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ORIGINAL ARTICLE

Initiation and propagation of coagulation from tissue factor-bearing cell monolayers to plasma: initiator cells do not regulate spatial growth rate*

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Summary. Exposure of tissue factor (TF)-bearing cells to blood is the initial event in coagulation and intravascular thrombus formation. However, the mechanisms which determine thrombus growth remain poorly understood. To explore whether the procoagulant activity of vessel wall-bound cells regulates thrombus expansion, we studied *in vitro* spatial clot growth initiated by cultured human cells of different types in contact pathway-inhibited, non-flowing human plasma. Human aortic endothelial cells, smooth muscle cells, macrophages and lung fibroblasts differed in their ability to support thrombin generation in microplate assay with peaks of generated thrombin of 60 ± 53 nmol L^{-1}, 135 ± 57 nmol L^{-1}, 218 ± 55 nmol L^{-1} and 407 ± 59 nmol L^{-1} (mean ± SD), respectively. Real-time videomicroscopy revealed the initiation and spatial growth phases of clot formation. Different procoagulant activity of cell monolayers was manifested as up to 4-fold difference in the lag times of clot formation. In contrast, the clot growth rate, which characterized propagation of clotting from the cell surface to plasma, was largely independent of cell type (≤ 30% difference). Experiments with factor VII (FVII)-, FVIII-, FX- or FXI-deficient plasmas and annexin V revealed that (i) cell surface-associated extrinsic Xase was critical for initiation of clotting; (ii) intrinsic Xase regulated only the growth phase; and (iii) the contribution of plasma phospholipid surfaces in the growth phase was predominant. We conclude that the role of TF-bearing initiator cells is limited to the initial stage of clot formation. The functioning of intrinsic Xase in plasma provides the primary mechanism of sustained and far-ranging propagation of coagulation leading to the physical expansion of a fibrin clot.

Keywords: factor VIII, procoagulant activity, regulation, thrombus growth.

Introduction

Tissue factor (TF), a cell surface transmembrane glycoprotein, is the principal initiator of blood coagulation *in vivo* and cells that bear TF play a central role in hemostasis and thrombosis. *In vitro* experiments with tissue extracts [1–3] and cultured cell monolayers [4,5] showed that the procoagulant activity of different cell types, i.e. their ability to clot plasma, is mediated by the cell surface TF and increases with its concentration. Histological analysis of human tissues using antibodies [6–8] or labeled FVIIa [1,2] revealed that under normal conditions TF-bearing cells are physically separated from blood but they 'envelope' the circulatory system. TF is absent on the surface of undisturbed endothelial cells, which form the inner lining of blood vessels, and its concentration increases from smooth muscle cells in the media to fibroblasts in the adventitia [3,6,8]. The highest levels of TF are constitutively expressed by cardiac myocytes, lung fibroblasts, cells within the brain and the placenta, i.e. at sites where protection against hemorrhage after tissue injury is critical [3,6,8]. Under pathological conditions the high level of TF expression by activated vascular cells [endothelial cells (ECs), smooth muscle cells (SMCs), macrophages (MPs)] determines the thrombogenicity of atherosclerotic plaques

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[2,9] and malignant tumors [1,10] and ultimately leads to intravascular fibrin deposition and thrombus formation.

While the role of TF in the initiation of coagulation is well established, the regulation of thrombus propagation by TF-bearing cells of the damaged vessel wall remains poorly understood. Exposure of TF to blood is immediately followed by the formation of a complex between TF and factor VIIa (FVIIa) (extrinsic Xase) that activates both FX and FIX leading to generation of thrombin and fibrin on the surface of TF-bearing cells. To what extent the factors generated on the surface of initiator cells regulate expansion of thrombus is a subject of debate, because the contribution of vessel wall-mediated reactions may be limited by several possible mechanisms. First, FXa generated by extrinsic Xase is rapidly inactivated by antithrombin and tissue factor pathway inhibitor [11,12]. Secondly, there is a high affinity binding between thrombin and fibrin and thus the formed fibrin clot may limit diffusion of thrombin from the cell surface [13,14]. Thirdly, blood flow dilutes activated coagulation factors and may effectively limit their diffusion [15]. Also, deposition of 'blood-borne' TF from flowing blood may support FIX and FX activation on the luminal surface of a growing thrombus independently of TF-bearing cells [16,17]. However, it remains unknown which of these mechanisms is operative *in vivo*. In particular, the relationship between the concentration of vessel wall-bound TF and the rate of thrombus growth has not been investigated.

The present study was designed to explore the role of the procoagulant activity of vessel wall-bound cells in thrombus growth. In our *in vitro* model, the clotting was initiated by various human TF-expressing cells, and expansion of clotting was monitored by a real-time videomicroscopy system, which we have described previously [18]. To highlight the contribution of the cell monolayer-mediated reactions, the clotting was studied in platelet-free plasma in the absence of flow. We found that even under these favorable conditions the initiator cells regulate the initiation but not the rate of clot growth. Experiments with coagulation factor deficient plasmas revealed that the primary mechanism of clot expansion is provided by the intrinsic (FIXa/FVIIIa) Xase complex in plasma. These results further indicate that the intrinsic pathway of blood coagulation may be a target for an effective and safe antithrombotic therapy.

Materials and methods

Materials

Plasma-derived FVIII was purified as described previously [19]. Plasma-derived FVII, activated FVII and corn trypsin inhibitor (CTI) were purchased from Enzyme Research Laboratories (South Bend, IN, USA). Human recombinant TF was purchased from Baxter (Innovin®, Deerfield, IL, USA); annexin V from Medical and Biological Laboratories (Nagano, Japan); and fluorogenic thrombin substrate Z-GGR-AMC from Bachem (Torrance, CA, USA). Human thrombin, D-Phe-Pro-Arg chloro-chloro-methylketone, lactic acid and AMC were from Sigma (St Louis, MO, USA). Inhibitory monoclonal antibody to TF (no. 4509) and Actichrome® TF chromogenic activity kit were from American Diagnostica Inc (Stamford, CT, USA). Frozen human pooled FVII, FVIII, FX, FXI and fibrinogen congenitally deficient plasmas and normal standard plasma, all obtained by apheresis procedure, were from George King Bio-Medical, Inc. (Overland Park, KS, USA).

