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Dynamics of clot growth induced by thrombin diffusing into nonstirred citrate human plasma

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Abstract

The dynamics of clot formation was studied in a two-compartment chamber designed to allow free diffusion of thrombin according to its concentration gradient into nonstirred citrate plasma or fibrinogen solution. Fibrin clots in fibrinogen solutions increased progressively until the substrate was depleted. In plasma, the clot weight dynamics significantly depended on the concentration of thrombin in the thrombin compartment. When the thrombin concentrations were extremely low (25–40 nM), the clot weight increased throughout the experiment (sometimes 20–24 h). At higher thrombin concentrations, the clot weight increased for 1–2 h and then stopped growing for the following 3–4 h. The clot weight observed at the plateau varied only slightly in the range of thrombin concentrations of 50–770 nM. In this range, high thrombin concentrations (250–770 nM) caused a second increase in the clot weight 4–8 h after the start of diffusion, which was followed by the second plateau in the curve of clot weight against time. The time to the plateau and the plateau duration decreased with increasing thrombin concentrations. The abundant plasma inhibitors of thrombin cannot account for these results. It was hypothesized that an as yet unknown mechanism is responsible for the inhibition of clot growth.

Keywords: Blood coagulation; Clot growth dynamics; Thrombin; Citrate plasma; Fibrinogen

1. Introduction

Thrombi formed in normal organisms are usually of limited size and strictly localized to the site of injury. Most of the current knowledge about regulation of coagulation was derived from studies of plasma or reconstituted systems made homogeneous by stirring [1–3]. In these systems, coagulation is generalized and involves the entire volume. In our previous study, we examined the spatiotemporal dynamics of blood and plasma clot growth in vitro in the absence of stirring [4]. Three patterns of clot growth were found. We observed clots that grew continuously, clots that began to grow and then abruptly stopped growing, and stratified clots whose growth was stopped and then resumed again. Although much is known about the reactions initiating of

Abbreviations: AMC, 4-methyl-7-aminocoumarin; APC, activated protein C; BOC-Ala-Pro-Arg-AMC, *t-N*-butoxycarbonyl-alanyl-prolyl-arginyl-4-methyl-7-aminocoumarin; CPD, citrate–phosphate–dextrose solution; CPDA-1, citrate–phosphate–dextrose–adenine solution; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; FITC–thrombin, thrombin conjugated to fluorescein isothiocyanate; PPP, platelet-poor plasma; PVC, polyvinyl chloride

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clot growth and the inhibitors of coagulation [5–7], a number of questions had arisen when we tried to understand how the clots in our experiments stopped growing. The involvement of an inhibitor of thrombin that is generated by thrombin itself was suggested. This inhibitor was suggested to be formed autocatalytically in coagulating plasma at concentrations of thrombin exceeding some threshold [4].

As a first step, we tried to understand whether that inhibitor whose chemical nature is unknown would be formed in citrate plasma in the presence of exogenous thrombin. Therefore, this study was designed to compare the dynamics of in vitro clot growth induced by thrombin diffusing into nonstirred human plasma and fibrinogen solutions free of ionized calcium.

2. Materials and methods

2.1. Materials

Random donor blood units stabilized with standard citrate anticoagulants CPD or CPDA-1 were obtained from the Blood Bank Department of our Center and centrifuged at $2400 \times g$ (22°C) for 20 min. The supernatant platelet poor plasma (PPP, platelet count less than $2 \cdot 10^{10}$/l) was transferred into a satellite PVC bag within the closed system, and the PPP bag was then sealed and separated from the system. PPP was stored at room temperature for no longer than 72 h from the moment of blood collection.

Human fibrinogen free of plasmin(ogen) (Sigma, USA) was used without further purification. Bovine thrombin (50 NIH Units/mg) was purchased from Merck (Germany). For a series of experiments, this thrombin was conjugated to fluorescein isothiocyanate (FITC; Sigma) at the Institute of Immunology (Ministry of Health, Moscow, Russia) according to the method described in [8].

Fibrinogen or thrombin solutions of desired concentrations were prepared in sterile 5% human albumin for i.v. infusions (Bogdanov Institute of Blood Transfusion, Research Center for Hematology, Russian Academy of Medical Sciences, Moscow, Russia).

