Effect of factor VIII on tissue factor-initiated spatial clot growth

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Summary

Using time-lapse videomicroscopy, we studied the role of coagulation factor VIII (fVIII) in tissue factor-initiated spatial clot growth on fibroblast monolayers in a thin layer of non-stirred recalcified plasma from healthy donors or patients with severe Haemophilia A. Analysis of temporal evolution of light-scattering profiles from a growing clot revealed existence of two phases in the clot growth-initiation phase in a narrow (0.2 mm) zone adjacent to activator surface and elongation phase in plasma volume. While the initiation phase did not differ in normal and haemophilic plasmas, the rate of clot growth in the elongation phase in haemophilic plasma constituted only 30% of that in normal plasma. Supplementation of haemophilic plasma with 0.05 U/ml fVIII restored the normal clot growth rate (44.9 ± 2.5 μm/min) at high but not at low fibroblast density. Our results indicate that the functioning of the intrinsic tenase complex is critical for normal spatial clot growth.

Keywords

Factor VIII, Haemophilia A, spatial clot growth, fibroblast, time-lapse light-scattering videomicroscopy

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Introduction

Factor VIII (fVIII) is an essential component of the blood coagulation cascade, since its congenitally or acquired low activity in blood is associated with a tendency to severe bleeding, Haemophilia A (1). It is acknowledged that initiation of blood clotting is mediated by the extrinsic pathway in which a complex of tissue factor (TF) with activated factor VII generates small amounts of activated factor X (2-5). On the other hand, the intrinsic tenase complex consisting of membrane-associated activated fVIII (fVIIIa) and activated factor IX (fIXa) was shown to maintain the clotting process *in vitro* by generating activated factor X (fXa) approximately 50 times more efficiently (6-11). When clotting is initiated via the extrinsic pathway, there are two ways of fIX activation: (i) by the fVIIa:TF complex (6, 12, 13) or (ii) by fXI activated by thrombin (7, 14). At present, however, it remains unclear in what situations each of these pathways of intrinsic tenase formation is operative and what their relative contribution is.

Analysis of accumulated experimental data and mathematical modeling of the spatial dynamics of blood coagulation (15, 16) led us to hypothesize that fVIII (or fIX) may be a limiting factor in clot formation if it proceeds in two spatially separated phases. The initiation phase takes place on the activa-

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tor surface and in a narrow adjacent layer (*in vivo*, at the exposed subendothelium at the site of vessel injury (2-4), from which the nascent clot advances into the bulk where none of the factors that have initiated clotting is present (15, 16). The clotting expansion away from the vessel wall (the elongation phase) is likely to be driven by assembly of the intrinsic tenase complex using fIX activated by either (i) fVIIa:TF complex or (ii) fXIa produced by thrombin activation of fXI.

Recently, we have compared the spatial dynamics of clot formation in normal plasma and plasma of patients with severe forms of Haemophilia A and B using a novel experimental system that allows continuous monitoring of the clot growth in a thin layer of non-stirred recalcified plasma (17). When clotting was initiated by contact with a fibroblast monolayer carrying TF on its surface, the rate of clot growth in patient plasma constituted one-quarter to one-half of that in normal plasma, indicating the role of the intrinsic tenase in the clotting expansion. In the present study, we used this experimental system to examine the dependence of the clot growth rate on fVIII content in plasma and on TF concentration on the activating surface.

Materials and methods

Subjects

A total of 21 plasma samples from patients with severe Haemophilia A (fVIII ≤1% of the normal level as determined by the one-stage fVIII:C assay) were studied. All patients had a history of frequent bleeding episodes and suffered from arthropathy of large joints (stages II-IV). The sampling time was chosen according to the ISTH recommendation (18). Blood (13.5 ml) was drawn no earlier than 4-5 days, usually 7 days or more, after the last administration of fVIII-containing blood products. All patients were assayed for the presence of fVIII inhibitors [the Bethesda method (19)] in the year preceding the experiment. One patient had a high fVIII antibody titre (20 Bethesda Units; BU); others exhibited no inhibitors (less than 0.5 BU).

A total of 11 pools of normal plasma were prepared, each pool containing plasma from two or three healthy donors with no history of coagulopathy. Their activated partial thromboplastin time (APTT) and prothrombin time (PT) values were within the normal range (28-40 s and 15-18 s, respectively).