Cell culture

Primary cultures of human aortic SMCs, human aortic ECs and human fetal lung fibroblasts (LFs) were purchased from Cambrex (Walkersville, MD, USA) and were used at passages 4–10. Cell monolayers were grown to 60–80% confluence in 96-well microtiter plates and on treated polyethylene terephtalate slips placed on the bottom of 12-well plates as previously described [18]. SMCs, ECs and LFs were propagated in SmGM-2 BulletKit complete medium supplemented with 10% fetal bovine serum (FBS, Cambrex), in EGM-2 BulletKit supplemented with 2% FBS and in FGM-2 BulletKit supplemented with 2% FBS, respectively, at 37 °C.

Human monocytes were isolated from mononuclear leukocyte preparations obtained by apheresis procedure performed under an approved Institutional Review Board protocol. The population of monocytes was enriched to 97% by positive selection on CD14 beads (Miltenyi, Auburn, CA, USA). Differentiation of monocytes into macrophages was promoted by addition of macrophage colony-stimulating factor (Sigma) in X-VIVO 15 growth medium (Cambrex) supplemented with 10% FBS [20]. Cells were seeded at 10^4 cells cm^2.

One day prior to experiments, cells were transferred to corresponding serum-free [for ECs, MPs and LFs] or low-serum (0.5% FBS for SMCs) medium. In some experiments, cells were preincubated with annexin V (100–500 nmol L^{-1}), anti-TF antibody (50 μg mL^{-1}) or FVIIa (25 pmol L^{-1} – 1.7 μmol L^{-1}) in washing buffer [10 mmol L^{-1} HEPES, 137 mmol L^{-1} NaCl, 4 mmol L^{-1} KCl, 2.5 mmol L^{-1} $CaCl_2$, 11 mmol L^{-1} alpha-D-glucose, 1 mg mL^{-1} bovine serum albumin (BSA)] for 40, 40 or 15 min, respectively. Cells were washed three times with washing buffer and used in experiments.

Blood collection and plasma preparation

Normal pooled plasma (NPP, three donors) was prepared from human blood under an approved Institutional Review Board protocol, American Red Cross. Approximately 450 mL of whole blood were collected by standard phlebotomy procedure into PL146 blood packs containing CPD anticoagulant (Baxter Healthcare, Deerfield, IL, USA). Blood was centrifuged twice at 2500 ***g*** for 15 min at room temperature to remove erythrocytes and platelets. Platelet-poor plasma was additionally centrifuged at 3000 ***g*** for 25 min to remove residual platelets. In some experiments, the plasma was depleted from

remnant platelets and cell-derived microparticles by ultracentrifugation at 100 000 ***g*** for 1 h at 21 °C [21,22]. The pH of the plasma was stabilized at 7.2–7.4 by lactic acid treatment [18], and the plasma was stored at −80 °C. After thawing, NPP was left to settle for 2–3 h at room temperature to avoid the effects of cold-induced contact activation of FXII and FVII [23]. Commercial frozen normal and deficient plasmas were subjected to a pH stabilizing procedure after thawing and treated similarly. Ten minutes prior to experiments, the plasmas were supplemented with CTI to a final concentration of 0.1–0.2 mg mL^{-1}, which was a saturation inhibitory concentration in activated partial thromboplastin time (APTT) assay. In some experiments, the plasma was supplemented with FVII, FVIII, NPP, annexin V, recombinant TF or anti-TF antibody. Total dilution of plasma with all compounds was < 10%.

Thrombin and fibrin generation assays

Thrombin and fibrin generation assays were performed in parallel experiments on cell monolayers grown in 96-well plates according to Hemker *et al.* [24] and Nagashima [25], with some modifications. Plasma (90 μL, 90% v/v) was supplemented with CTI (2%, final concentration 0.1–0.2 mg mL^{-1}), fluorogenic substrate Z-GGR-AMC (1.25%, 800 μmol L^{-1}), $CaCl_2$ (2.5%, 20 mmol L^{-1}) and buffer (20 mmol L^{-1} HEPES, 0.15 mol L^{-1} NaCl, 0.1% BSA, pH 7.4). The reaction was started by adding plasma to wells ($t = 0$). The fluorometric measurements (λ_{ex} 380 nm, λ_{em} 440 nm, each 40 s) were performed in a SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA, USA) and photometric measurements (410 nm) in a SpectraMax 384 plus (Molecular Devices) microplate reader at 37 °C. Thrombin concentration was calculated from the rates of substrate cleavage using a calibration curve. The rate of substrate cleavage was linearly proportional to dilution of active site titrated thrombin [25] in the range of 0.1–1200 nmol L^{-1}. The lag time of fibrin generation was defined as the time interval required for the solution turbidity to reach the half-maximal value. The peak of thrombin concentration and fibrin generation lag time were calculated for each well independently, and the mean values for four replicates for each condition were calculated. A control experiment was performed to prove that the presence of fluorogenic substrate does not alter the sensitivity of the assays to the concentration of TF. Plasma was supplemented with varying concentrations of fluorogenic substrate, and the clotting was initiated by addition of diluted lipidated TF (Innovin). The relative ratio of thrombin peaks measured for different concentrations of TF was not dependent on the substrate concentration in the plasma (200–1600 μmol L^{-1}).

Spatial clot growth

Spatial parameters of clot formation were studied using light-scattering videomicroscopy system [18]. A microchamber was assembled in a 35-mm polystyrene Petri dish (Corning, NY, USA) around a 1 mm-thick microscope glass slide (Fisher, Vernon Hills, IL, USA) fixed to its bottom. The glass slide edge, which formed a vertical wall of a chamber, was wrapped around with a cell-coated polyethylene terephtalate slip and covered with a piece of black polystyrene which formed the upper surface of the microchamber. Recalcified ($CaCl_2$, 20 mmol L^{-1} final concentration) human plasma was transferred into the assembled chamber ($t = 0$), and the dish was sealed and placed to a temperature-controlled water jacket (37 °C). The microchamber was illuminated from below with red light-emitting diodes (L-1543-E, peak wavelength 660 nm; Kingbright, Tokyo, Japan) at 135° to the direction of recording. The light-scattering image from a 7.2 × 5.4-mm microchamber area was recorded every 30 s by OS-75D camera (Mintron Enterprise, Hsinchuang, Taiwan) coupled to a EZ98 framegrabber (Lifeview Inc., Freemont, CA, USA) and processed as described below.