ε-Aminocaproic acid was from Chemapol (Czech Republic). Imidazole was from Reanal (Hungary). Fluorescein and 4-methyl-7-aminocoumarin were purchased from Sigma. Polyethylene glycol 6000 was from Serva (Germany). High-purity grade NaCl was a domestic product. A specific substrate for thrombin *t-N*-butoxycarbonyl-Ala-Pro-Arg-4-methyl-7-aminocoumarin (BOC-Ala-Pro-Arg-AMC) [9] was synthesized at the Institute of Biological and Medical Chemistry (Russian Academy of Medical Sciences, Moscow, Russia). The substrate was stored as a 10 mM stock solution in DMSO (Merck) at −20°C until the experiments were carried out. The kinetic parameters of the substrate K_m and k_{cat} were determined to be 13 μM and 120 s^{-1}, respectively.

2.2. Dynamics of clot growth: design of experiments

Experiments were performed in chambers whose construction allowed thrombin to freely diffuse into plasma or fibrinogen solution. Their schematic drawing is shown in Fig. 1. Each chamber consists of two tightly fitted Teflon compartments (0.75 ml), which communicate through a round window in the wall. Between the compartments (in the window), a thin nuclear membrane made of a polyethylene terephthalate film with 5-μm pores (Joint Institute for Nuclear research, Dubna, Moscow oblast, Russia) was placed. The chambers were preheated at 37°C for approximately 30 min. One compartment was filled with 0.65 ml of human plasma treated with lactic acid or 0.65 ml of 4 mg/ml human fibrinogen in 5% albumin. If no leakage through the membrane was noticed, the second compartment was filled with bovine thrombin of a desired concentration in

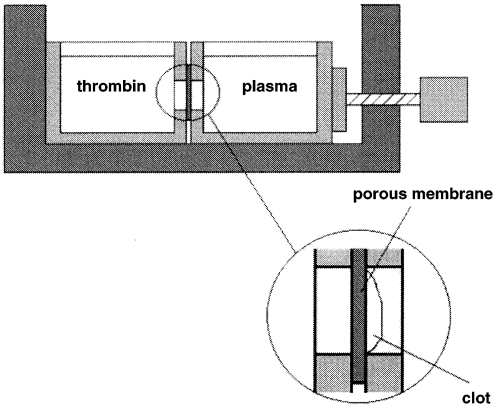

Fig. 1. Schematic drawing of two-compartment chamber for thrombin-diffusion studies.

5% albumin. (Albumin was used to equalize oncotic pressure on both sides of the membrane.) The filled chambers were sealed with Parafilm and placed into a temperature-controlled cabinet (37°C) for various time intervals (10 min–24 h). Thrombin that diffused to the plasma compartment caused the formation of a clot at the membrane separating the compartments.

Every hour, the thrombin compartment was carefully agitated with a pipette tip, and its content was assayed for the activity of thrombin. A decrease in its activity due to the opposing diffusion of plasma inhibitors was compensated for by the calculated additions of thrombin of high activity. The volume of each addition was equal to that of a sample drawn for thrombin assay (15–25 μl). Therefore, total volume in the compartment (0.65 ml) did not change. Precautions were made to leave the plasma compartment undisturbed throughout the experiment.

After incubation, both the thrombin and plasma were removed carefully and immediately analyzed for the thrombin activity (and the concentrations of FITC in experiments with FITC–thrombin). The chamber was taken apart, and the membrane with a clot on it was removed, rinsed in distilled water, and then submerged into 0.2 M NaOH (0.6 ml) for several hours at room temperature in order to dissolve the clot. The clot weight was estimated from the protein concentration in the solutions obtained. Protein was determined by the biuret method at 540 nm on a spectrophotometer (Specol, Germany).

In fibrinogen solutions, fibrin clots had a loose structure. In order to avoid losses of the coagulated material with rinsing water, this procedure was performed in a funnel equipped with a filter support containing a 5-μm-pore membrane. After the membrane removed from a chamber was rinsed in this funnel, the content of the fibrinogen compartment was also transferred into this funnel, attached to a syringe. Both membranes, with the clot and the material remaining after the filtration, were transferred into 0.2 M NaOH and then processed as described above.