Blood collection and plasma preparation

Plasma was obtained and treated as described elsewhere (17) with some minor modifications. Briefly, patient or normal blood was collected into 3.8% sodium citrate (pH 5.5), with a blood to anticoagulant ratio of 9/1. The blood was centrifuged at 3000 × g for 25 min. Platelet-free plasma was obtained by centrifuging the supernatant at 10,000 × g for 5 min. The pH value of the platelet-free plasma was stabilized at 7.2–7.6 by lactic acid treatment (20). All the experiments were performed on freshly prepared plasma kept at room temperature within 24-36 h after blood collection. As we previously showed, no changes in the plasma clotting ability (as evidenced by APTT, PT and clot growth rate values) occur within this time period (17). Freezing/thawing and cooling was avoided to prevent cold promoted spontaneous clotting (21, 22).

Experiments with the corn trypsin inhibitor

The fXIIa inhibitor (corn trypsin inhibitor, CTI) was prepared according to the procedure described by Hojima et al. (23) with minor modifications. To suppress contact activation, 0.2 mg/ml CTI was added to freshly prepared normal or patient plasma 2 h prior to the experiment. At this concentration, CTI efficiently inhibited fXIIa in normal plasmas, as evidenced by 2-2.5-fold prolongation of their APTTs.

Factor VIII

Plasma-derived fVIII was purified from a commercial AHF concentrate as described elsewhere (24). FVIII solution at a concentration of 1.59 μg/ml was prepared in 20 mM HEPES containing 0.15 M NaCl, 5 mM KCl, 5 mM $CaCl_2$, and 0.01% Tween 20, aliquoted and kept frozen until use in the experiments. The specific activity of this fVIII preparation was 1600 U/mg.

Supplementing human plasma with exogenous fVIII

FVIII solution (1.59 μg/ml, or 2.5 U/ml) was added to freshly prepared haemophilic plasma (40 or 20 μl per ml) to a final concentration of 0.1 and 0.05 U/ml, respectively. These supplemented plasmas were serially diluted (each time twofold) with patient plasma to give from 0.1 to 0.000078125 U/ml FVIII. The dilutions and plasma containing no added factor were kept under the same conditions (room temperature) in Eppendorf tubes until measurements. Cooling was avoided to prevent cold promoted spontaneous clotting (21, 22).

Activated partial thromboplastin and prothrombin time tests

Each patient plasma or NPP was characterized using the prothrombin time (PT) and activated partial thromboplastin time (APTT) tests. The PT reagent (Renam, Moscow, Russia) was a lyophilized rabbit brain extract. The APTT kit (Renam, Moscow, Russia) consisted of a light fraction of kaolin and membrane phospholipids from outdated human erythrocytes. Both clotting times were recorded using a computer-assisted Biola laser photometer (model #320LA, Biola Ltd., Moscow, Russia). With these reagents, the PT and APTT normal ranges were 15-18 and 28-40 s, respectively.

Fibroblast culture

A human fetal lung fibroblast line was from the Research Institute of Virology (Russian Academy of Medical Sciences,

Moscow, Russia). Cells (10^5/ml) were grown on polyethylene terephthalate (PET) film slips (Joint Institute for Nuclear Research, Dubna, Russia) as described elsewhere (17). In most experiments, spatial clot growth was initiated by contact of plasma with confluent monolayers with an average cell density of 1500 cells/mm^2. In some experiments, very sparse monolayers (5-50 cells/mm^2) were used to activate clotting. They were obtained by decreasing the seeding density from 10^5 cells/ml (confluent monolayers) down to 10^3 cells/ml. Sparse monolayers appeared as single fibroblasts randomly scattered over film slips. For each plasma, control experiments were conducted where plasma without exogenous fVIII was used and clotting was initiated by contact with PET slip without fibroblasts. Clotting started 35-70 min after recalcification in five patient plasmas and later than 1.5 h in other 15 patient plasmas. In normal plasma, clotting on PET started 7-15 min after beginning of the experiment.