Image processing

The parameters of spatial clot formation were determined from the experimental image series as described in [18,26]. Briefly, clot formation in the microchamber was considered as a process spatially separated into the initiation and growth phases. The initiation phase was defined as the kinetics of fibrin formation in the close vicinity to the cell monolayer. A 0.2 mm × 0.2 mm area adjacent to the cell surface was selected on the first frame, and the mean light scattering intensity within this square area was plotted vs. time. From this plot the lag time of clot formation was defined as the time interval required for the mean light scattering intensity to reach the half-maximal value. The growth phase of clot formation was characterized by the rate at which the fibrin network extends away from the cell monolayer. For every frame, the clot size was determined as a distance between the initiator cells and the edge of the clot (the clot edge was defined as the point where the light scattering intensity was equal to the half-maximal value [18]). Then, the steady-state rate of clot growth was derived from the clot size vs. time curve as a mean rate in the range of 10–35 min following the onset of clotting. The described measurements were performed in three different parts of image for each set of experimental images.

Results

Different procoagulant activity of cell monolayers

For studies of the regulation of clot growth by TF-bearing cells (initiator cells), we cultured intravascular and extravascular cell types, which were previously shown to express markedly different levels of TF. We characterized the procoagulant activity of human aortic ECs (minimal TF expression [1,6,27]), SMCs and MPs (medium TF expression [7,27]) and LFs (highest TF expression [3,4,6,8]) by the ability of cell monolayers to induce thrombin generation and coagulation in human plasma containing a fluorogenic substrate for thrombin. Figure 1 depicts a representative microplate assay experiment showing time courses of generation of free thrombin

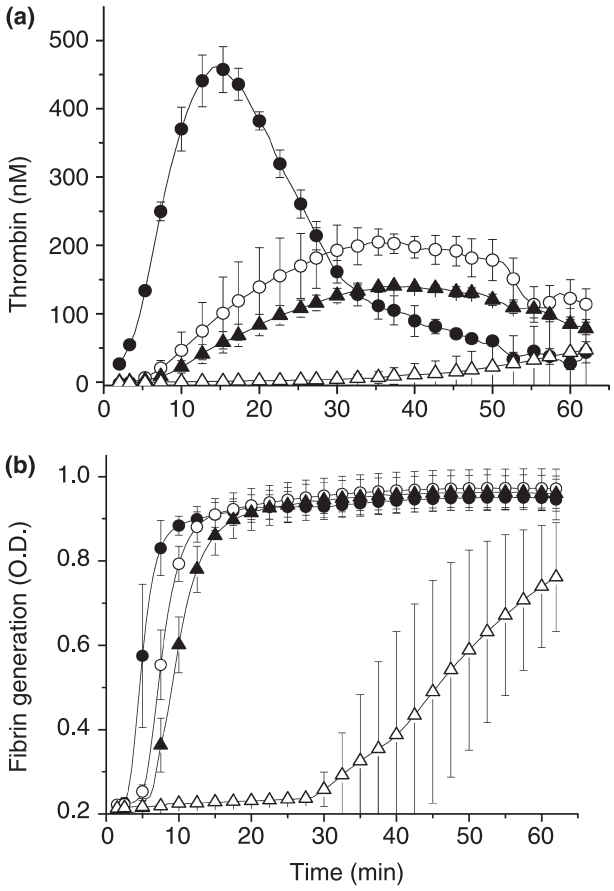

Fig. 1. Assessment of procoagulant activity of cell monolayers. CTI-inhibited human normal pooled plasma containing fluorogenic substrate for thrombin (ZGGR-AMC) was brought into contact with monolayers of human LFs (●), MPs (○), aortic SMCs (▲) and ECs (△) grown in microtiter plates. Thrombin generation (a) and fibrin formation (b) were monitored in parallel experiments on photometric and fluorometric readers as described in Materials and methods. Symbols correspond to each fifth point from kinetic data (mean ± SD, $n = 3$). Figure is representative of four to eight independent experiments for each condition.

(Fig. 1a) and fibrin formation (Fig. 1b). The contact of plasma with cells initiated thrombin generation, the rate of which was maximal with LFs and minimal with ECs. Time-to-peak thrombin concentration was significantly different between LFs (15.2 ± 0.9 min, mean ± SD, $n = 4$), MPs (36.3 ± 10.4 min) and ECs (65.6 ± 12.3 min), while there was no significant difference between MPs and SMCs (see Table 1). Peak thrombin concentration also depended on the cell type, with LFs and ECs showing the highest (407 ± 59 nmol L^{-1}) and the lowest (60 ± 53 nmol L^{-1}) levels of thrombin, respectively (see Table 1). The specificity of these assays to the TF-mediated procoagulant activity (PCA) was confirmed by a virtual absence of coagulation on SMCs and MPs when FVII-deficient plasma was used instead of normal pooled plasma (data not shown).

As follows from the comparison of Fig. 1a and b, the lag times of clot formation correlated inversely with peak thrombin concentrations. The shortest and the longest lag times were registered for LFs and ECs (4.2 ± 0.8 min vs. 43.1 ± 11.7 min, respectively) in accordance with their highest and lowest thrombin-generating activities (see Table 1).

Procoagulant activity of cell monolayers has a minor effect on clot growth

Growth of the fibrin clot from various cell monolayers into non-flowing human normal plasma was monitored in real-time using light-scattering videomicroscopy [18]. To ensure that coagulation was induced exclusively by the TF-bearing cell surface, plasma was supplemented with CTI, an inhibitor of the contact pathway. Significant suppression of contact-driven coagulation was confirmed by the absence of clotting during a 30-min interval in control experiments under the same conditions in the absence of cells (data not shown).

Typical light scattering images of spatial clot growth are presented in Fig. 2a. For the characterization of coagulation events occurring on the cell surface, we monitored an increase in the light scattering in 0.2 mm square areas immediately adjacent to the cell monolayer. The kinetics of clotting on the cell surface (Fig. 2b) was similar to that observed in the fibrin generation microplate assay (Fig. 1b). The lag time was used as a parameter describing the initiation phase of spatial clot growth (Table 1). The shortest lag time was observed for LFs (3.1 ± 0.1 min, mean ± SD, $n = 3$) and the longest lag time

Table 1 Effect of cell monolayer PCA on parameters of clotting in microplate and clot growth assays

	Microplate assays			Clot growth	
	Fibrin generation lag time, min***	Peak thrombin activity, nmol L^{-1}	Time-to-peak of thrombin activity, min	Clot formation lag time, min***	Clot growth rate, μm min^{-1}
LFs	4.2 ± 0.8	407 ± 59	15.2 ± 0.9	3.1 ± 0.1	39.4 ± 4.0
MPs	7.6 ± 1.1*	218 ± 55*	36.3 ± 10.4*	5.5 ± 1.0*	35.2 ± 3.8**
SMCs	9.6 ± 3.0*	135 ± 57*	41.4 ± 8.3*	4.8 ± 1.9	35.5 ± 1.7**
ECs	43.1 ± 11.7*	60 ± 53*	65.6 ± 12.3*	11.6 ± 6.1**	29.5 ± 3.4**