2.3. Assay for thrombin activity

Thrombin activity was measured as the hydrolysis rate of BOC-Ala-Pro-Arg-AMC in a sample. An aliquot of each sample was added to a reaction mixture containing 50 mM imidazole (pH 7.4), 145 mM NaCl, and 0.1% polyethylene glycol 6000, which prevented thrombin adsorption to cuvette walls. The reaction was started with addition of 10 μl of 10 mM BOC-Ala-Pro-Arg-AMC. The total volume of the reaction mixture was 2 ml. The fluorescence of AMC cleaved from the substrate by thrombin was recorded at 37°C and continuous stirring on a JY 3-D spectrofluorometer (Jobin Yvon, France). The excitation and emission wavelengths were 380 and 440 nm, respectively. The signals recorded were calibrated by comparison with the signal from 1 μM AMC in the same reaction mixture containing no substrate. Thrombin concentrations were calculated from the initial hydrolysis rates of the substrate and its known kinetic constants (see above).

2.4. Lactic acid treatment of plasma

Lactic acid (10%) was added into plasma (5–25 μl per ml) under conditions of a large area of contact with air at 37°C for 1–1.5 h. Later, no alkaline shift in the plasma pH caused by continuous CO_2 loss was observed, and pH of lactic acid-treated plasma was stabilized at a constant level for the subsequent 24 h. The final concentration of lactic acid was selected so that the pH of plasma took values of 7.1–7.4. Only lactic acid-treated plasma was used in experiments.

2.5. Clot permeability to thrombin

The clot permeability to FITC–thrombin was estimated from the rate of its accumulation in the plasma compartment. The fluorescence of FITC–thrombin was excited at 490 nm and determined at 520 nm. The signals were compared with a calibration signal of 0.1 μM fluorescein.

To estimate the clot permeability to thrombin, clots were formed for 24 h in chambers with 1 mg/ml thrombin in one compartment and citrate plasma in the other. The plasma and thrombin were then carefully removed, and both compartments were rinsed with buffer (50 mM imidazole, 145 mM NaCl, pH 7.4). Buffer was then added into the former plasma compartment and 490 nM thrombin (dissolved in buffer) into the other compartment. The thrombin was allowed to diffuse into the buffer compartment for various time intervals. The accumu-

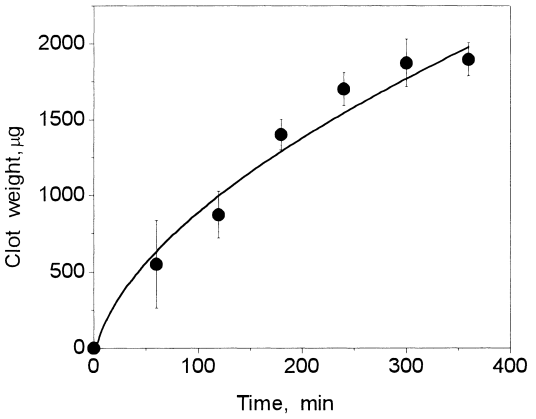

Fig. 2. Dynamics of the clot growth induced by thrombin diffusing into fibrinogen solutions. Data are means ± S.E.M. ($n = 3$). Fibrinogen and thrombin concentrations were 11.1 μM and 126 nM, respectively.

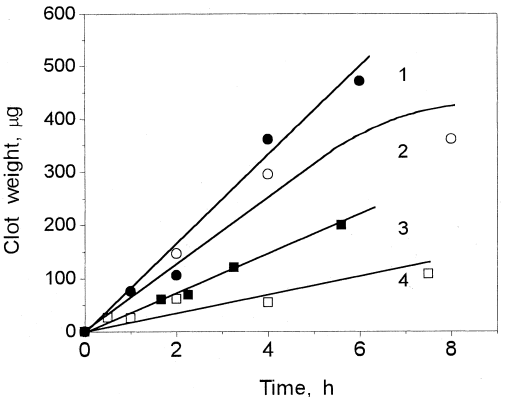

Fig. 3. Dynamics of the clot growth at low concentrations of thrombin diffusing into plasma: (1) 40.3 nM, (2) 36.8 nM, (3) 32.5 nM, and (4) 25 nM.

lation of thrombin activity was determined by measuring the hydrolysis rate of the fluorogenic substrate as described above.

2.6. Adsorption of FITC–thrombin to clots

In experiments with FITC–thrombin, the clots were thoroughly rinsed with water in order to remove free FITC–thrombin and then dissolved in 0.6 ml of 0.2 M NaOH. The concentration of FITC–thrombin in these solutions was determined from FITC fluorescence as described above. It was shown preliminarily that the fluorescence signals from FITC–thrombin in buffer and 0.2 M NaOH do not differ.

