Hemophilia A and B are associated with abnormal spatial dynamics of clot growth

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Abstract

To gain greater insight into the nature of the bleeding tendency in hemophilia, we compared the spatial dynamics of clotting in platelet-free plasma from healthy donors and from patients with severe hemophilia A or B (factor VIII:C or IX:C < 1%). Clotting was initiated via the intrinsic or extrinsic pathway in a thin layer of nonstirred plasma by bringing it in contact with the glass or fibroblast monolayer surface. The results suggest that clot growth is a process consisting of two distinct phases, initiation and elongation. The clotting events on the activator surface and the preceding period free of visible signs of clotting are the initiation phase. In experiments with and without stirring alike, this phase is prolonged in hemophilic plasma activated by the intrinsic, but not the extrinsic pathway. Strikingly, both hemophilia A and B are associated with a significant deterioration in the elongation phase (clot thickening), irrespective of the activation pathway. The rate of clot growth in hemophilic plasma is significantly lower than normal and declines quickly. The resulting clots are thin, which may account for the bleeding disorder.
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1. Introduction

Hemophilia is a common hereditary disease characterized by the uncontrolled and, often, life-threatening bleeding tendency. Hemophilia A and B are caused by severe deficiencies in plasma factors VIII and IX, respectively [1]. Less common is hemophilia C (1–3% of all hemophilic patients [2]), which is associated with a factor XI deficiency. Significantly, all the three factors are not involved in the extrinsic coagulation pathway, which is thought to be central to thrombin generation [3–5]. Here, we consider two hypotheses explaining this apparent paradox and test them in a novel experimental system that allows direct examination of the spatial aspects of clot growth.

The extrinsic (or tissue factor (TF)) pathway is activated when circulating plasma factor VIIa comes in contact with TF exposed at the site of injury [3,5]. This complex activates circulating factor X. Its active form, factor Xa, in a complex with phospholipids can activate factor VII, thereby boosting its own production. More importantly, factor Xa activates a limited amount of prothrombin, which in its active form cleaves fibrinogen to form a clot. These reactions are accelerated more than 100 000-fold by the prothrombinase complex, which is formed on a phospholipid membrane via the assembly of factors Xa and Va in the presence of calcium ions [6]. Thrombin activates its production even further through activation of factor V and subsequent formation of the additional prothrombinase complexes.

Factors VIII, IX, and XI, which are affected in hemophiliacs, are involved in the intrinsic (or contact) coagulation pathway. This pathway is thought to play a marginal role in activation of blood coagulation [7] because deficiencies in the factors initiating this pathway, such as factor XII, high-molecular-weight kininogen, or prekallikrein, are not associated with bleeding disorders or thrombosis [8–10]. Following their activation in the presence of calcium ions, factors VIIIa and IXa assemble on a phospholipid membrane to form a tenase complex. This complex is 50 times more active in activating factor X than the VIIa–TF complex [6]. Tenase resembles the prothrombinase complex, both structurally [11] and functionally [6]. Like cofactor V, cofactor VIII is activated by thrombin. The tenase proteolytic component, factor IX, can be activated by three different mechanisms. One of them, via contact activation, cannot account for bleeding disorders because of the insignificance of this pathway in activating coagulation in vivo [4,7]. Clearly, the issue of the nature of bleeding disorders in hemophilic patients is tightly linked with identifying the major pathway of factor IX activation. We will, therefore, consider two hypotheses explaining the bleeding tendency in hemophilia, which are based on two different but not mutually exclusive mechanisms of factor IX activation.

The first hypothesis relies upon the cross talk between factor IX and the extrinsic pathway. The surface-associated VIIa–TF complex can activate factor IX, in addition to factor X [12–14]. Only the latter reaction is thought to be physiologically relevant in activating the coagulation cascade [5,15–17]. However, it is conceivable that this activation loop (VIIa–TF complex–factor IXa–tenase–factor Xa) provides an important contribution to the blood clotting process. If so, a deficiency in either factor IX or VIII will lead to a decrease in the tenase production and thereby to a bleeding tendency. The VIIa–TF complex is restricted to the activating surface, and factor IXa produced by this complex (and thrombin then produced by factor Xa) can spread away from the site of their formation only by diffusion. This model, therefore, predicts that the initiation of clotting via the extrinsic pathway should be prolonged in hemophilic plasma, and both the clot growth rate and final size would decrease proportionally to the extent of this prolongation (because of the proportionality between the concentration and diffusion).

The second hypothesis takes into account slow activation of factor XI by thrombin [18,19]. This positive feedback loop leads to self-sustained production of thrombin. The rate of this reaction is very low, and, against a background of highly active VIIa–TF, tenase, and prothrombinase complexes, its contribution is almost negligible in stirred in vitro systems [20,21]. However, as we showed previously using mathematical modeling, inclusion of this reaction into the model significantly alters the spatial parameters of simulated clot growth, e.g., the clot growth rate [22,23]. In the absence of this reaction, factor Xa and thrombin spread away from the activating surface only by diffusion. Obviously, a deficiency in either factor XI, IX, or VIII may disrupt

self-accelerated thrombin production off the activating surface. This model predicts that the initiation of clot growth in hemophilic plasma via the extrinsic pathway is not very different from normal, but the clot growth proper (i.e., thickening) is severely disrupted.

To discriminate between these two models, we experimentally recorded the dynamics of clot growth on different activating surfaces in nonstirred hemophilic and normal plasma and compared the clotting initiation times (IT), the clot growth rates, and the sizes and the shapes of growing clots. As in a previous work carried out in the homogeneous system, we found that the initiation of clot growth in hemophilic plasma of any type was significantly impaired when the intrinsic, but not the extrinsic pathway was activated. Strikingly, we also observed that a deficiency in either factor VIII or IX dramatically affected the clot thickening (elongation) phase, irrespective of the activation pathway. Apparently, the initiation stage of clotting in glass- or fibroblast-monolayer-activated hemophilic plasma proceeds normally, but the resulting clots are thin, which probably accounts for the bleeding tendency. We propose that, whatever the activation pathway, a weak positive feedback loop whereby thrombin activates factor XI, and, hence, factors IX and X, plays an essential role in clot thickening.

2. Materials and methods

2.1. Subjects

A total of seven pools of normal plasma were prepared, each containing plasma from three healthy donors with no history of bleeding. All pools were tested for the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time. None of the hemophilic patients had antibodies against clotting factors. Five of them (aged 19–21) had hemophilia A, and five (three patients were 18–21, one 30, and one 61 years old) had hemophilia B with factor VIII:C or IX:C < 1% of the norm, as was determined by one-stage clotting assay. All had a history of frequent bleeds and suffered from arthropathy of large joints (stages II–IV). The sampling time was chosen according to the ISTH recommendation [24]. Blood samples were taken during bleed-free periods, not earlier than 4–5 days (hemophilia A) or 7–10 days (hemophilia B) after the last administration of the factor VIII- or IX-containing blood products, so that the factor VIII or IX activity in the samples was < 1%. The APTT was 60–111 and 60–86 s in the plasma from patients with hemophilia A and B, respectively, as compared to 26–39 s in the normal plasma pools.