Parameters of fibrin and thrombin generation and of clot growth were determined as described in Materials and methods. For each parameter, mean ± SD for independent experiments ($n = 4$–8) is shown. *,** Differences between LFs and other cells were assessed by unpaired t-test with P-values of < 0.003 (*) and < 0.03 (**). ECs were significantly different from MPs ($P < 0.03$). *** Control microplate experiment with varying plasma sample volume revealed the dependence of the lag time on the ratio of plasma volume to the cell monolayer surface. This ratio was higher in a microtiter well than in a microchamber, thus accounting for longer lag times in microplate assays in comparison with the clot growth experiments.

for ECs (11.6 ± 6.1 min) that is consistent with the procoagulant activity of the initiator cells.

Growth of the fibrin clot from the cell monolayers into the plasma volume was characterized by an increase of clot size plotted vs. time (Fig. 2c). As evidenced from the slopes of the curves, the initial clot growth rate depended on the procoagulant activity of initiator cells, being 2-fold higher for LFs than for ECs. However, the steady-state clot growth rate in normal plasma, which was reached within 10–15 min, proved to be very close for all cell types, irrespective of their different procoagulant activity (Table 1). Thus, 3–7-fold differences in the parameters of thrombin and fibrin generation on the cell surface resulted in only a 1.3-fold difference in the rates of spatial clot growth, indicating that clot expansion is not primarily regulated by the procoagulant activity of initiator cells.

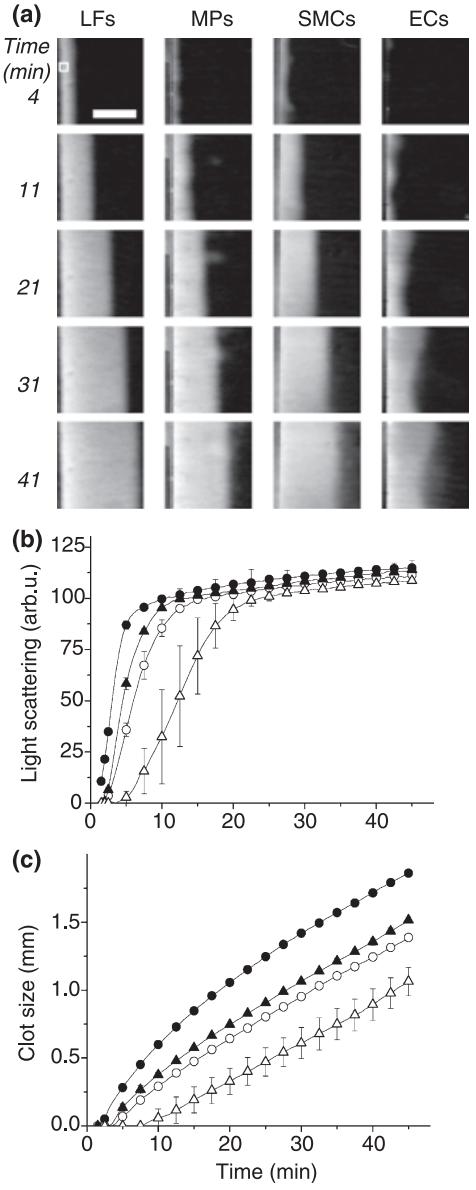

Fig. 2. Effect of cell procoagulant activity on spatial clot growth in normal plasma. CTI-inhibited plasma was brought into contact with monolayers of human LFs, MPs, aortic SMCs and ECs grown on polyethylene terephtalate slips. (a) Light-scattering time-lapse images of clot growth initiated by indicated cell monolayers. Cell-coated slip is seen as a vertical black strip on the left side of each image. For visualization, light-scattering data [about 120 arbitrary units (arb. u.) of digital brightness] were linearly extended to full grayscale range (256 arb. u.) uniformly for each image in set. Bar: 1 mm. (b) Initiation phase of clot formation. Light-scattering in the 0.2 mm × 0.2 mm area adjacent to the cell monolayer [example shown in the first frame of (a)] plotted vs. time. (c) Growth phase. Clot size is plotted vs. time. The symbols in (b) and (c): (●) – LFs (○) – MPs (▲) – SMCs (△) – ECs. Symbols correspond to each fifth point from kinetic data (mean ± SD, $n = 3$). Figure is representative of four to eight independent experiments for each condition.

Fibrin formation does not regulate clot growth

Previous experimental studies suggested that fibrin(ogen) may regulate the propagation of the coagulation process via a high affinity binding of generated thrombin and FXa to the formed fibrin clot [13,14]. Therefore, the limited effect of initiator cells on the clot growth rate might be attributed to the inhibition of thrombin diffusion from the cell surface by the fibrin clot. To test this mechanism, we studied clot growth in plasma containing varying concentrations of fibrinogen to model different binding capacities of clots. Clot growth was initiated by LFs, MPs and ECs in fibrinogen-deficient plasma supplemented with normal standard plasma (George King Bio-Medical, Inc.) as a source of fibrinogen. As follows from Fig. 3a, the light-scattering of the growing clots was linearly proportional to the fibrinogen concentration reflecting different densities of formed fibrin clots. The linear dependence of the clot optical density on fibrinogen concentration was further confirmed in an independent control microplate assay (Fig. 3a, ×). For all cell types, the clot density did not affect the lag times of clot formation (Fig. 3b). Importantly, the rates of clot growth did not increase with a decrease of fibrinogen concentration to 10% of the normal plasma value (Fig. 3c). These results indicate that binding of thrombin to fibrin(ogen) can not explain the limited effect of the TF-mediated PCA on clot propagation.

Different roles of extrinsic and intrinsic coagulation pathways in clot growth

The limited effect of PCA of initiator cells on the clot growth rate may also indicate that clot growth is governed by the coagulation reactions that are not associated with TF-bearing cells. Therefore, we studied clot growth in plasmas deficient in individual coagulation factors to identify these essential regulatory pathways.