3. Results

3.1. The dynamics of clot growth in fibrinogen solutions and citrate plasma

In fibrinogen solutions, the clot weight continuously increased with time until fibrinogen converted to fibrin completely, and the entire volume of the compartment was polymerized (Fig. 2). The rate of clot formation was higher at higher thrombin concentrations (data not shown).

In citrate plasma, the pattern of clot formation upon thrombin diffusion was significantly different. The clots were limited in size. Most of the plasma volume in compartment remained liquid even 24 h after the start of thrombin diffusion. However, this liquid portion of the plasma coagulated 5–60 min after being transferred (by pipetting) into a tube for analysis. The dynamics of changes in the clot weight depended on the thrombin concentration used.

Low concentrations of thrombin caused the clot to grow continuously for 5–6 h (for 22.5 h in a particular experiment). Fig. 3 shows the dynamics of clot growth for thrombin concentrations from 25 to 40 nM. The initial rate of clot growth depended nonlinearly on the thrombin concentration in this range (Fig. 4). Note that at thrombin concentrations close to 40 nM, the rate of clot growth began to decrease 5–6 h after the start of the diffusion experiment.

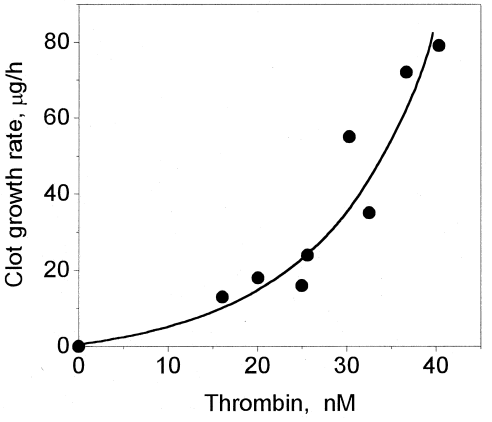

Fig. 4. Dependence of the clot growth rate on the thrombin concentration observed in the range of low concentrations.

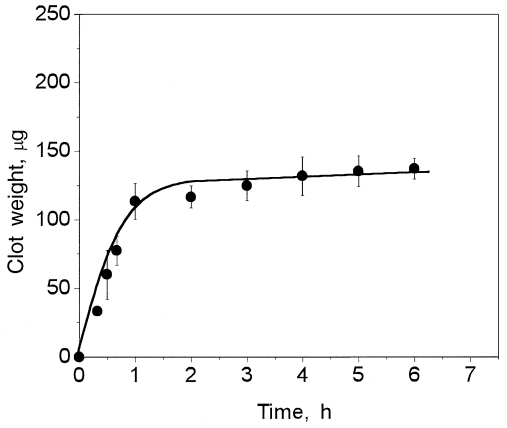

Fig. 5. Clot growth during the first 6 h after the start of diffusion of thrombin (60–420 nM) into plasma. Data are means ± S.E.M. of 20 experiments.

A further increase in thrombin concentrations from 40 to 770 nM resulted in that the clot weight rapidly reached a certain value 1–2 h after the start of the experiment and remained at this level for the subsequent several hours (Fig. 5). The clot weight corresponding to this plateau only slightly depended on the thrombin concentration. Fig. 5 shows the results averaged over 20 experiments in which thrombin varied from 60 to 420 nM. At high concentrations of thrombin (250–770 nM), a second rapid increase in the clot weight was observed 4–8 h after the start of diffusion. This increase was followed by a second plateau in the curve of clot weight against time. Fig. 6 shows the pooled data of three long-term experiments with high thrombin concentrations. The time to the plateau (whether first or second) and the plateau lengths decreased with increasing thrombin concentrations.

Also unexpected was that the clot weight corresponding to the first plateau in experiments with a high thrombin concentration could be lower than the clot weight observed at the same time and in the same plasma in experiments with a low concentration of thrombin (at which the clot weight continuously increased for the first several hours). Fig. 7 shows how the clot weight changed with time in an experiment performed on the same plasma for two thrombin concentrations (40 and 770 nM) in the thrombin compartment. After 3–5 h of thrombin diffusion, the clot weight observed with higher thrombin appeared less than that with lower thrombin. This effect seems to be observable in a narrow range of thrombin concentrations individual for each plasma. At low concentrations of thrombin, a clot increased in weight continuously, but slowly, remaining smaller that the clots corresponding to the plateau throughout the time of observation. At higher thrombin concentra-