2.2. Blood collection and plasma preparation

Donor blood was collected into 3.8% sodium citrate (pH 5.5). Platelet-poor plasma was obtained by centrifuging the
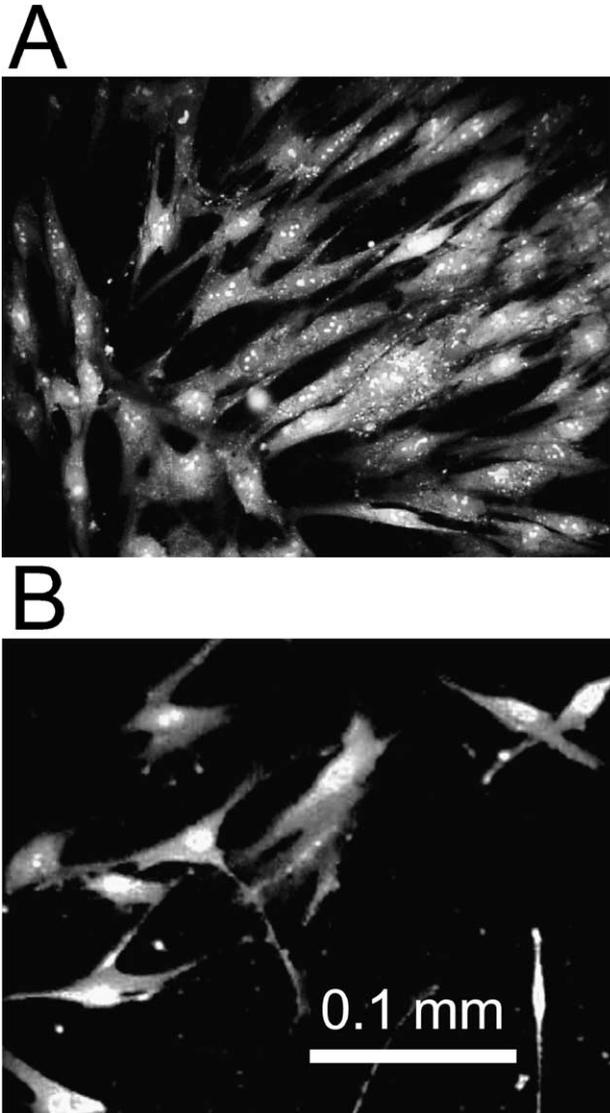
Fig. 1. Fibroblast monolayers grown on PTF: (A) 1400 cells/mm^2 (confluent) and (B) 200 cells/mm^2. To determine the density, cells were stained with acridine orange and counted using a Leica DM/RBE fluorescent microscope.

blood at $2400 \times g$ for 15 min, followed by $10\,000 \times g$ for 5 min. The pH of supernatant plasma was stabilized at 7.2–7.6 by lactic acid as described elsewhere [25]. The plasma contained 10 μM sodium azide to prevent bacterial growth and was kept overnight sealed in polystyrene 5-ml tubes at room temperature. The overnight incubation [26] was necessary to minimize the formation of spontaneous clots, which grow in freshly prepared recalcified plasma presumably activated during collection and fractionation. As we previously showed, spontaneous clotting begins 15–25 min (depending on the plasma pool) after recalcification, and the number of such foci is proportional to the platelet concentration [22]. Storage of plasma at room temperature for up to 24 h does not affect the clotting factor concentrations, except for factor VIII, which decreases by 10–30% [27,28]. We determined the APTT and PT in each plasma

pool on the day of its preparation and on the next day immediately before the experiment. The clotting times remained essentially constant during plasma storage ($P < 0.01$).

2.3. Fibroblast culture

A human fetal lung fibroblast line was from the Ivanovskii Research Institute of Virology (Russian Academy of Medical Sciences, Moscow, Russia). Cells (10^5/ml) were grown at 37 °C in 50-ml flasks (Nunk, Denmark) containing 5 ml of a complete 199 medium (Sigma, MO, USA) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., VA, USA), 10 μg/ml gentamicin (Flow Laboratories), and 300 μg/ml L-glutamine. Cells were passaged every 3–4 days (upon reaching confluence). Briefly, after being incubated for 10 min in 0.125% trypsin and 0.01% EDTA preheated to 37 °C, cells were resuspended by gentle shaking in 10 ml of the fresh culture medium. Cell concentration was determined using a hemacytometer. To grow fibroblast monolayers of different densities, we prepared a series of dilutions (from 2×10^3 to 100×10^3 cells/ml) in
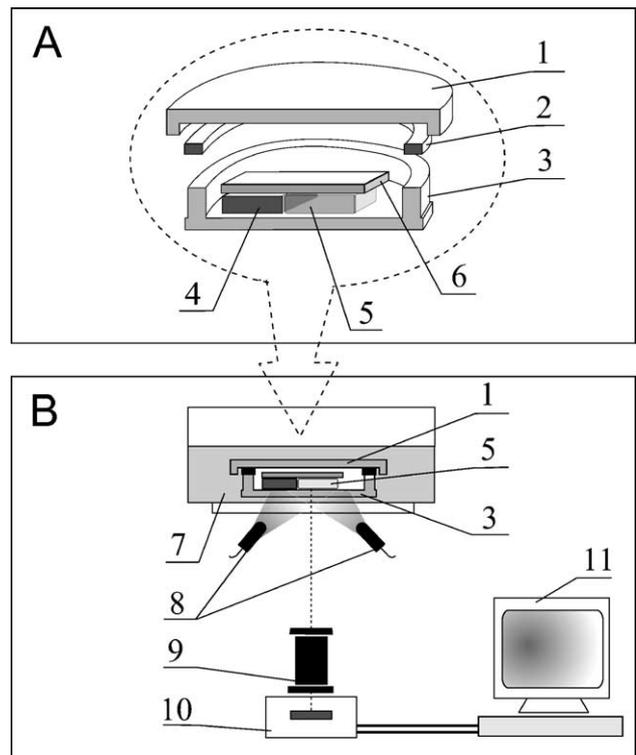

Fig. 2. Schemes of (A) the chamber and (B) the experimental setup. The chamber was assembled in a polystyrene Petri dish (chamber bottom; 3), with activator (4) as a lateral wall and a polystyrene plate (6) at the top. Then, it was filled with plasma (5) and covered by a lid (1) with a sealing gasket (2). The sealed chamber was placed inside the transparent temperature-controlled (37 °C) water jacket (7) and illuminated from below with light emitting diodes (8). A set of lenses (9) focused the images of plasma (5) and activator (4) onto the matrix of a cooled digital CCD camera (10), connected to a computer (11). For more details of the experimental setup, see Ref. [29].

HEPES-buffered RPMI (Sigma) supplemented as above. The dilute suspension was added in 1-ml aliquots into wells of a 24-well plate (Flow Laboratories) with a 10-μm-thick polyethylene terephthalate film (PTF, Joint Institute for Nuclear Research, Dubna, Moscow oblast, Russia) slip 2 cm^2 in area on the bottom. The plate was placed in a CO$_2$ incubator at 37 °C for 24 h to allow fibroblasts settled onto slips to reach confluence. The cell density in monolayers was determined by counting acridine orange stained cells using a Leica DM/RBE (Leica Gmbh, Germany) fluorescence microscope (see Fig. 1). For example, to grow a confluent monolayer uniformly covering the entire slip (1000–1500 cells/mm^2), we plated $(80–100) \times 10^3$ cells/well (Fig. 1A). At lower seeding concentrations, the cell density was 1–1000 cells/mm^2 (Fig. 1B).