Figure 4a shows selected frames of clot growth following contact of deficient plasmas with monolayers of MPs. The initiation of clotting was inhibited in the absence of FVII and FX but was unaffected in the absence of FVIII and FXI (Fig. 4b). The clot growth rate was reduced by 7–10-fold in FVII- and FX-deficient plasmas, by 2-fold in FVIII-deficient plasma and was only slightly reduced in FXI-deficient plasma compared to normal plasma (Fig. 4c). Thus, initiator cell-independent generation of thrombin via FXI-mediated

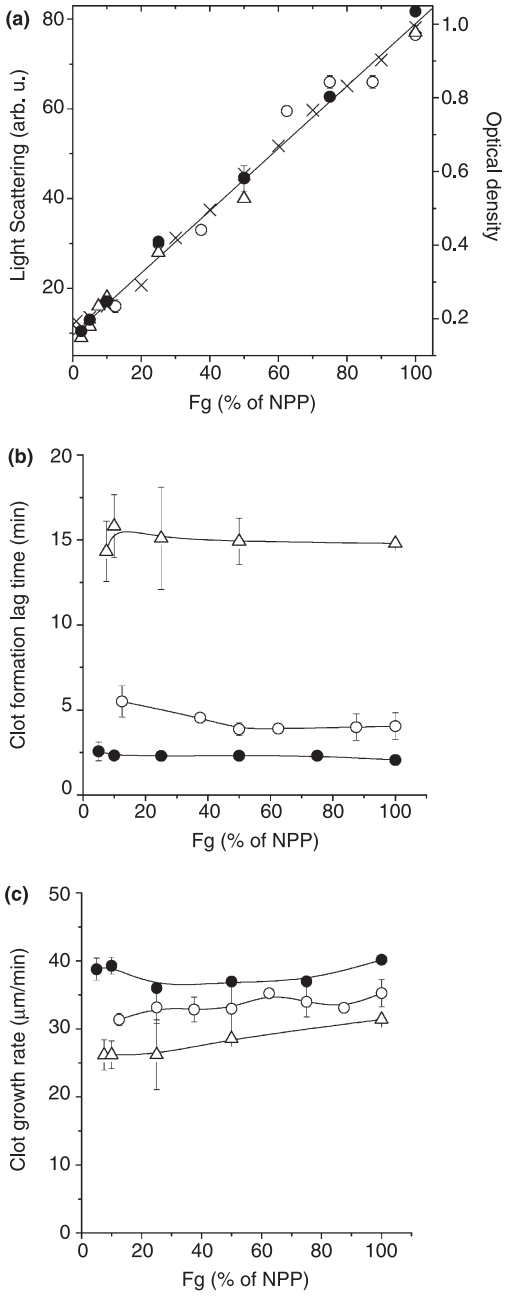

Fig. 3. Effect of fibrin(ogen) concentration on kinetics of clot growth. CTI-inhibited fibrinogen-deficient plasma (Fg concentration < 15 mg dL^{-1}) was supplemented with normal standard plasma (Fg concentration 320 mg dL^{-1}) to provide increasing concentrations of fibrinogen. Clotting was initiated by monolayers of LFs (●), MPs (○) and ECs (△). (a) Effect of fibrinogen concentration on the light-scattering of growing fibrin clots 40 min after initiation of clotting. (×) – optical density of fibrin clot in control microplate experiment with plasma mixed with diluted thromboplastin (1 : 20 000). (b, c) Lag times and steady-state rates of clot growth; mean ± SD.

pathways (FXIIa- or thrombin-driven activation of FXI) does not significantly contribute to clot formation in this experimental system. In contrast, the initiation of clot formation on TF-bearing cells is solely regulated by plasma components of the extrinsic Xase complex (TF/FVIIa/FX). For spatial clot growth, components of both extrinsic and intrinsic (FIXa/FVIIIa/FX) Xases are essential.

In recent years it has been established that plasma contains 'blood-borne' TF and this TF may support clot propagation [16]. The TF activity (measured by Actichrome TF functional assay) was comparable for healthy donor plasmas (7.3 ± 2.4 pmol L^{-1}, $n = 6$), FVIII-deficient plasmas (6.1 ± 0.1 pmol L^{-1}, $n = 2$) and FVII-deficient plasma (5.8 ± 0.01 pmol L^{-1}, $n = 1$), suggesting that TF content is not responsible for the decreased clot growth rate in deficient plasmas. Furthermore, preincubation of normal plasma with an inhibitory anti-TF antibody no. 4509 (50 μg mL^{-1}) had no effect on the clot growth rate, whereas preincubation of initiator SMCs with this antibody resulted in a 2-fold prolongation of the clotting lag time. To assess the contribution of plasma TF in clot propagation, we studied clot growth on monolayers of LFs and ECs in plasma supplemented with increasing concentrations of lipidated TF (Innovin). Exogenous TF stimulated the cell-independent clotting in plasma in a dose-dependent manner throughout the microcuvette but the initiation and growth phases of the cell-initiated clotting remained unchanged (data not shown). Collectively, these data show that plasma-associated TF does not contribute to the propagation of clotting in these experiments.

To explore specifically the contribution of initiator cell-bound extrinsic Xase, we studied spatial clot growth in FVII-deficient plasma under conditions when the TF/FVIIa complex was preassembled on the cell surface. In control experiment, no clotting was observed in normal plasma for as long as 30 min when slips without cells were preincubated with activated FVII (240 nmol L^{-1}, data not shown). As follows from Fig. 5a, non-treated SMCs did not initiate clot formation in FVII-deficient plasma but preincubation of the cells with FVIIa restored both the initiation and the growth of fibrin clots in a dose-dependent manner. Normalization of the lag time was observed at approximately 10 nmol L^{-1} of FVIIa, which is the mean concentration of fVII in normal plasma (Fig. 5b). In another control experiment, supplementation of FVII-deficient plasma with purified FVII at 1 U mL^{-1} restored the clot growth parameters on non-treated cells to the values observed in normal plasma (Fig. 5b,c). Thus, cell surface-assembled extrinsic Xase is indispensable for the initiation of clotting on TF-bearing cells and contributes to spatial clot growth.

To study the role of intrinsic Xase in spatial clot growth, we performed experiments in FVIII-deficient plasma supplemented with increasing concentrations of FVIII (Fig. 6). In accordance with the procoagulant activity of cell monolayers (Fig. 1), the clotting lag times in FVIII-deficient plasma were shortest for LFs and longest for ECs. Compensation for FVIII deficiency had no effect on the lag time values (Fig. 6a). In contrast to the initiation phase, the steady-state clot growth rate correlated with PCA of initiator cells only in the absence of fVIII being 10-fold higher for LFs compared to ECs (Fig. 6b). Compensation for FVIII deficiency led to a dose-dependent increase in the rate of clot growth for all types of initiator cells.