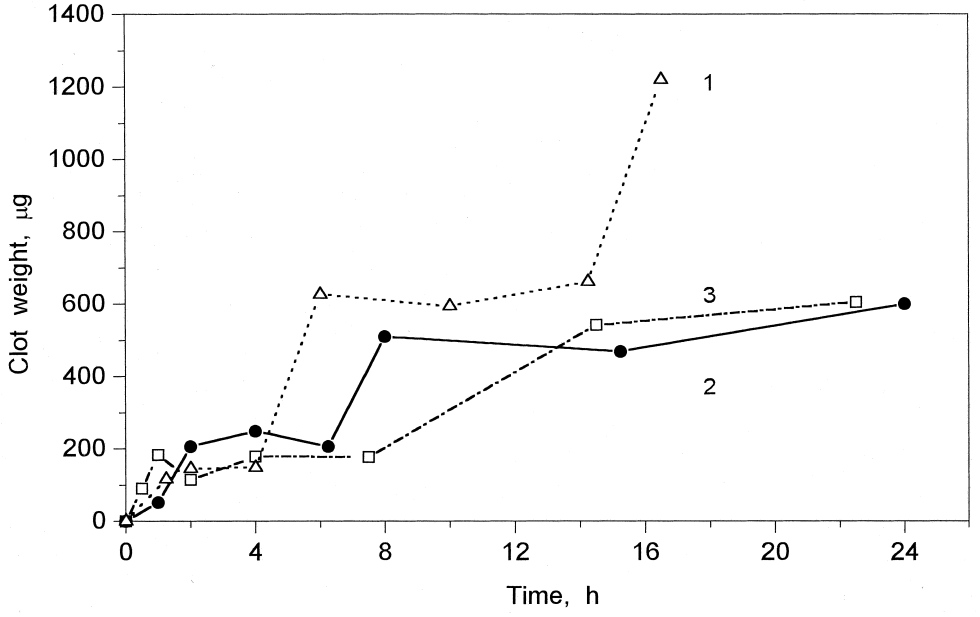

Fig. 6. Dynamics of the plasma clot growth induced by diffusing thrombin at concentrations of (1) 770 nM, (2) 725 nM, and (3) 250 nM, respectively.

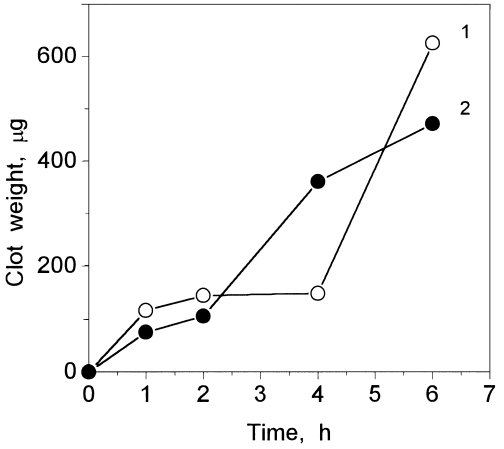

Fig. 7. Dynamics of the clot growth in the same plasma induced by diffusing thrombin at concentrations of (1) 770 nM and (2) 40 nM, respectively.

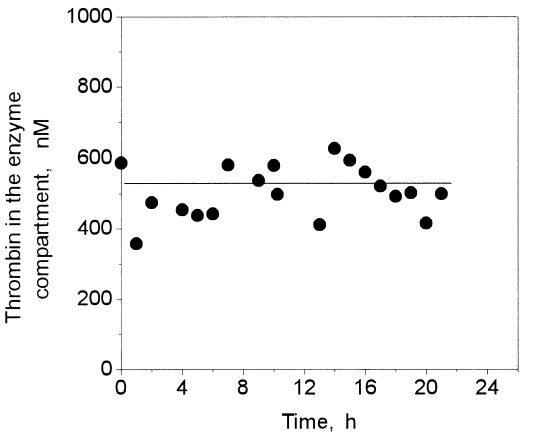

Fig. 8. Monitoring of active thrombin concentration in the thrombin compartment in one arbitrary chosen experiment.

tions, the pattern of clot weight growth changed: clots rapidly grew for a while and then stopped growing (Figs. 3 and 5).

3.2. Thrombin activities in the plasma and thrombin compartments

The activity of thrombin in the thrombin compartment was monitored in all experiments. The results of this monitoring can be exemplified by the data of one experiment (Fig. 8), which demonstrate that the activity of thrombin in the thrombin compartment was maintained constant throughout the experiment to an accuracy of 20%.