2.4. Experimental chamber

A 1-mm-thick glass slide (Fig. 2A,(4); #48300-25, VWR Scientific Inc., PA, USA) was fixed with a double-stick Scotch tape between the bottom of a 40-mm polystyrene Petri dish ((3); Medpolimer, St. Petersburg, Russia) and a polystyrene plate (6). The glass slide edge, which formed a vertical wall of the chamber, was wrapped around with a cell-coated PTF slip rinsed three times in a Hanks solution. In control experiments, PTF slips without cells were used or the glass wall served as an activator of clotting.

The experiment was started by adding 20 μl of 1 M CaCl$_2$ per 1 ml plasma (final Ca^{2+} concentration 1.2–2.0 mM) preheated at 37 °C for 30 min. The plasma (0.4 ml) was rapidly transferred into the preassembled chamber. The Petri dish with the filled chamber was then hermetically sealed with a lid (1) and temperature-controlled in a water jacket (7). First frame could be recorded as early as ~ 1 min after recalcification. All experiments with plasma from the same patient or donor pool were usually performed in duplicate or triplicate on the same day.

2.5. Recording the clot growth

The spatial dynamics of clot growth was optically monitored using a computer-assisted laboratory setup (Fig. 2B) [29]. The chamber (1–6) in a water-jacketed Petri dish was illuminated from below with red-light-emitting diodes ((8); $\lambda_{peak} = 660$ nm, L-1543-E, Kingbright, Japan) at 135° to the direction of recording by a digital CCD camera ((5); EDC 1000 D, Electrim Corp., NJ, USA) [30] with a custom-made cooling system (-5 °C). We used the software based
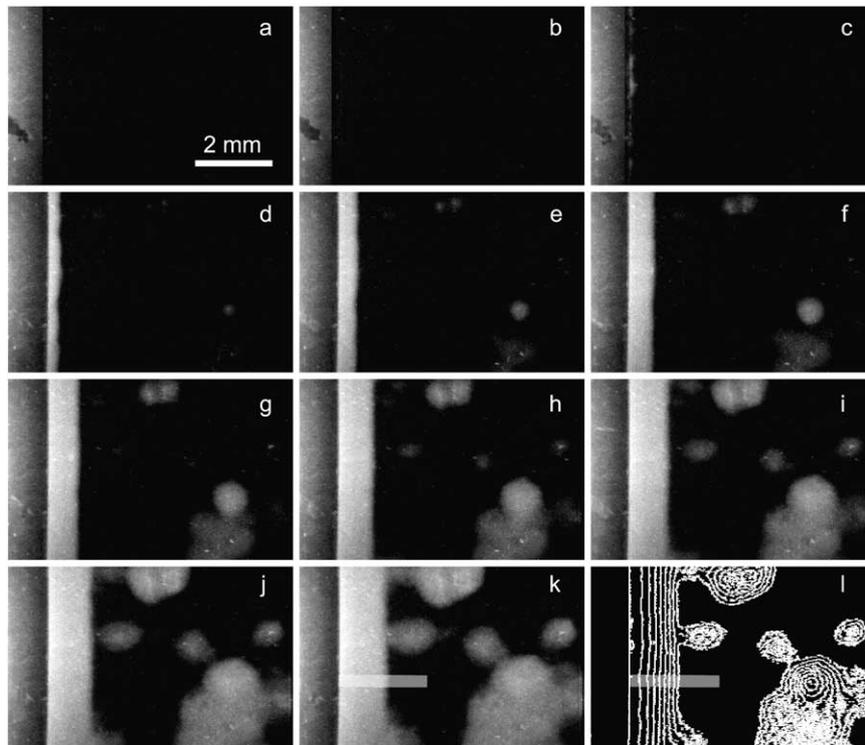

Fig. 3. Time-lapse digital imaging of the dynamics of clot growth in normal plasma activated by glass. Selected frames are shown, but the whole series is available upon request. The first frame (a) was taken 1 min after recalcification, all other frames (b–k) at 3-min intervals. Exposure time, 600 ms. The last frame (l) represents a family of the clot contours; the leftmost vertical line corresponds to the activator edge; the first line to the right of it is the contour of the clot shown in (d), the subsequent contours are separated by 2-min intervals. The contour of a clot is a set of image pixels, where the light scattering intensity is half-maximal. Clotting first appears along the activator surface; however, other clots, e.g. as in (e), become visible 15–26 min later [22]. Image analysis was carried out for a 0.2-mm-wide area free of spontaneous clots. This area is indicated by semitransparent horizontal bars in (k) and (l). For every time point, the light scattering intensities within this area were averaged in the direction parallel to the activator surface in order to increase the signal to noise ratio. The resulting profiles of the light scattering intensities are shown in Fig. 4C.

on standard drivers supplied by Electrim, which allowed automated data acquisition and preliminary processing. The light scattering data from a 7.2×5.4 mm area were recorded every 30 s as .TIF files and additionally processed as described in the legend to Fig. 3.

3. Results

3.1. Activation of clotting via the intrinsic pathway

3.1.1. Clot growth in normal plasma

When plasma is contact activated, the first signs of clotting are detected after some lag-period, thereafter called the clotting IT. In plasma from healthy donors activated by glass, the IT was 7.9 ± 2.6 min (Fig. 3c). The wide front of the growing clot propagated parallel to the activator surface (Fig. 3e–k). Spontaneous clots began to form 10 to 25 min (depending on the plasma pool) after recalcification (Fig. 3d–f), and filled the entire plasma in 40–60 min. The contours of the clots for different moments can be superimposed in one image, allowing pictorial visualization of the dynamics of clot growth (Fig. 3l). More detailed information is obtained by scanning the area indicated by a horizontal bar in Fig.

3k. The resulting profiles can be presented as plots of the light scattering intensity versus distance from the glass surface (Fig. 4). In normal plasma activated by glass, such profiles have a shape of a step-function: while being even and maximal within the clot, the light intensity drops steeply at its interface with liquid plasma (Fig. 4C). The rate of propagation of the light scattering front was assessed by monitoring its point at the half-maximal height (Fig. 4C, dashed line). The distance from the glass surface to the half-intensity point plotted versus time is shown in Fig. 5A, filled squares). As estimated from the slope of this line, the rate of clot growth was maximal immediately after beginning of clotting, but then slowly decreased, and 5–7 min later became quasistationary. The average quasistationary rate of clot growth was 0.040 ± 0.006 mm/min; the average clot size 40 min after recalcification was 1.72 ± 0.25 mm (Table 1).