Measurements of spatial clot growth

The experimental system used was described in detail elsewhere (17, 25). Briefly, a microchamber was assembled in a 40-mm polystyrene Petri dish (Medpolimer, St. Petersburg, Russia) around a 1-mm-thick glass slide (VWR Scientific Inc., PA, USA) fixed to its bottom. The glass slide edge, which formed a vertical wall of a chamber, was wrapped around with a cell-coated PET slip preliminarily rinsed twice in Hanks solution. In control experiments, PET slips without cells were used to activate clotting. All experiments were conducted at 37° C. Plasma was recalcified by adding $CaCl_2$ and was rapidly transferred into the assembled chamber (time $t = 0$). The final concentration of free calcium was 1-1.2 mM according to measurement using AVL 984-S electrolyte analyzer (AVL Scientific corp., GA, USA). The spatial dynamics of clot growth was optically monitored using a computer-assisted laboratory setup (17). In some experiments, plasmas were preicubated with 0.2 mg/ml of corn trypsin inhibitor (CTI) for 2 h. The light-scattering data from a 7.2 ×5.4-mm area were recorded every 30 s and additionally processed as described below. Since only areas free of spontaneous clots are suitable for data processing in determining the clot growth rate, the experiments were stopped if spontaneous clots had formed in front of the clot advancing into the bulk from the fibroblast monolayer.

Image processing

Automated data acquisition and preliminary processing was performed using a software (17) supplied with an EDC 1000 D computer camera (Electrim Corp., NJ, USA). The light scattering profile was obtained by scanning the light scattering intensity along the line drawn perpendicular to the activator surface in areas free of spontaneously growing clots in every frame. For each profile, the coordinate of a point where the light scattering intensity was half-maximal ($D_{1/2}$) was determined. The $D_{1/2}$ coordinates obtained for time series of profiles were plotted against time using Microcal Origin 6.0 (Microcal Software Inc., MA, USA). A linear portion of the plot, which corresponded to clot growth at a constant rate, was chosen and approximated with a straight line using the least squares method. The clot growth rate was determined from the slope of this line and expressed in μm/min.

Results

Effect of purified factor VIII on spatial clot growth

The typical patterns of clot growth in normal or haemophilic plasmas brought in contact with a confluent fibroblast monolayer (1500 cells/mm^2) are shown in Fig. 1. In normal plasma, the clotting could be detected as early as 1 min after recalcification (Fig. 1A, frame a), and the light scattering rapidly increased within 3 min in a narrow zone near the activator surface (data not shown). Thereafter, a wide front of the growing clot advanced into the bulk of plasma remaining parallel to the fibroblast monolayer (activator surface) (Fig. 1A, frames b-d). Noteworthy, as we reported earlier (16, 17), along with the clot advancing into the bulk from the activator surface, we also observed spontaneous clot formation in normal plasma and random distribution of formed clots in the plasma volume (Fig. 1A, frames b-d), the first spontaneous clot appearing in 7.3 ± 2.0 min (mean ± S.D.).

To exclude the contribution of contact, factor XIIa-driven activation on clot growth in normal plasma, we performed the experiments in the presence of the corn trypsin inhibitor (CTI), a potent inhibitor of factor XIIa. Preincubation of freshly drawn citrated plasma with 0.2 mg/ml CTI did not change noticeably the spatial dynamics of clot growth indicating that contribution of the contact activation is negligible (Fig. 1B). Notably, no spontaneous clotting in the plasma volume was observed in normal plasma under these conditions for 30-40 min. In another control experiment, normal plasmas without CTI were brought in contact with PET slips without cells. While no clotting was observed during first 10 ± 2 min, both spontaneous clotting and clotting on PET slips were registered at later time intervals (data not shown). Time required for the emerging of spontaneous clots and their number was similar to that in experiments with fibroblast activation. In contrast, in the presence of CTI no spontaneous clots were observed throughout the experiment (40 min).

We found that in plasma from patients with severe Haemophilia A the clot growth rate away from the confluent fibroblast monolayers constituted approximately 30% of that observed in normal plasma and no spontaneous clots were formed (Fig. 1C). Unlike in normal plasma, in haemophilic plasmas PET slips without fibroblasts (control) did not activate the clotting for 35 min or even longer.