Fig. 9 shows the thrombin activity in plasma determined immediately after it had been removed from the plasma compartment after completion of incubation. This activity increased linearly with time. However, its maximum value even in experiments with high thrombin concentrations corresponded to that of 1–1.5 nM thrombin. Thrombin in complex with α_2-macroglobulin retains to a great extent its activity towards small substrates; therefore, the observed activity may be that of the complex. Note, however, that the fluorogenic substrate used in this study was not tested with α_2-macroglobulin–thrombin complex. Active thrombin was also present in the plasma, because it coagulated with time after being removed from the chamber by pipetting (i.e., after it was stirred).

3.3. Assessment of the clot permeability to thrombin and the thrombin adsorption capacity of clots

Preliminary experiments showed that (1) the activities of thrombin and FITC–thrombin towards plasma fibrinogen were similar, and (2) the curves of clot weight dynamics obtained with thrombin and FITC–thrombin taken at concentrations giving similar activities towards the fluorogenic substrate were also similar (data not shown). FITC is known to bind to proteins via ε-amino groups of lysine residues [10]. Hence, the active site of thrombin can scarcely be disturbed by its binding to FITC. However, the binding site to fibrinogen contains lysine residues and is likely to be affected in heavily labeled thrombin.

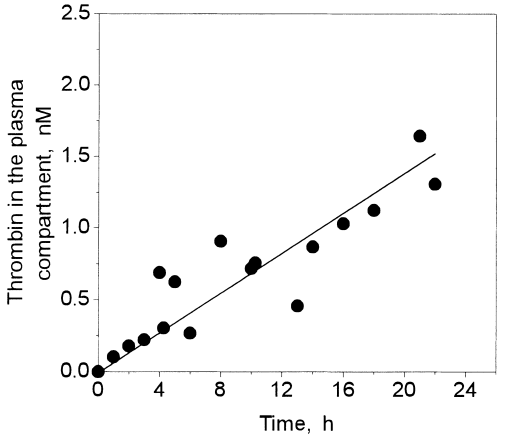

Fig. 9. Active thrombin concentration in the plasma compartment (averaged over its entire volume) for the experiment shown in Fig. 8.

Fig. 10 allows the rates at which thrombin and FITC–thrombin used in this study permeated plasma clots to be compared. Fig. 10a presents the pooled data of five experiments on accumulation of FITC–thrombin in the plasma compartment: it increased linearly with time throughout the experiment. Consequently, the clot permeability to FITC–thrombin did not change as the clots grew. Fig. 10b shows the accumulation of thrombin activity in the buffer compartment separated from the thrombin compartment by a beforehand formed clot attached to the membrane (see Materials and methods). Thrombin activity began to increase linearly after some lag (because thick 24-h clots were used in these experiments). The rate of thrombin accumulation in the buffer compartment was about half the rate of FITC–thrombin accumulation in the plasma compartment. Hence, the use FITC–thrombin resulted in underestimating the clot permeability to thrombin (and thrombin adsorption) only by a factor of 2.0.

Fig. 11 shows the results of an experiment designed to estimate the adsorption capacity of a clot for thrombin. FITC–thrombin concentration in the thrombin compartment was 380 nM, and the clot weight reached plateau values 1 h after the start of the diffusion experiment. Under the plateau conditions, the amount of adsorbed thrombin did not change, accounting for 0.2% of FITC–thrombin in

