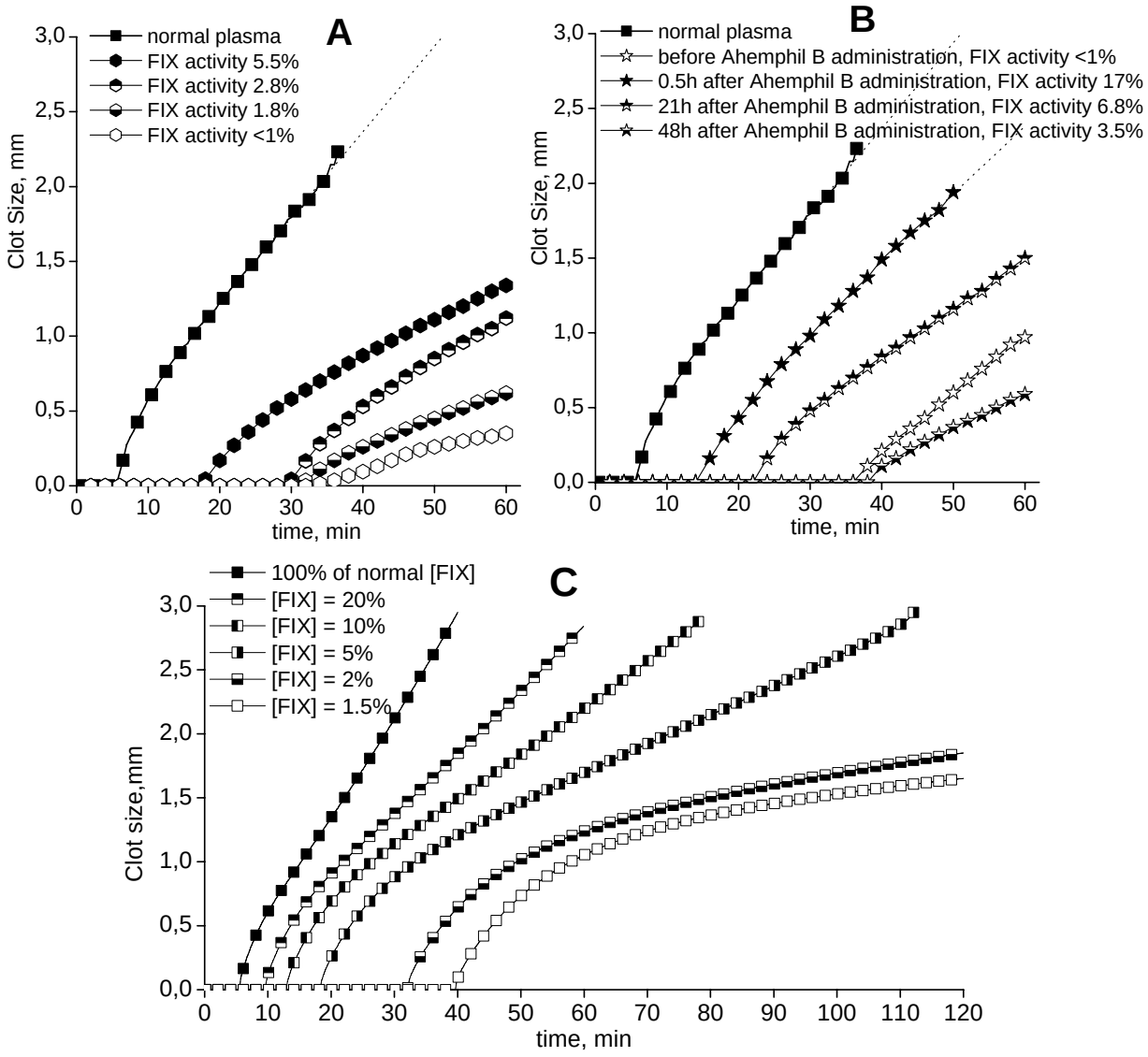

Figure 8. Influence of FIX clotting activity on clot growth kinetics in glass-activated plasma in experiment (A, B) and model (C). Note the lag-phase (clot size=0) followed by the growth phase. Experimental curves are extrapolated by the dot lines if the spontaneous clotting didn't allow to determine the clot size at t=60 min.