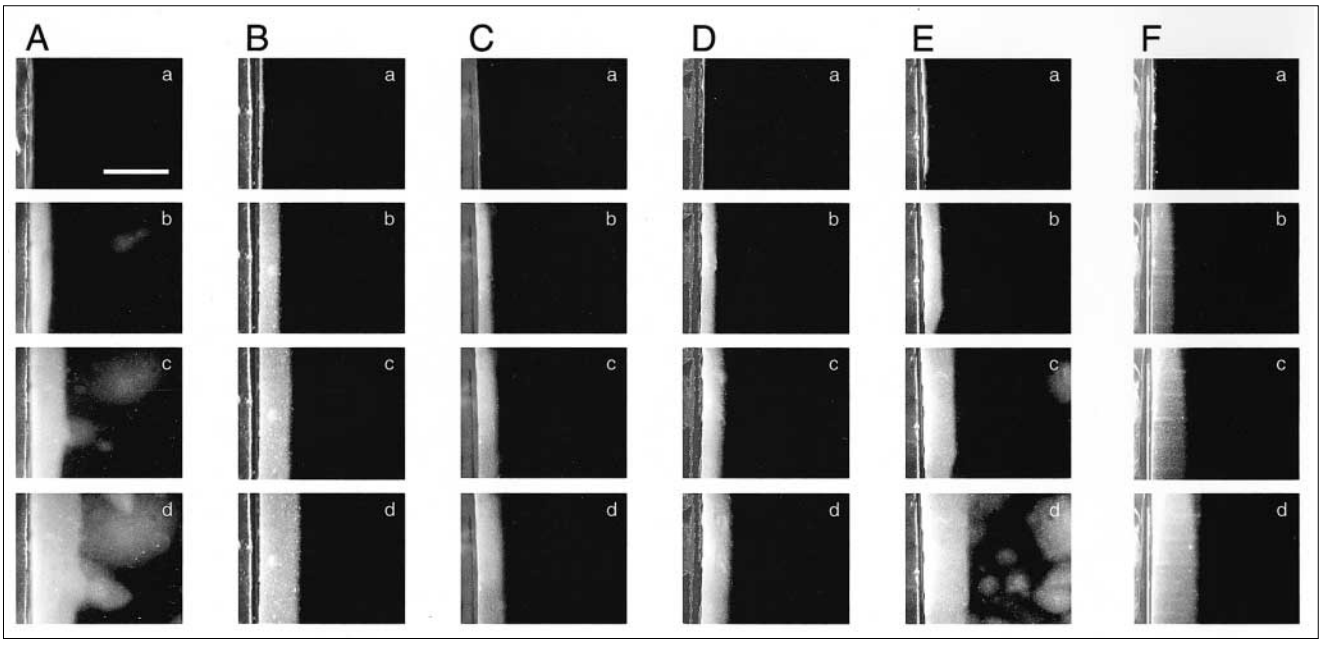

Figure 1: Time-lapse digital imaging of clot growth in normal and haemophilic plasmas. Clot growth in normal plasma *(column A)* and in normal plasma in the presence of 0.2 mg/ml CTI *(column B)*; in haemophilic plasma (fVIII <1% of the normal level) in the absence of exogenous fVIII *(column C)* or supplemented with 0.0003 U/ml *(column D)* and 0.05 U/ml fVIII *(column E)* without CTI; in haemophilic plasma supplemented with 0.05 U/ml fVIII in the presence of 0.2 mg/ml CTI *(column F)*. The clotting was induced by a confluent TF-expressing fibroblast monolayer grown on a PET slip as described under Materials and Methods. The surface of the PET slip is seen as a bright line at the left side of each image. In each series, the light-scattering images of the clot were taken 1.5 min after recalcification *(frames a)* and subsequently at 8 min intervals (*frames b, c* and *d*). Exposure time, 600 ms. Scale bar, 2 mm.

Remarkably, purified fVIII added to haemophilic plasmas normalized the clot formation in a dose-dependent manner. An increase in the clot growth rate became detectable at fVIII concentration of 0.00015-0.00031 U/ml (Fig. 1D). A steady increase in the clot growth rate was seen at fVIII concentration of 0.05 U/ml (Fig. 1E) and up to 0.1 U/ml, the highest fVIII concentration used in our experiments. In parallel with normalization of the clot growth rate, supplementation of patient plasma with purified fVIII above 0.000625–0.00125 U/ml also led to appearance of foci of spontaneous clotting. Preincubation of haemophilic plasmas (supplemented with 0.05 U/ml fVIII) with 0.2 mg/ml CTI had almost no effect on clot growth and on normalization of the clot growth rate by fVIII but completely abolished spontaneous clotting (Fig. 1F).

Figure 2 presents the time courses of evolution of light-scattering profiles obtained from clots growing on the surface of confluent fibroblast monolayer under different conditions. For a clot growing in normal plasma without CTI, the light-scattering intensity rapidly reaches its maximum near the activator surface and steeply declines at the clot interface with liquid plasma, suggesting that the clot has a sharp edge (Fig. 2a). As the clot increases in size, the descending part of the profile moves further away from the activator surface with a constant speed. The presence of CTI in normal plasma did not change the pattern of evolution of light-scattering profiles with time (Fig. 2b). While the time courses of evolution of light-scattering profiles in haemophilic plasmas with or without CTI had patterns similar to those in normal plasma, the rates of clot growth in patient and normal plasma were different (Figs. 2c and 2d, respectively).