Similar experiments were carried out to examine the dynamics of clot growth activated by an PTF. The beginning of clotting was slightly delayed and usually started after 10–13 min. All other parameters of clot growth, such as the maximal light scattering intensity, steepness of the front (Fig. 4D), and the clot growth rate (Fig. 5B, filled squares), were very similar to those observed after activation by glass (Table 1).
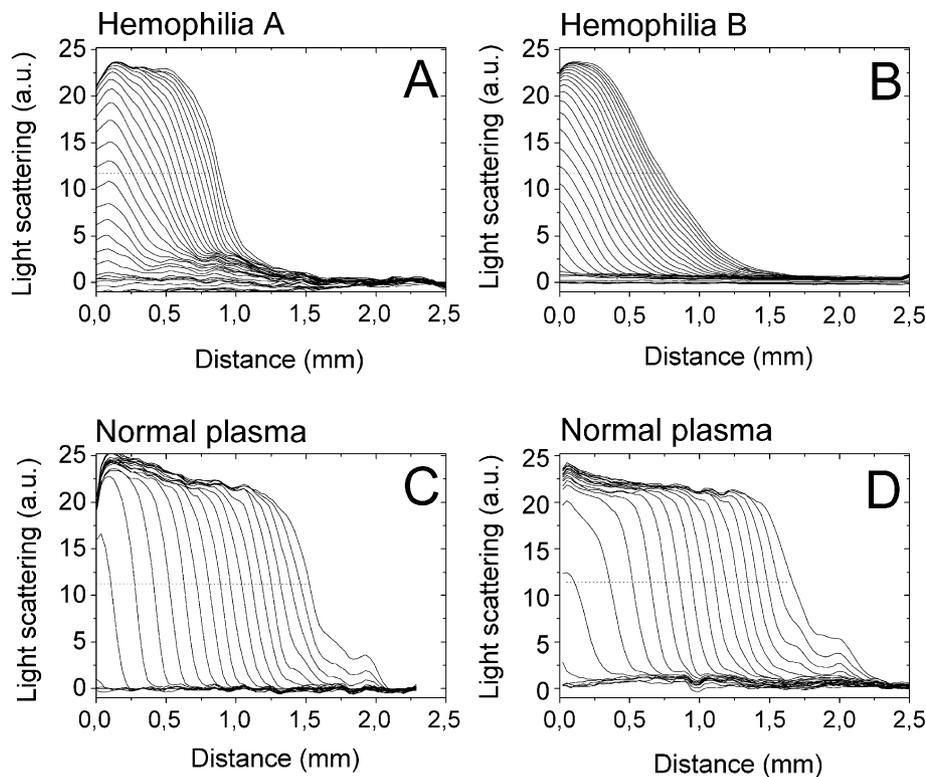

Fig. 4. Evolution of light-intensity profiles of clots growing in (C,D) normal and (A,B) hemophilic plasma. Clotting was activated by (A–C) glass or (D) PTF. Each curve (profile) corresponds to a different moment of time during clot growth, with the leftmost lowest curve corresponding to 1 min after recalcification; the subsequent curves are separated by 2-min intervals. As the clot grows and its size increases, the curves shift upward (implying that the clot becomes denser) and to the right (wider clots). Clot size and growth rate were measured along the half-maximal intensity line (dashed).
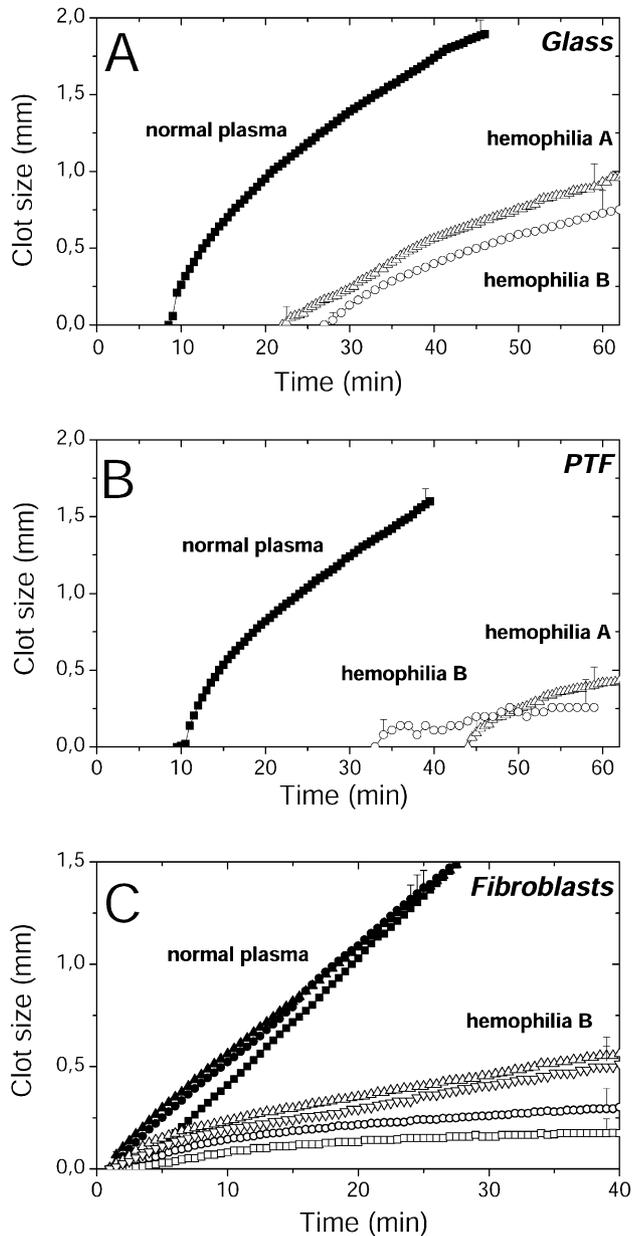
Fig. 5. Clot size versus time in normal pooled and hemophilic plasma activated by (A) glass, (B) PTF, or (C) fibroblast monolayers. Panels A and B (■) normal pooled plasma, (△) hemophilia A, and (○) hemophilia B; panel C: (■) normal plasma, 1 to 2 cells/mm²; (●) normal plasma, 50 cells/mm²; (▲) normal plasma, 450 cells/mm²; (□) hemophilia B, 3–5 cells/mm²; (○) hemophilia B, 50 cells/mm²; (▽) hemophilia B, 150 cells/mm²; and (△) hemophilia B, 1000 cells/mm². The clot size was estimated along the half-maximal intensity line. Clots grow significantly more slowly in hemophilic plasma than in normal plasma.

3.1.2. Clot growth in hemophilic plasma

The beginning of clotting on the glass surface was significantly delayed (up to 20–30 min after recalcification) in hemophilic plasma. The average IT was 35.4 ± 2.8 min for hemophilia A and 20.2 ± 3.9 min for hemophilia B (Table 1). The clot grew more slowly than in normal plasma (see below), and no spontaneous clots were observed throughout the experiment (40–90 min and longer). In general, the dynamics of clot formation on the glass surface were similar in plasma of patients with hemophilia A and B. However, in two out of five patients with hemophilia A, no clotting could be detected throughout the experiment (IT>70 min).

To compare the glass and PTF surfaces in the strength of activation of clotting, we monitored clot growth in hemophilic plasma exposed to these two activators simultaneously. Part of the glass surface was wrapped with the PTF, allowing a direct comparison of these activators. Irrespective of hemophilia type, clots started to form 20–40 min later on the PTF than on the glass surface (Fig. 5B, open triangles and circles; Table 1).