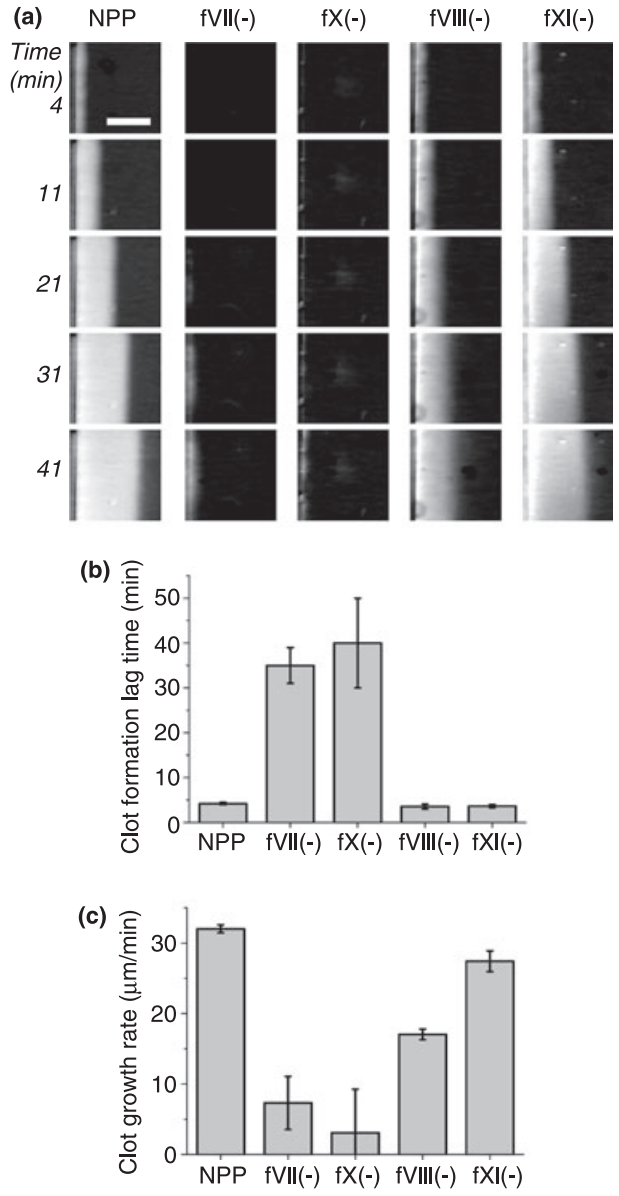

Fig. 4. Effect of deficiency of single coagulation factor on spatial clot growth. Clot growth in CTI-inhibited normal plasma and plasmas congenitally deficient in the indicated coagulation factor was initiated by monolayers of MPs. (a) Light-scattering time-lapse images of clot growth. Bar: 1 mm. (b, c) Lag times and steady-state rates of clot growth; mean ± SD ($n = 2$).

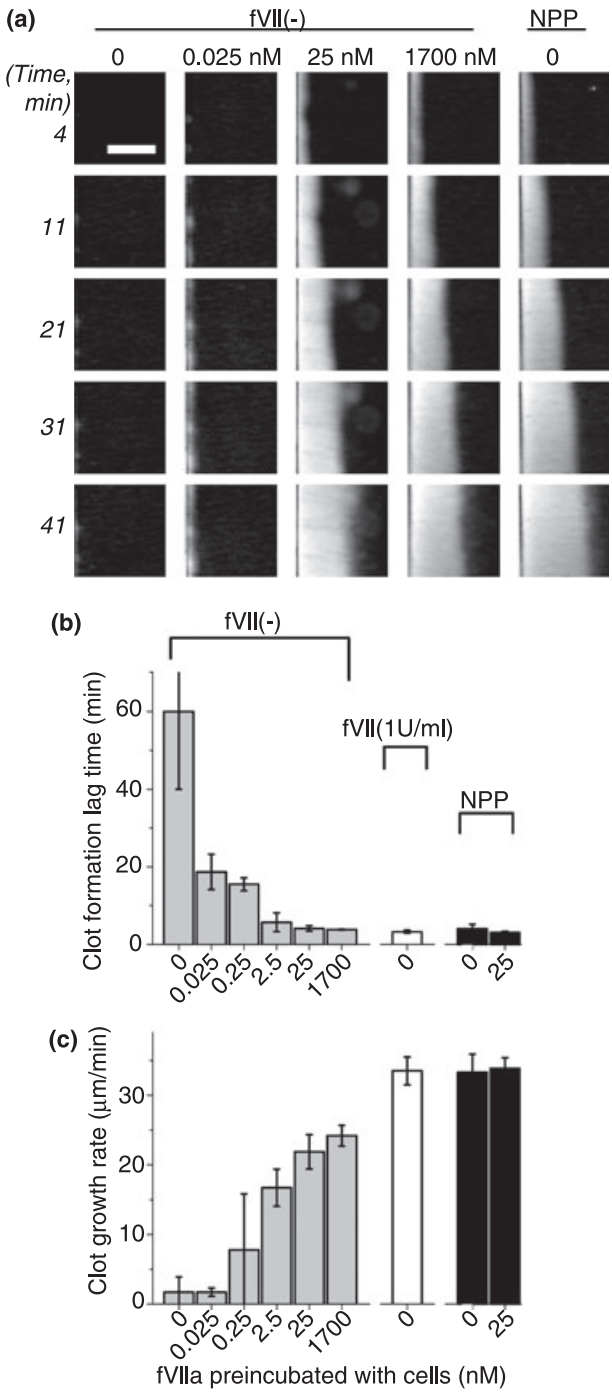

Fig. 5. Regulation of clot growth by cell-bound extrinsic Xase. Extrinsic Xase (TF/FVIIa complex) was preassembled on the surface of SMCs by preincubating cell monolayers with the indicated concentrations of FVIIa. (a) Light-scattering time-lapse images of clot growth in CTI-inhibited FVII-deficient or normal pooled plasma. Bar: 1 mm. (b, c) Lag times and steady-state clot growth rates in FVII-deficient plasma (gray bars), FVII-deficient plasma supplemented with purified FVII (white bars) and normal pooled plasma (black bars); mean ± SD ($n = 2$).