As can be seen from the curves showing growth of the clot size (Fig. 3a), within first 4-6 min after activation, the rate of clot growth in haemophilic plasma did not depend on the concentration of added fVIII. This dependence on fVIII concentration became appreciable at later time periods: at low fVIII concentrations, the clot growth rate decreased with time, reaching the steady-state level by 10-15 min (Fig. 3a, diamonds). At fVIII concentrations higher than 0.00125 U/ml, the clot growth rate gradually increased, and at 0.05 U/ml fVIII the pattern of clot growth approached that registered in normal plasma (Fig. 3a, squares). Noteworthy, inhibition of the contact activation by CTI had no appreciable effect on the growth of clot size (Fig. 3b). This visual conclusion was further confirmed by determination of the steady-state clot growth rates in the absence or presence of CTI at increasing concentrations of exogenous fVIII added to haemophilic plasma. As follows from Table 1, at each concentration of added fVIII, the clot growth rates in the absence and in the presence of CTI had similar values confirming that the contribution of the contact pathway to the clot growth is negligible.

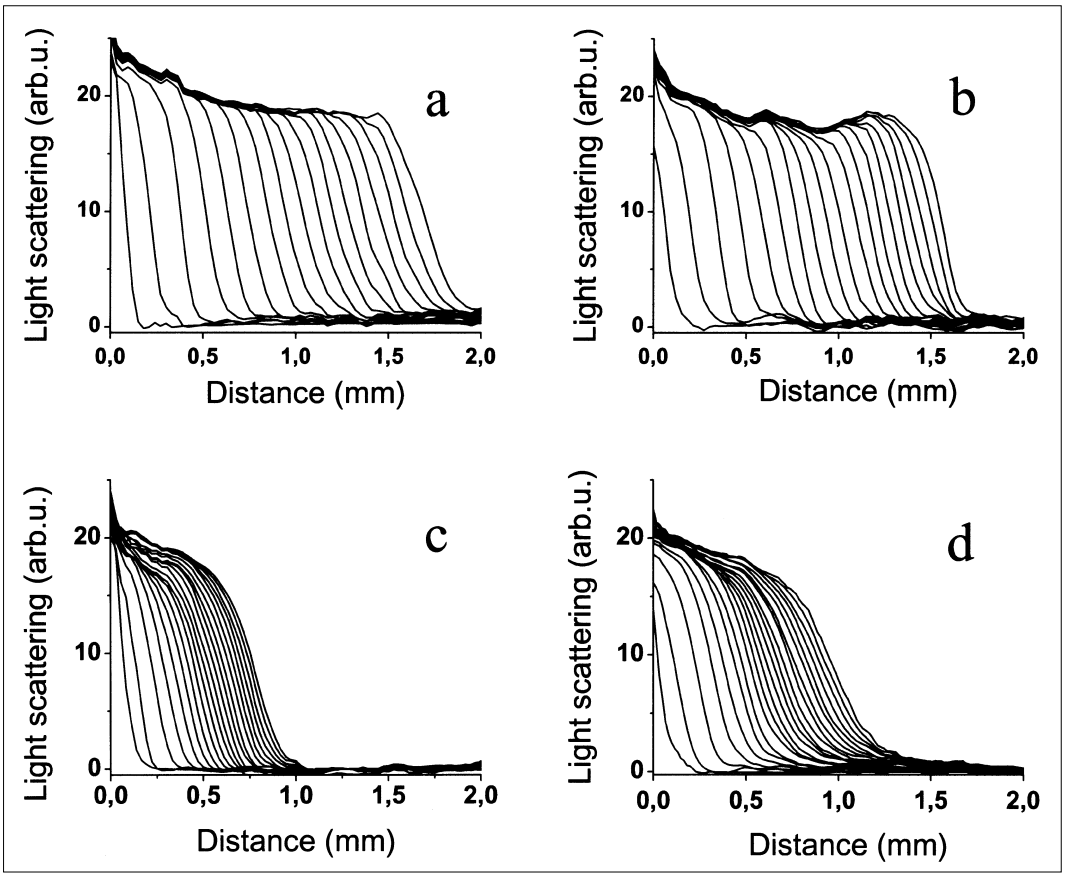

Figure 2: Time course of evolution of light scattering profiles of the clots. Clots were formed on the surface of a confluent fibroblast monolayer and were growing into the bulk of normal plasma *(panels a, b)* or haemophilic plasma (fVIII <1% of the normal level, *panels c, d*) in the absence of CTI *(panels a, c)* or in the presence of 0.2 mg/ml CTI *(panels b, d)*. Plots of the light scattering intensity versus distance from the activator surface were obtained from a complete series of light scattering images as described in Materials and Methods. Each profile series consists of a set of individual curves corresponding to different time-points of clot growth, with the leftmost curve corresponding to 1 min after recalcification and subsequent curves separated by 2-min intervals. As a clot grows and its size increases, the curves shift to the right (indicating that clot becomes larger).