In hemophilic plasma, clots grew four to five times more slowly than in normal plasma. The light scattering profiles for a clot growing on the glass surface in plasma from a patient with hemophilia B are shown in Fig. 4B. The clot edge is fuzzy, and the clot density decreases gradually with time, so that the respective profiles are less steep than in normal plasma. It is, therefore, difficult to unambiguously determine the rate of clot growth. We arbitrary set the scan line in the same way as for the "step" profiles in normal plasma, i.e., at the half-maximal height (Fig. 4B, dashed line). The movement of a representative point along this line is four times slower than in normal plasma (Table 1). After 40 min, the maximal clot size in the patient plasma was <0.7 mm.

The light scattering profiles in plasma from a patient with hemophilia A plasma behaved similarly (Fig. 4A). The rate of glass-activated clot growth was only about 26% of that in normal plasma (Fig. 5A, open triangles). The PTF-activated clot growth was even slower: it was about twice slower than on the glass surface, and about four to seven times slower than clotting on the PTF surface in normal plasma (Fig. 5B, open triangles).

The delay in clot growth in nonstirred hemophilic plasma was correlated well with the prolonged clotting in fully stirred plasma. To compare the spatial dynamics of clot growth with the kinetics of clotting in a stirred system, we determined the APTT in the same plasma that was used in the spatial experiments (Table 1). This allows direct comparison of the APTT with the parameters of clot growth. Normal or hemophilic plasma was induced to clot by stirring in the presence of kaolin, a strong activator of the intrinsic pathway. The APTT in all hemophiliacs was prolonged and correlated well with the increased IT in hemophilic plasma activated by glass or PTF. However, in all cases, the APTT was significantly shorter than the IT, presumably because kaolin is a much stronger activator of clotting than glass or PTF. If the APTT is taken as unity, then the ITs for glass and PTF in normal plasma are 14 and 20.2, respectively (1:14:20.2 ratio, Table 1). The analogous ratio is 1:11.5:15.5 in hemophilia A, and 1:16.6:34 in hemophilia B. It is easy to see that the deficiency in either factor is not associated with considerable deviations of this ratio from its value in normal plasma.

Table 1
Plasma clotting parameters for healthy donors and patients with severe hemophilia A or B

Activator		Normal plasma	Hemophilia A	Hemophilia B
Kaolin	APTT (s)[a]	33.6 ± 3.8	90.7 ± 21.0	73.1 ± 14.3
Glass	Clotting initiation time (min)	7.9 ± 2.6	17.4 ± 0.9[b]	20.2 ± 3.9
	Quasistationary rate of clot growth (mm/min)	0.040 ± 0.006	0.011 ± 0.008[b]	0.009 ± 0.005
	Clot size 40 min after recalcification (mm)	1.72 ± 0.25	0.79 ± 0.18[b]	0.52 ± 0.24
PTF	Clotting initiation time (min)	11.3 ± 3.5	23.5 ± 4.4[b]	41.3 ± 1.1
	Quasistationary rate of clot growth (mm/min)	0.043 ± 0.010	0.008 ± 0.005[b]	0.002 ± 0.002
	Clot size 40 min after recalcification (mm)	1.50 ± 0.36	0.24 ± 0.08[b]	0.03 ± 0.04
Thromboplastin	PT (s)[a]	16.1 ± 0.6	16.3 ± 0.4	16.2 ± 0.5
Fibroblast monolayer	Clotting initiation time (min)	<1	<1	<1
	Quasistationary rate of clot growth (mm/min)	0.039 ± 0.005	0.013 ± 0.004	0.010 ± 0.002
	Clot size 40 min after recalcification (mm)	1.74 ± 0.27	0.90 ± 0.12	0.74 ± 0.15

To determine the spatial parameters of clot growth, clotting was activated by glass, a PTF, or a fibroblast monolayer as described in Section 2. The clotting IT is the lag-period between plasma recalcification and a detectable increase in the light scattering on the activator surface. The clot size and the growth rate were determined by monitoring the coordinates of the half-intensity point (intersection of the half-maximal intensity line and the light scattering profile). Values are means $\pm$ S.E.

  [a] The kinetic parameters were determined in stirred plasma activated by kaolin or thromboplastin.
  [b] Two out of the five patients with Hemophilia A were excluded because their ITs were >70 min.

3.2. Activation of clotting via the extrinsic pathway

3.2.1. Clot growth in normal plasma

To examine the spatial dynamics of clot growth activated via the extrinsic pathway, we used fibroblasts grown in monolayer cultures. These cells constantly express TF, an activator of the extrinsic pathway, on their surface [31]. When pooled plasma from healthy donors was brought in contact with a confluent fibroblast monolayer, clotting could be detected as early as within 1 min after recalcification. We observed no delays in the initiation of clot growth, which were typical of the activation by glass or PTF. It, therefore, appears that such a fast response is characteristic of the activation via the extrinsic pathway. Other clotting parameters were very similar, implying that the spatial dynamics of clot growth in normal plasma is largely independent of the activation pathway. For example, the light scattering profiles in normal plasma activated by a fibroblast monolayer (Fig. 6C, panel b) and by glass (Fig. 4C) were very similar. Some differences were detected in the shape of the clot size versus time plots (Fig. 5). The clot size linearly increased with time, indicating a constant rate of clot growth. The quasistationary rate of clot growth activated on a fibroblast monolayer was 0.039 ± 0.005 mm/min, which did not differ statistically significantly from the rate of glass-induced clot growth (Table 1). The average size the clot reached 40 min after recalcification was 1.74 ± 0.27 mm.

Clot growth in normal plasma was almost independent of the fibroblast density. The dynamics of clot growth was studied as a function of the activator strength by varying the fibroblast density in monolayers from 1 or 2 to up to 450 cells/mm². Fig. 7C(a–c) shows the propagation of the light scattering fronts recorded in normal plasma from the same pool for three different cell densities. In all three experiments, the clot edge appeared relatively flat and continuous, the light scattering profiles were similar, and clots grew to almost the same size (compare panels a and b in Fig. 6C). No significant differences were detected in the IT, which was <1 min in all experiments, except when clotting was activated by a low-density monolayer (1 or 2 cells/mm²). Fig. 5C (filled symbols) shows the plots of the clotting front position versus time: clearly, the clot growth rates were also similar at all the cell densities used.