Importantly, similar rate values were achieved at the normal level of fVIII (1 U mL^{-1}): the difference between the growth rates for LFs and ECs was only 30%. Thus, in the presence of the functionally active intrinsic Xase, the clot growth rate becomes independent of PCA of the initiator cells.

Clot growth is regulated by plasma-associated lipid surfaces

The assembly of coagulation complexes requires a phosphatidyl serine (PS)-containing lipid surface, which is provided by TF-bearing cells and cells or lipid vesicles present in the circulation. To localize the surfaces that are essential for clot growth in our experimental system, we used annexin V to

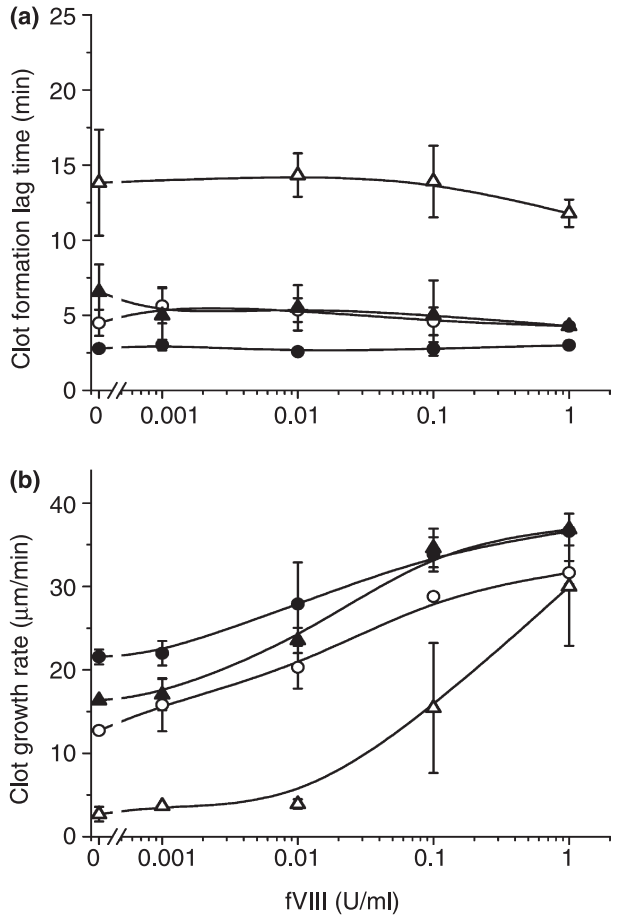

Fig. 6. Effects of fVIII level on initiation and spatial clot growth. (a, b) Dependence of lag times and steady-state rates of clot growth on FVIII concentration; mean ± SD ($n = 2$). Clotting was initiated by monolayers of LFs (●), MPs (○), SMCs (▲) and ECs (△) in CTI-inhibited FVIII-deficient plasma supplemented with indicated concentrations of FVIII.

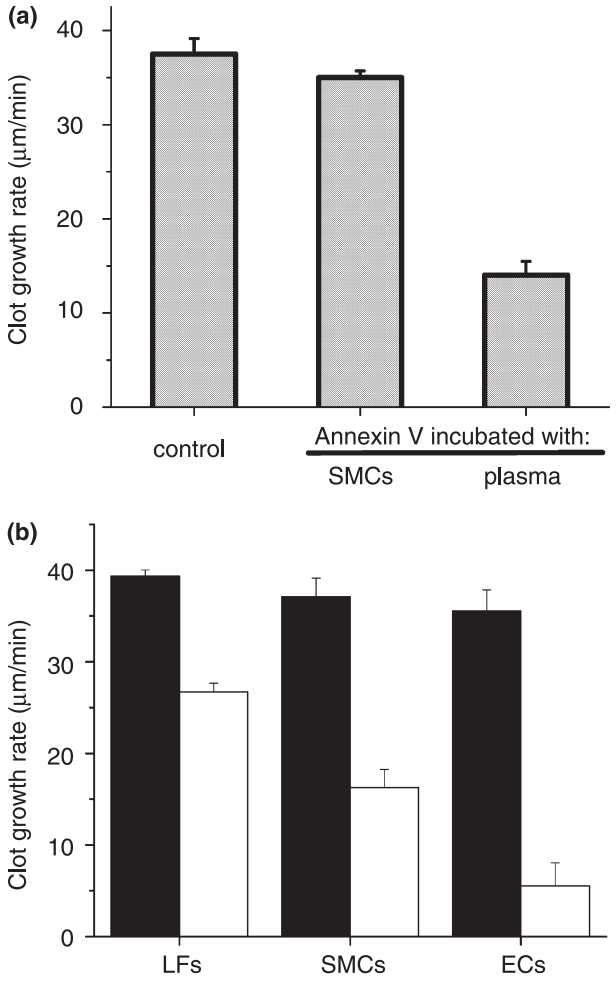

Fig. 7. Role of cell monolayer- vs. plasma-associated coagulation reactions in spatial clot growth. (a) Effect of blocking of TF-bearing cell- and plasma-associated phospholipids by annexin V on the clot growth rate initiated by SMCs. Either SMC monolayer or CTI-inhibited plasma was incubated with annexin V (100 nmol L^{-1}) for 40 min before experiment. (b) Effect of the removal of remnant platelets and cell-derived lipid microparticles on clot growth initiated by the indicated cell monolayers. Black bars: normal pooled plasma; open bars: plasma after ultracentrifugation procedure; mean ± SD ($n = 2$).

selectively block PS either on cell monolayers or in plasma (Fig. 7a). Preincubation of SMC monolayers with up to 500 nmol L^{-1} of annexin V did not affect the propagation of clotting and only slightly prolonged the initiation phase (less than 1.4-fold as compared to the control non-treated cells). In contrast, preincubation of plasma with a lower concentration of annexin V (100 nmol L^{-1}) inhibited the clot growth rate by 60% (Fig. 7a) but had a minor effect on the initiation (not shown). These results suggested the dominant role of plasma lipids in the propagation of clot growth.

In further experiments, remnant platelets and cell-derived microparticles, which provide a considerable amount of phospholipids in platelet-free plasma, were sedimented by ultracentifugation [21,22]. This resulted in a significant reduction of the steady-state rate of clot growth from monolayers of LFs, SMCs and ECs. Importantly, in lipid-depleted plasma the clot growth rate correlated with PCA of the initiator cells (Fig. 7b). We conclude that spatial growth of fibrin clot is significantly supported by coagulation reactions that proceed on plasma phospholipid surfaces but not on TF-bearing cells.