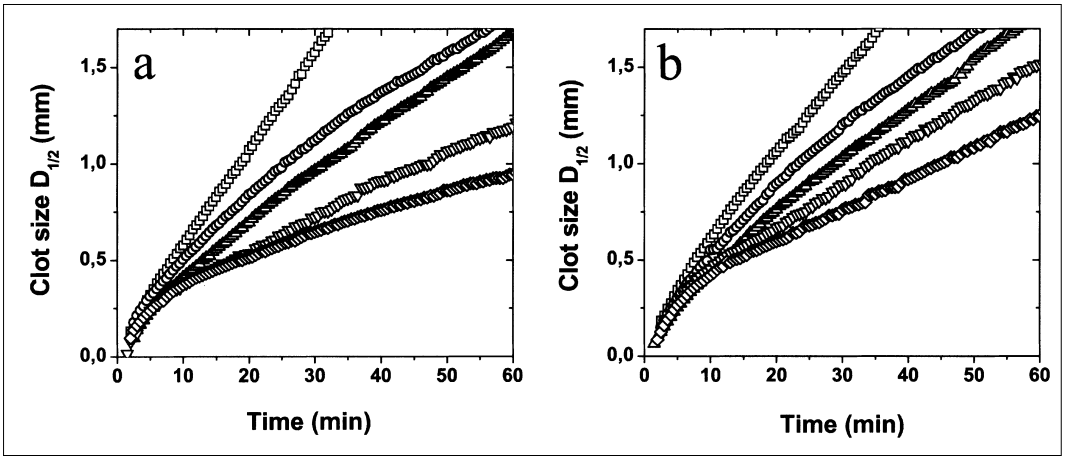

Figure 3: Effect of added fVIII on clot growth in haemophilic plasma. Haemophilic plasma (fVIII <1% of the normal level) was brought in contact with a confluent fibroblast monolayer and the experiments were performed as described in Materials and Methods in the absence *(panel a)* or in the presence of CTI *(panel b)*. Clot growth was studied in haemophilic plasma in the absence of exogenous fVIII *(diamonds)* or supplemented with 0.0003 *(down triangles)*, 0.005 *(up triangles)*, 0.0125 *(circles)* and 0.05 *(squares)* U/ml of fVIII.

We next quantitatively assessed the effect of fVIII concentration on the steady-state rate of clot growth in haemophilic plasmas on the surface of a confluent fibroblast monolayer. In 11 haemophilic plasmas tested, the clot growth rate appeared to be proportional to the logarithm of fVIII concentration (Fig. 4a, circles), and at 0.05 U/ml of added fVIII the rate of clot growth was close to that in normal plasma. Notably, addition of purified fVIII to normal plasma had no effect on the clot growth rate. Indeed, in NPP without added fVIII and in NPP supplemented with 0.05 U/ml fVIII the clot formed on the fibroblast monolayer grew at similar rates of 44.7 ± 5.9 μm/min and 45.7 ± 5.2 μm/min (n = 3), respectively.

Among the patients with severe Haemophilia A, only one was found to have a fVIII inhibitor with a titer of 20 BU/ml, as determined by the Bethesda assay (19). Noteworthy, incubation of this plasma sample with 0.05 U/ml fVIII for 3 h failed to restore the normal spatial dynamics of clot growth and also had no effect on the clot growth rate (data not shown).