3.2.2. Clot growth in hemophilic plasma

The initiation phase of clot growth was unaffected in hemophilic plasma. In plasma from patients with hemophilia A or B activated by a confluent fibroblast monolayer, clot growth began almost immediately (<1 min after recalcification), as in normal plasma. The undelayed activation was observed in a broad range of cell densities (from 5 to 2000 cells/mm²), suggesting that this response is largely independent of the strength of the activating signal. The IT for activation by a fibroblast monolayer can be directly compared with the PT in stirred system, because both tests rely on the use of the same activating agent, i.e., thromboplastin [3,14]. We determined that normal and hemophilic plasma in our experiments had
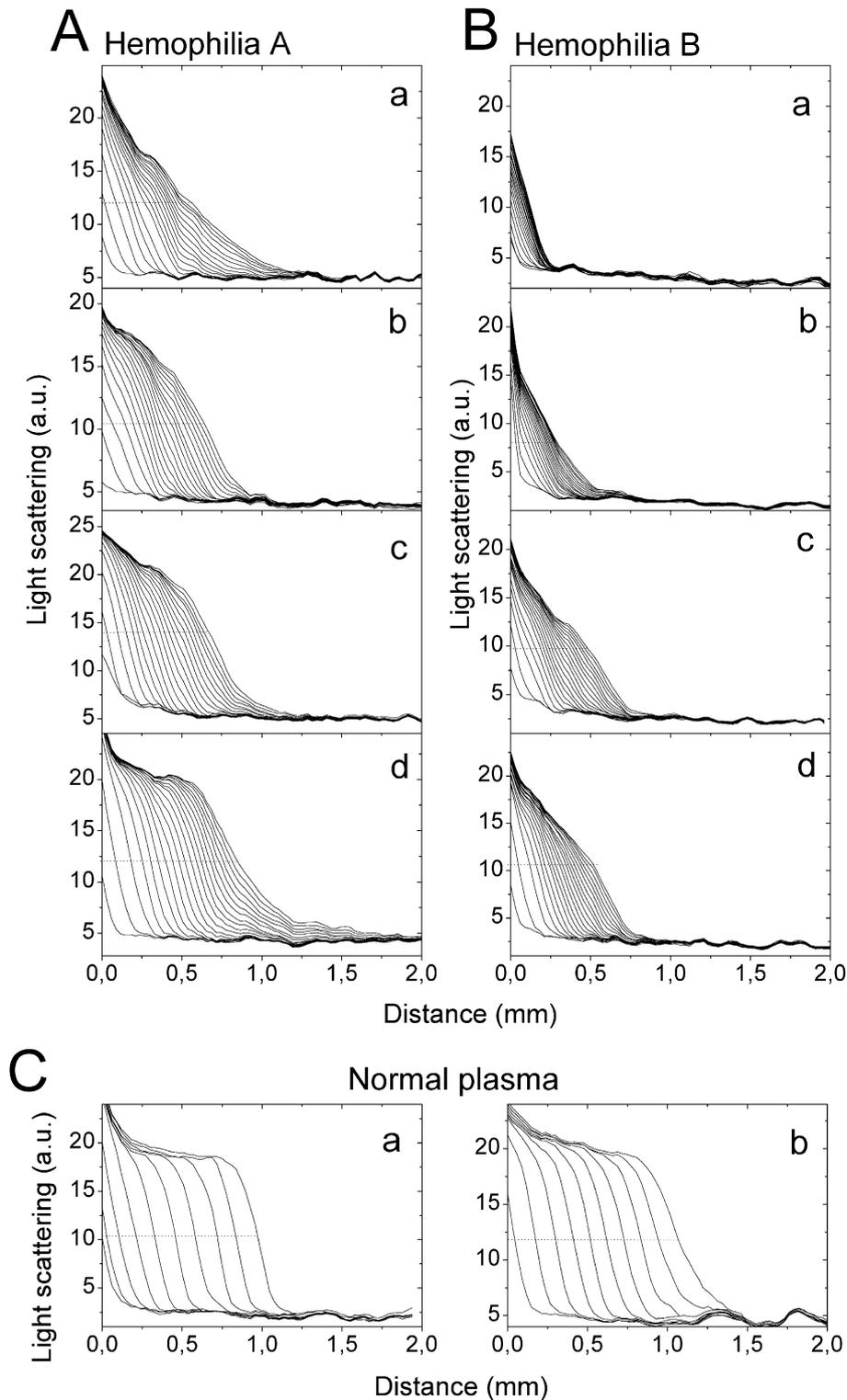
Fig. 6. Evolution of the light-intensity profiles of clots growing in normal and hemophilic plasma on the surfaces of fibroblast monolayers of different densities. (A) Hemophilia A: (a) 5 cells/mm^2; (b) 50 cells/mm^2; (c) 150 cells/mm^2; (d) 1000 cells/mm^2. (B) Hemophilia B: (a) 5 cells/mm^2; (b) 50 cells/mm^2; (c) 150 cells/mm^2; (d) 1000 cells/mm^2. (C) Normal pooled plasma: (a) 1 to 2 cells/mm^2; (b) 450 cells/mm^2. The profiles are separated by 2-min intervals.

the PT of ∼16 s (Table 1), which is shorter than the time required to begin recordings of clot growth (see Section 2.4). We, therefore, concluded that the ITs in hemophilic and normal plasma were comparable, but their

accurate determination was not possible in our experimental system.

It is unlikely that the IT in our experiments might be influenced by the activation produced by a PTF slip itself.
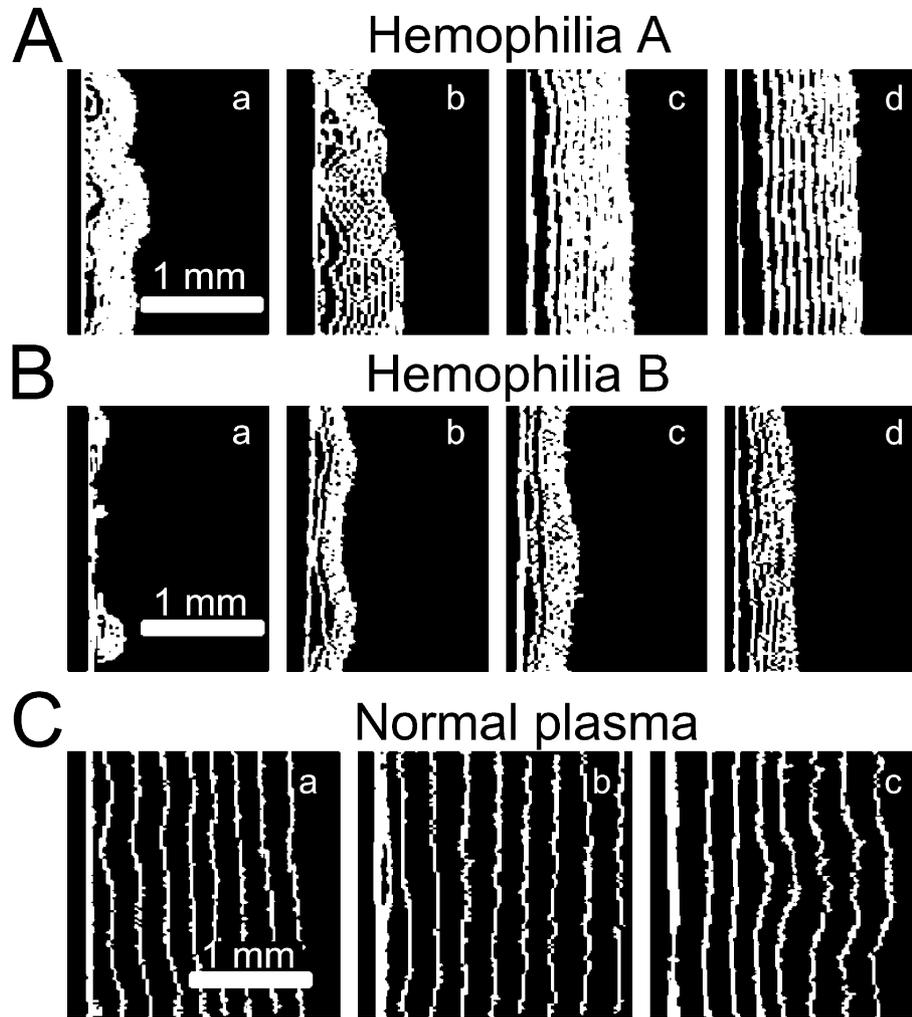
Fig. 7. Dynamics of the clot growth in normal and hemophilic plasma activated by fibroblast monolayers of different densities. Time series of clot contours (see legend to Fig. 3 for the definition of the clot contour). In each panel, the leftmost vertical line corresponds to the activator (fibroblast monolayer) surface; the clot contours are separated by 4-min intervals. (A) Hemophilia A: (a) 5 cells/mm^2; (b) 50 cells/mm^2; (c) 150 cells/mm^2; (d) 1000 cells/mm^2. (B) Hemophilia B: (a) 5 cells/mm^2; (b) 50 cells/mm^2; (c) 150 cells/mm^2; (d) 1000 cells/mm^2. (C) Normal plasma: (a) 5 cells/mm^2; (b) 50 cells/mm^2; (c) 450 cells/mm^2.