5. Discussion

It is a common practice to study clotting activity in homogeneous systems, i.e. systems with full stirring. However, clot growth *in vivo* is a principally spatial process: it is normally localized near the place of injury while uniform clotting throughout the vascular system is incompatible with life. Consequently, focusing on the spatial aspects of clotting allows us to make a principal step from homogeneous studies to the situation *in vivo*.

Three different phases in clot growth can be distinguished: initiation, propagation (or growth) and termination [4]. During the initiation phase, products (active factors) of reactions proceeding on the activation surface or near it accumulate in this region. These are FIXa, FXa and thrombin if the extrinsic pathway is activated or FXIa, FIXa, FXa and thrombin in the case of the intrinsic pathway activation (fig. 1). In both cases, accumulated thrombin activates FXI of the intrinsic pathway, thereby increasing its own production further independently of the activation signal. This positive feedback loop allows clotting to propagate from the activator in a self-sustaining manner, essentially resembling the autowave propagation in active media [4,11].

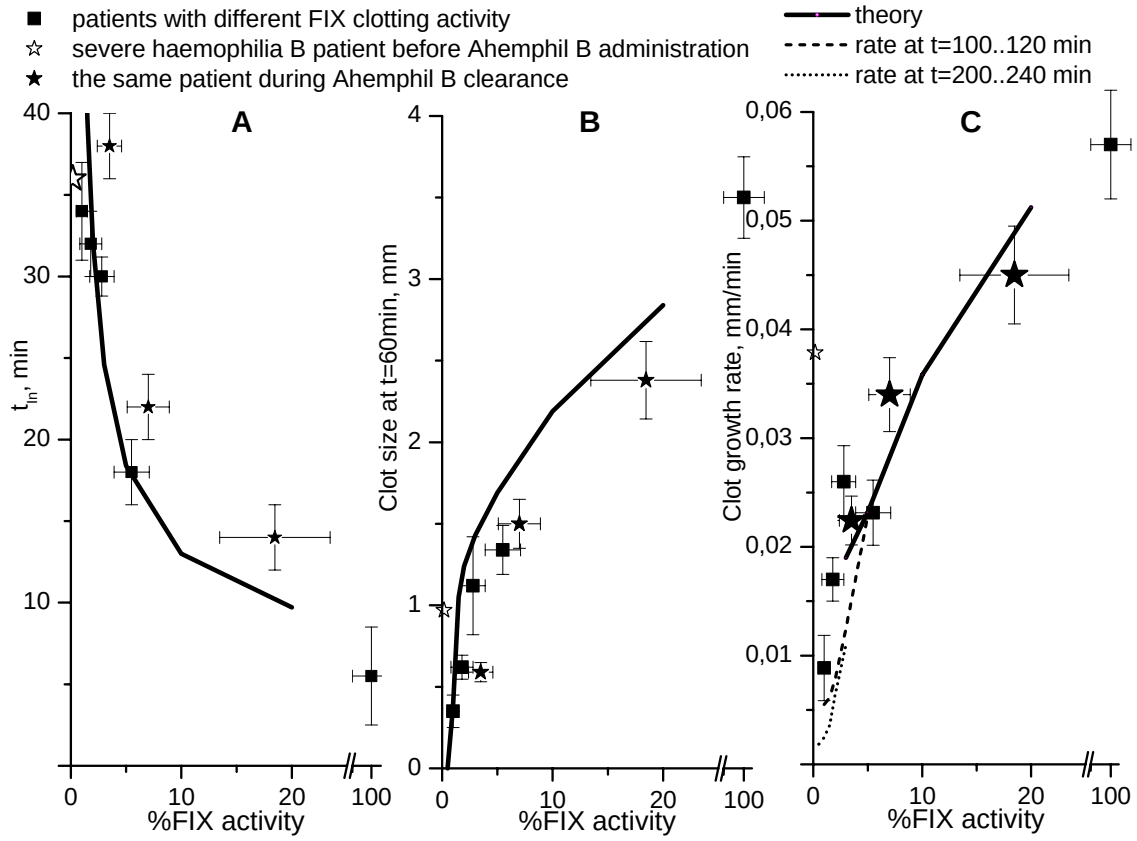

Figure 9. Parameters of clot growth kinetics in experiment (points) and model (lines): (A) time of clot growth initiation t_{in}, (B) clot size at t=60min, (C) activator-distanced rate of clot growth. For experiment, error bars are shown. Solid, dashed and dotted lines show rate calculated further and further from the activator: at time intervals 50..60, 100..120 and 200..240 min, respectively. Combined these lines show the steady clot growth rate in the model.

As can be seen in fig. 8 A-B, in the *in vitro* experiment the rate of clot growth becomes steady in approximately 10 minutes after the fibrin front began propagation. At this time fibrin concentration profile also sustains its shape (fig. 4A). Simulation shows that thrombin concentration profile also becomes steady after the initial phase completion (fig. 2B). Thus, an auto-wave-like regime of spatial propagation of clotting settles in some distance from the activator [4,11].

Severe deficiency in any of the components of intrinsic pathway (haemophilia A, B or C if FVIII, FIX or FXI is absent) affects the course of initiation phase if clotting was initiated by the intrinsic pathway and propagation phase if it was initiated by any of these two pathways [6]. Physiological activation proceeds via the extrinsic pathway (TF-bearing cells contacting with blood upon the vessel wall injury), so the effect of this deficiency just on the propagation phase is the most critical for situation *in vivo* [6,7].