Discussion

In the present study we attempted to explore to what extent the procoagulant activity of vessel wall-bound cells regulates thrombus expansion, an important phenomenon of the blood coagulation process. Using an *in vitro* model of thrombus formation, we showed that the rate of clot growth is not determined by TF-bearing initiator cells.

In our experimental system, coagulation in non-flowing plasma was induced by monolayers of human vascular and extravascular cells representing different physiological sources of TF. Lung fibroblasts were chosen as one of cell types expressing the highest TF levels *in vivo* and *in vitro* [3,4,6,8], whereas aortic endothelial cells are known to lack TF antigen *in vivo* under normal conditions [1,6] and only a minimal

TF concentration is associated with these cells in culture [27]. Consistent with different levels of TF expression, the four cell types used in this study demonstrated an up to 6-fold difference in their thrombin generating ability. Importantly, although the markedly different procoagulant activity was manifested as a 4-fold earlier onset of clotting on highly procoagulant LFs than on ECs, the difference in respective rates of clot propagation did not exceed 30%.

Previously, several mechanisms were suggested that may 'mask' the role of vessel wall-bound cells in the formation of large thrombi. Therefore, in our experimental design we excluded blood flow to avoid dilution of activated coagulation factors and deposition of blood-borne TF from the circulation to the luminal surface of the thrombus. Plasma without platelets was used to further highlight the initiator cell-driven coagulation reactions. We also showed that a 10-fold reduction of the fibrin clot density had no effect on the clot growth rate for all cell types. In similar experiments with plasma from fibrinogen knockout mice, Kerlin *et al*. [28] observed that this low concentration of fibrin binds less than 10% of thrombin activity. Thus, the sequestering of thrombin by a fibrin clot [13,14] was not the factor limiting the contribution of TF-bearing cells in clot expansion.

The current study suggests that initiator cells do not determine the rate of clot propagation because the contribution of coagulation reactions, which involve intrinsic Xase complex and occur in plasma, prevails. This conclusion is based on the following observations. First, in the absence of either FVIII (Fig. 5b) or plasma phospholipids (Fig. 7b) the clot growth rate was low and correlated well with the procoagulant activity of the cell monolayers. Importantly, compensation for FVIII deficiency led to the very close clot growth rates for all cell types independently of their procoagulant activity. Secondly, experiments with annexin V demonstrated a predominant role of plasma-associated phospholipid surfaces in clot propagation over that of cell surface phospholipids. Blocking TF-bearing cell surface phospholipids with annexin V had no effect on the clot growth rate, while blocking/removal of phospholipids from plasma resulted in up to 80% inhibition of the clot growth rate. It should be noted that phospholipid surfaces in platelet-free plasma are mainly presented by cell-derived microparticles and lipoproteins, which are all able to support coagulation reactions. However, annexin V is unable to block a population of very small-size lipid vesicles (diameter < 0.1 μm) [29] and ultracentrifugation cannot effectively remove such small vesicles and lipoproteins [21]. These trace phospholipid surfaces possibly accounted for the residual clot growth rate in the ultracentrifuged or annexin V-treated plasma (see Fig. 7). Finally, we demonstrated that intrinsic Xase regulates clot growth but is not critical for the initiation of clotting. Collectively, these observations show that generation of coagulation factors in plasma by the intrinsic Xase complex provides a mechanism for sustained and far-ranging propagation of clotting from TF-bearing cells. Although our experimental *in vitro* system did not contain platelets, which under physiological conditions provide the major procoagulant surfaces, we believe that our results will be also valid for the *in vivo* situation. Indeed, the presence of platelets will further facilitate plasma-based reactions thus making their role in thrombus growth even greater.

Our results support and further develop the 'cell-based' model of hemostasis [30]. This model postulates that TF-bearing cells provide the stimulus for the initiation of coagulation but they can not support generation of hemostatically sufficient amounts of thrombin [31]. In contrast, a critical role is attributed to activated platelets which provide numerous specific receptors for coagulation factors and in this way support generation of the bulk of thrombin on their surface. While the 'cell-based' model ascribes an essential role to 'transmission' of coagulation reactions from TF-bearing cells to the surface of platelets [12], it does not specify the mechanisms of the spatial clot growth.

We take into consideration that *in vivo* TF-bearing cells are mainly confined to the damaged vessel wall. Therefore, propagation of thrombin generation reactions from the TF-bearing vessel wall to platelets could in fact be the mechanism of the physical expansion of a fibrin clot [26,32]. In our 'spatial' concept, formation of a fibrin clot proceeds through two spatially separated phases: (i) the initiation phase, which occurs on the surface of TF-bearing cells confined to the vessel wall; and (ii) the growth phase, which proceeds within the vessel lumen away from TF-bearing initiator surface, i.e. on activated platelets and cell-derived microparticles. The principal novelty of our model is that it further elucidates the significance of thrombin generation on platelets over that on TF-bearing cells: activated platelets (cell-derived microparticles in our system) support thrombin generation in plasma volume near the luminal surface of growing thrombus. Importantly, both the 'cell-based' and the 'spatial' model agree on the role of intrinsic Xase in normal clot formation. FXa generated on the surface of TF-bearing cells is rapidly inactivated by antithrombin and tissue factor pathway inhibitor thus limiting the contribution of cell surface-bound extrinsic Xase in spatial propagation of thrombin generation. In contrast, more stable FIXa [11] may diffuse to platelets (or other plasma-associated phospholipids) and bind FVIIIa so that assembled intrinsic Xase will provide sustained thrombin generation away from TF-bearing cells [12,31] ('cell-based' model) leading to clot expansion in size ('spatial' model).

Recent reports suggest that specific inhibition of the intrinsic pathway of blood coagulation may be a successful and safe way for the management of thrombosis [33–36]. In animal thrombosis models, blockage of intrinsic Xase assembly suppressed thrombus formation without causing an overt bleeding tendency [33,36]. In *ex vivo* models of human blood coagulation, inhibition of components of the intrinsic pathway selectively suppressed the propagation phase of thrombin generation on platelets [34] as well as platelet and fibrin deposition from flowing blood [35]. Our experimental results also favor the inhibition of intrinsic Xase as a safe method of antithrombotic therapy. We showed that a decrease in FVIII level significantly reduces the rate of spatial clot growth without

affecting the onset of clotting on the cell surface whereas the absence of FVII completely abolishes coagulation. Thus, blockage of intrinsic Xase complex may selectively suppress development of occlusive thrombi without preventing formation of a nascent hemostatic clot.

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