As shown above, the PTF is relatively ineffective in activating the clotting response: when induced by PTF, clot growth began 11 min after recalcification or even later. Obviously, the PTF cannot account for the fast response.

The spatial dynamics of clot growth in hemophilic plasma activated via the extrinsic pathway was significantly different from normal. One of the most pronounced differences between hemophilic and normal plasma was in the rate of clot thickening. Clots in hemophilic plasma grew significantly more slowly than in normal plasma (Fig. 5C). In all experiments, the quasistationary rate of clot growth was two to six times lower than normal, and the resulting clots were only 0.5–1.0 mm thick 40 min after recalcification (Table 1). The differences between normal and hemophilic plasmas were even more dramatic when clotting was examined at different fibroblast densities (Fig. 7). The clot growth rate decreased and the clot shape deteriorated with the decreasing cell density. When the cell density was <15 cells/mm^2, the front of the growing clot was no longer flat;

instead, it appeared fragmented into several separate clots. The light scattering profiles of the clots in hemophilic factor-IX-deficient plasma lacked the characteristic "step-function" shape and had less steep slopes (Fig. 6). For all cell densities, these profiles were similar to the profiles seen in glass-activated plasma from a patient with hemophilia B (Fig. 4B). At low cell densities, light scattering profiles of the growing clots were similar in hemophilia B and hemophilia A. However, clotting in plasma from hemophilia A patients improved at high (>130 cells/mm^2) cell densities (compare frames a–d in Fig. 6A). We concluded that both the rate of clot growth and the clot density were considerably decreased in hemophilic plasma.

3.3. Combining hemophilic plasma with normal plasma

If the defective clot growth in hemophilic plasma is due to the deficiencies in clotting factors, then adding a small amount of healthy donor plasma should restore the spatial

characteristics of clotting. To test this suggestion, we examined how clotting proceeded in hemophilic plasma supplemented with up to 10% of normal plasma. In the supplemented plasma, the shape of the light scattering profiles improved and gained more resemblance to normal. Spontaneous clots were also seen as in normal plasma. The average rate of clot growth in the supplemented plasma became 65% of normal.

4. Discussion

Bleeding disorders in hemophilic patients suggest essential, yet unidentified roles for plasma factors VIII and IX in clotting activated by the extrinsic pathway. To dissect their putative roles in determining the dynamic parameters of clot growth, we took advantage of the recently developed experimental system, which allows examination of clot growth in a thin layer of nonstirred plasma [22,29]. Although this approach provides previously unavailable information (e.g., the rate of clot growth), some of the results obtained can be compared with the data provided by homogeneous (stirred) experimental systems. To this end, we will consider clot growth to be a process consisting of two distinct phases, initiation and elongation.

(1) The *initiation phase* is characterized by the IT, which is the lag-period between activation of clotting and its first visible manifestations (fibrin polymerization). By definition, this phase is also characterized by the kinetics of the increase in the light scattering intensity in the immediate vicinity of the activating surface. Since this phase includes the events occurring virtually on the activating surface, the role of diffusion is almost negligible. The characteristics of this phase are, therefore, equivalent to the parameters determined using the conventional homogeneous system, in which the activator is evenly distributed throughout the bulk of plasma. Specifically, an analogy can be drawn between the IT and the "initiation phase," and between the kinetics of an increase in the light scattering and the "propagation phase," introduced by Mann et al. [5,32,33] to describe clotting in homogeneous systems.

(2) The *elongation phase* is characterized by the evolution of the density of a growing clot (this process can be visualized by observing changes in the light scattering intensity [22,29]) and by the rate of clot growth. These parameters are unique and do not have analogues in traditional experimental systems.

4.1. The initiation phase

As previously, we find that the IT for normal plasma activated via the extrinsic pathway is very short (<1 min) and independent of the activity of clotting factors VIII and IX. Indeed, several studies have shown that activation of factor IX by TF does not contribute significantly to the initiation of clotting via the extrinsic pathway [16,17,

20,34,35]. However, contact activation is slower and depends on the activator strength even in normal plasma; the more so for hemophilic plasma deficient in either factor VIII or IX (Table 1). The data are consistent with the well-established roles for these factors in conveying the activation signal from factor XIIa. After a lag-period, clotting in the immediate vicinity of the activator can be detected as an increase in the light scattering intensity. The average light scattering intensity in a 0.2-mm area adjacent to the activator can be plotted versus time to reveal the kinetics of an increase in the clot density in the immediate vicinity of the activator (Fig. 8). In normal plasma, the density of fibrin polymer in this area increases rapidly and levels off in about 5 min after beginning of clotting, whatever the activation pathway. The light-intensity profiles in hemophilic plasma have gentler slopes (implying lower growth rates) and are more dependent on the nature of the activator and its strength (fibroblast density; Fig. 8). These results agree well with a considerable reduction in the fibrinopeptide production in plasma of hemophilia A patients [20].

4.2. The elongation phase

The IT for normal plasma activated via the extrinsic (versus intrinsic) pathway is >10 times shorter. Strikingly, despite this significant difference in timing, the parameters of the elongation phase are remarkably similar for both pathways. Indeed, the light scattering profiles appear identical for activation by glass or PTF (Fig. 4C,D) and by a fibroblast monolayer (Fig. 6C). In normal plasma, a steep front of the clot area of high density progressively advances into the bulk. It appears that the density of fibrin polymer within the clot area is constant. The rate of clot edge movement is 0.039–0.043 mm/min, irrespective of the activation pathway (the difference in the rates is within the experimental error (0.005 mm/min). Moreover, the characteristics of clot growth, including its rate, are almost independent of the amplitude of the activating signal: a 200-fold increase in the fibroblast density does not alter significantly either the light scattering profiles (Fig. 6C(a,b)), or the clot growth rate (Fig. 5C). Together, these data suggest that the kinetics of the initiation phase of clotting is a highly variable parameter, but the characteristics of the elongation phase are fixed. Once initiated, clot growth proceeds by the same scenario, whatever the strength and the nature of the activating signal [22].