In this study, we examined the effect of FIX deficiency on the spatial clotting dynamics in the range of 1-100% of the normal value of FIX activity. Plasma used was obtained from haemophiliacs with different extent of the disease and from severe haemophiliacs treated with FIX concentrate (Ahemphil B) before administration and during its subsequent clearance. Results obtained show that the most pronounced changes in clotting kinetics occurred at FIX activity less then 10% (fig. 9, points).

To simulate these experiments, a previously published mathematical model [3] was adapted to the clotting condition used, i.e. activation by glass instead of TF-bearing surface. Detailed description of this activation would be extremely difficult due to complexity and lack of kinetic data for surface-dependent reactions of intrinsic pathway activation. It was found that active

forms of contact factors were localized on the activating the intrinsic pathway surface or near it [2]. This allowed us [3] to mimic small artificial contact activation from plastic material of the experimental chamber assuming that constant amount of FXIa per surface area unit was located on the chamber top and bottom surfaces and cleaved FIX to FIXa with effective kinetic constant $k_{9,V}$ (3.5). It is unknown whether this assumption could be applied to the initial chamber wall-localized clotting activation by glass. In this study the description of intrinsic contact activation was extended to the glass activating surface composed one wall of the chamber (3.6), and this hypothesis was tested.

Kinetic constants $k_{9,S}$ and $k_{9,V}$ were determined separately by simulating clotting in normal plasma. Further, good agreement with experiment was found in the entire range of FIX activities when spatial clotting in haemophilia B was simulated (fig. 9). This agreement was in the duration of lag-phase, rate of clot growth far from the activator (these parameters separately characterize the initiation and propagation phases) and clot size at the end of experiment (1h). Nevertheless, one can see that some difference of the initial rate of clot growth (at $t \geq t_{In}$), i.e. in the very beginning of the propagation phase, remained in the case of severe haemophilia B (compare panel C with A and B on figure 8). It is likely that constant FXIa surface density assumption is appropriate for initiation and propagation phases taken separately and for the whole clotting process only if FIX activity is above 3%. Transition clotting kinetics description in severe haemophilia B requires taking into account intrinsic pathway components surface kinetics.

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