Effects of fibroblast monolayer density on the clot growth rate at increasing fVIII concentration

In homogeneous systems, i.e. when plasma is stirred and thereby is continuously exposed to a TF-expressing activator surface, the clotting depends on fVIII only at low tissue factor concentrations (6, 7, 12). In our experiments, haemophilic plasma did not differ from normal plasma in the prothrombin time (17.1 ± 0.4 s vs. 16.9 ± 0.7 s, mean ± SD, n = 16 and n = 11, respectively) because this test is performed at a high thromboplastin concentration. The prothrombin time did not depend on the concentration of added fVIII either.

Next, in our non-stirred system, we studied dependence of the spatial dynamics of clot growth on the tissue factor concentration using either confluent (1500 cells/mm^2) or sparse (<50 cells/mm^2) fibroblast monolayers to model different TF concentration on the activator surface. Haemophilic plasma was similar to normal plasma in that the clotting initiated by contact with the surface of a fibroblast monolayer, irrespective of its density, could be detected in the first frame taken 1.5 min after recalcification. However, the subsequent clot growth proceeded differently: while in normal plasma the clot growth rate did not depend on the fibroblast density (data not shown), in haemophilic plasma the clots grew more slowly at low than at high fibroblast density (Fig. 4a). Consistent with our previous results (17), the rate of clot growth in haemophilic plasma (endogenous fVIII <1%) without added fVIII at low fibroblast density was 1.7 times lower than that at high cell density. Upon addition of fVIII, the clot growth rate increased proportionally to the logarithm of fVIII concentration at both high and low fibroblast densities. However, the clot growth rate at low cell density remained 1.5 to 1.7 times lower (Fig. 4a, squares) than that at high fibroblast density (Fig. 4a, circles). While at the concentration of added fVIII of 0.1 U/ml the rate of clot growth at high fibroblast density was completely normalized, it constituted only 67% of the normal value at low fibroblast density. In control experiments, we compared haemophilic plasmas used in experiments with confluent and sparse monolayers by a standard APTT test. As follows from Fig. 4b, both plasma series showed

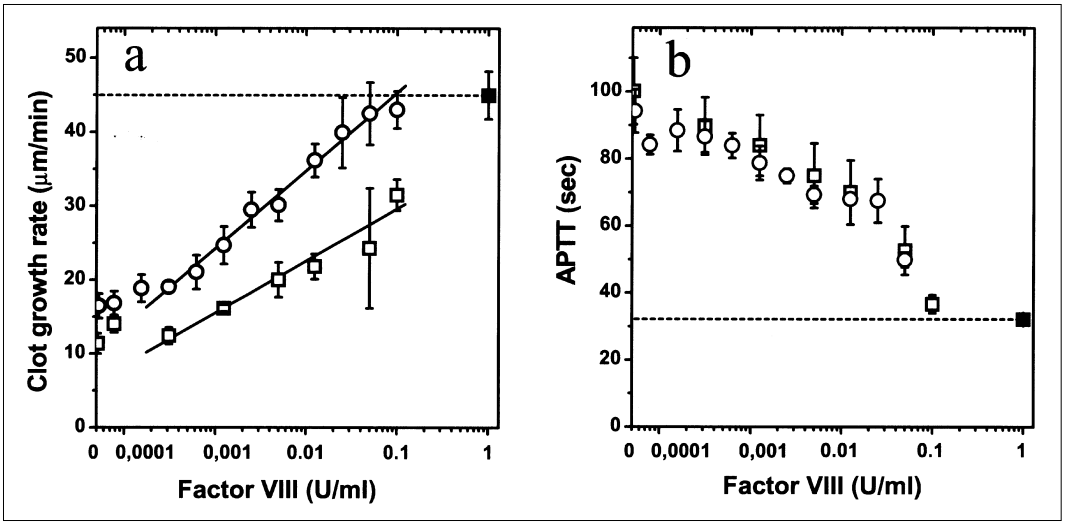

Figure 4: Effects of purified fVIII on the clot growth rate at high and low fibroblast density. *Panel a:* Haemophilic plasmas were brought in contact with either confluent (1500 cells/mm^2; circles; n = 11) or sparse (<50 cells/mm^2; squares; n = 7) fibroblast monolayers at increasing concentrations of added exogenous fVIII. *Panel b:* Results of a control APTT experiment on two series of plasma samples used in experiments shown in *panel a*. Clot growth rate *(panel a)* and APTT values *(panel b)* are presented as a function of logarithm of final fVIII concentration. Filled squares and horizontal dashed lines show the mean clot growth rate and the APTT value in normal plasma (n = 11). Each data point represents the mean value ± SEM.

a similar dependence of the APTT values on fVIII concentration thus confirming their identity.