The elongation phase of clot growth in hemophilia A and B was significantly abnormal, and the clot grew by a significantly different scenario. The deficiency in either factor VIII or IX is associated with the gradually sloping profiles of light scattering, with the maximum density of fibrin polymer being at the activator surface. The clot grows by broadening the polymerized area, but the maximum density remains at the site of activation and the clot edge appears fuzzy at any time. Addition of 10% normal plasma corrects these defects almost completely, indicating the
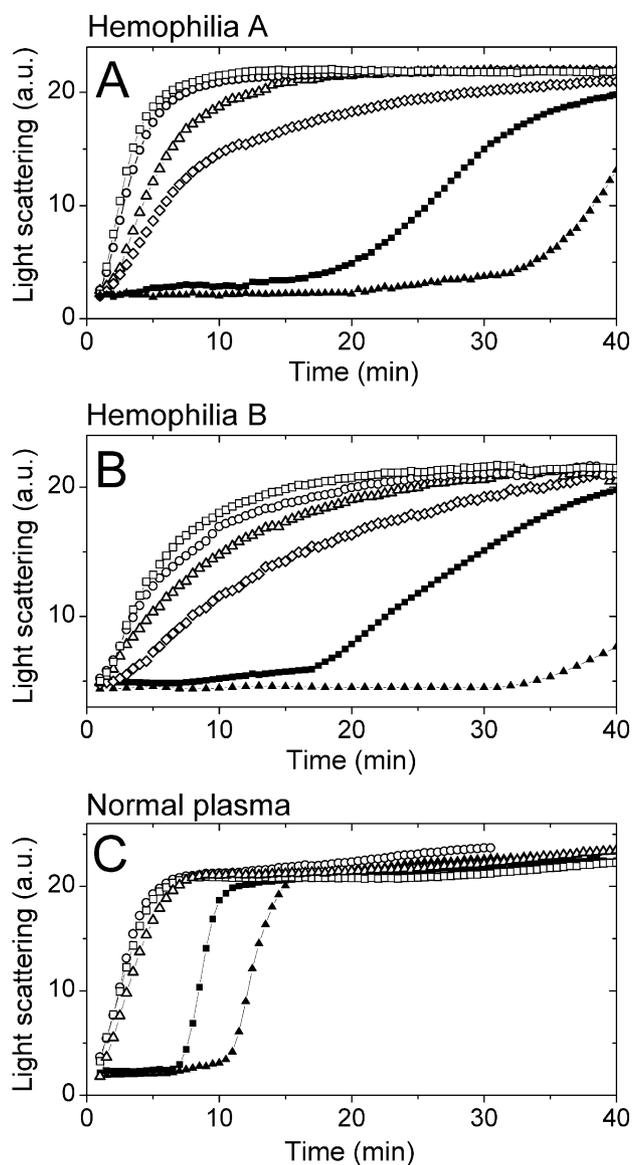
Fig. 8. Average light scattering intensity in a 0.2-mm area adjacent to the activator plotted vs. time. (A,B) Clotting in plasma of patients with hemophilia (A) A or (B) B was activated either by fibroblast monolayers containing (◇) 3–5 cells/mm^2; (△) 50 cells/mm^2; (○) 150 cells/mm^2; or (□) 1000 cells/mm^2 by (■) glass, or by (▲) PTF. (C) Clotting in normal plasma was activated either by fibroblast monolayers containing (△) 5 cells/mm^2; (○) 50 cells/mm^2; (□) 450 cells/mm^2, by (■) glass, or (▲) PTF.

specificity of the observed effects. Strikingly, abnormal clot growth in hemophilic plasma is independent of the activation pathway. Clots grown on the glass or fibroblast monolayer surface were thinner and lighter than normal. These results strongly suggest that both activation pathways require the tenase activity during the elongation phase.

4.3. Role of factor XI activation by thrombin

To gain greater insight into the mechanisms underlying the bleeding tendency in hemophilia, we formulated two testable

hypotheses based on different mechanisms of factor IX activation. Bleeding might be caused by failure of the VIIa–TF complex to produce tenase because of severe deficiency of factor IX or factor VIII [12–14]. Although our results do not rule out some role for this activation loop, this hypothesis is inconsistent with the absence of a delay in the clotting via the extrinsic pathway activated by fibroblast TF in hemophilic plasma. Since factor IX activation is surface-dependent, this hypothesis also cannot explain the abnormal elongation phase in hemophilic plasma. The second model has been suggested by the results of mathematical analysis of the spatial distributions of clotting factors during clot growth [22,23]. Modeling of clotting reactions has revealed the important role for the positive feedback loops of thrombin activation in determining the characteristic parameters of the elongation phase of clot growth. Thrombin is generated at the activator surface and diffuses into the bulk of liquid blood. However, its concentration at some distance away from the activator is higher than expected by diffusion alone. Three positive feedback loops—(1) thrombin-factors XIa–IXa–Xa–thrombin; (2) thrombin-factor VIIIa– tenase–factor Xa–thrombin; and (3) thrombin–factor Va– prothrombinase–thrombin—ensure additional thrombin generation in areas that are not in contact with the activating surface. These positive feedbacks are, therefore, responsible for the rapid spreading of clotting reactions into the liquid blood. Importantly, the last two feedbacks depends on the first, because both tenase and prothrombinase complexes require factors IXa and Xa for their activities. This dependence determines the essential and indispensable role for the factor XI activation by thrombin.

This model explains the observed independence of the parameters of the elongation phase in normal plasma from the activation pathway: clot thickening is determined by the positive feedback reactions and is independent of the initial activation reactions, which occur on the activator surface. According to our hypothesis, a deficiency in any of the factors involved in the feedback loops (such as factors VIII and IX) disrupts the self-sustained thrombin propagation. In hemophilic plasma, thrombin is produced only at the activator surface and spreads out solely by diffusion. The resulting clots grow more slowly, because the rate of diffusion is lower than the rate of self-sustained propagation. The resulting clot shape is determined by the thrombin distribution: the clot density gradually decreases farther away from the surface due to thrombin dilution; therefore, clots are formed thin and with fuzzy edges. In contrast, the contact activation factors (e.g., factor XII) are not involved in the positive feedbacks, and clot thickening should be normal in their absence. Consistent with this prediction, the deficiency in any of these factors is not associated with bleeding disorders [7–10]. In patients with hemophilia A, the initial stages of clot growth appeared quite normal for strong activating signals (high-density monolayers). It is likely that, with a strong activator, the amount of thrombin produced was so great that its propagation solely by diffusion was sufficient to cause normal clotting up to

~ 0.8 mm away from the surface (Fig. 6A(c,d)). However, at longer distances, thrombin dilution became more significant, so that the respective light scattering profiles became less steep, and the clot edge fuzzier.

In summary, the hypothesis of self-sustained thrombin production describes well the presented data on the parameters of clot growth in normal and hemophilic plasma. However, full description of clot growth will certainly be more detailed and complex. For example, the mechanisms responsible for the termination of clot growth remain largely undetermined. Further investigation into the spatial dynamics of the clotting reactions will be required to solve these problems.

Acknowledgements

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