Discussion

Using the new experimental system to analyze the spatiotemporal dynamics of clot formation induced by a fibroblast monolayer, we have recently shown that spatial clot growth proceeds in two phases – initiation and elongation (17). The initiation phase includes all the events on the activator surface and in the 0.2 mm-area near it. This phase spans the period between the onset of activation and the appearance of the first signs of fibrin formation. The initiation phase is significantly prolonged in haemophilic plasmas activated via the intrinsic pathway, and its duration correlates with the results of the APTT test.

It is known that clotting in stirred plasma with a high concentration of thromboplastin does not depend on the presence or absence of fVIII (4, 6). The results of our experiments in non-stirred system, where plasma is brought in contact with a fibroblast monolayer representing a TF-bearing activator surface, clearly demonstrate different sensitivity of the initiation and elongation phases to fVIII concentration in plasma. Independently of fVIII concentration (Fig. 3a) and the density of a fibroblast monolayer both normal and haemophilic plasmas began to clot on the monolayer surface within 1 min of their contact. Thus, the duration of the initiation phase is comparable for haemophilic and normal plasma.

Strikingly, it was the elongation phase, or clot thickening, that was markedly impaired in Haemophilia A. In severe Haemophilia A, the rate of TF-induced clot growth constituted only one-third of that observed in normal plasma. The rate of clot growth remained unaffected at up to 0.00015 U/ml of added fVIII. At higher fVIII concentrations, the clot growth rate increased with the increase of fVIII concentration, reaching its normal value in plasma supplemented with 0.05-0.1 U/ml of purified fVIII (Fig. 4a). Noteworthy, this is the threshold level below which preventive treatment for Haemophilia is recommended to be commenced (26). On the other hand, the decrease of fVIII activity to 0.1 U/ml by monoclonal antibody mAb-LE2E9 was shown to be sufficient to inhibit thrombosis without causing an overt bleeding tendency (27).

In our experimental system, the polysterene and PET surfaces of the microchamber caused only minor activation of the contact pathway. Indeed, suppression of this activation by CTI did not affect the spatial dynamics of clot growth (Figs. 1A and 1B, Figs. 1E and 1F), growth of clot size (Fig. 3) and the steady-state clot growth rate (Table 1). FXII-dependent pathway was in part responsible for the spontaneous clot formation (compare Figs. 1A and 1B, Figs. 1E and 1F).

In the present study, using confluent and sparse fibroblast monolayers to model different concentrations of tissue factor on the activator surface, we found a marked effect of TF activity on the clot growth rate (Fig. 4a). While at both high and low fibroblast densities the clot growth rate increased proportionally to the concentration of added fVIII starting with 0.00015 U/ml, at low fibroblast density the clot growth rate remained ~1.5 times lower than that at high cell density. This result is consistent with the earlier reports that a reduced prothrombinase activity is observed at low TF concentrations due to reduced formation of the functional tenase complex (6, 7, 13, 28). The marked effect of TF activity on the clot growth rate detected in the present study supports the hypothesis that the clotting expansion away from the vessel wall is likely to be driven by fIXa diffusion away from TF-bearing cells (10, 29), at least in Haemophilia A conditions. In conclusion, based on the established dependence of clot growth rate on fVIII concentration, our results indicate that the proper functioning of the intrinsic tenase complex is critical for normal spatial clot growth away from the activator surface.

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Abbreviations

APTT, activated partial thromboplastin time; CTI, corn trypsin inhibitor; NPP, normal pooled plasma; TF, tissue factor; PT, prothrombin time; PET, poly(ethylene terephthalate).

Table 1: Effect of fVIII on clot growth rate in the absence and in the presence of CTI. Haemophilic plasmas supplemented with increasing concentrations of exogenous fVIII were brought in contact with confluent fibroblast monolayers in the absence or in the presence of 0.2 mg/ml CTI. The clot growth rate was determined from a series of light scattering profiles as described under Materials and Methods. Each data point represents the mean value ± S. D., n = 2.

	fVIII, U/ml	5.3125E-5	7.8125E-5	3.125E-4	0.00125	0.005	0.0125	0.1
without CTI	V, μm/min	16.5±8.1	16.6±1.0	18.3±2.4	20.7±2.0	24.8±0.1	30.7±5.4	45.1±9.3
with CTI	V, μm/min	15.9±0.5	14.0±1.1	18.7±3.7	18.0±3.0	24.2±1.6	28.0±3.8	38.8±15.1

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