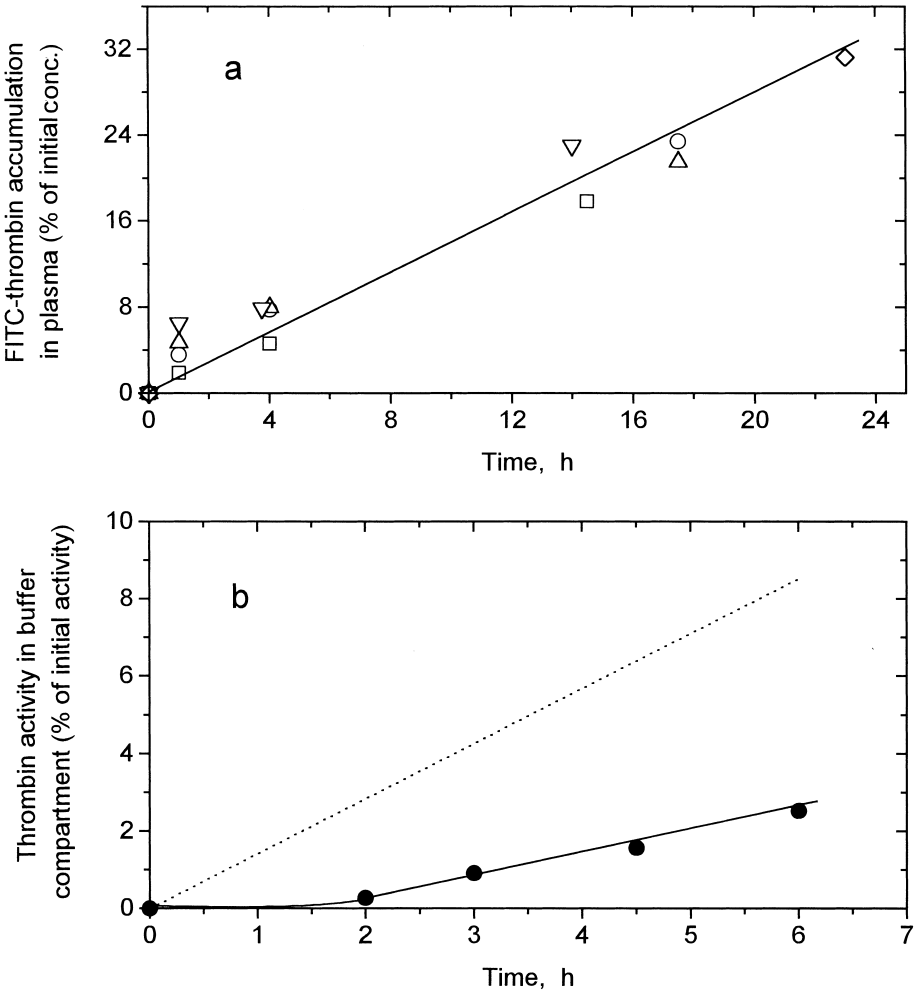

Fig. 10. Accumulation of (a) FITC–thrombin and (b) thrombin permeating plasma clots in plasma and buffer, respectively. Different symbols correspond to the different plasma samples. For an experiment shown in panel b, plasma clots were formed for 24 h and each chamber was then refilled. Imidazole buffer (pH 7.4) instead of plasma and thrombin dissolved in buffer were used for refilling. For comparison, the FITC–thrombin kinetics are given in panel b by a dotted line.

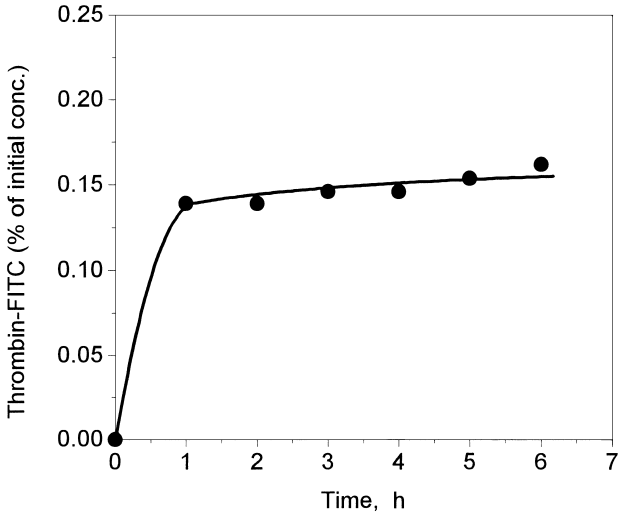

Fig. 11. FITC–thrombin accumulation in the plasma clot during the first 6 h after the start of thrombin diffusion. FITC–thrombin concentration in the thrombin compartment was 380 nM.

the thrombin compartment, or 5–10% of the FITC–thrombin that had diffused to the plasma compartment by the time.

3.4. Effects of fibrinolysis inhibition

Fig. 12 shows how the clot weight changed with time in the absence and presence of 10^{-3} M ε-aminocaproic acid. Consistently with the data described in [11], ε-aminocaproic acid taken at this concentration inhibited fibrinolysis completely, but did not change the activity of thrombin (data not shown). Thus, inhibition of fibrinolysis had no effect on the dynamics of clot growth in the experimental system used.

4. Discussion

Fibrin clots in fibrinogen solutions increased progressively until the substrate was converted completely. Obviously, a clot growing on a membrane between the compartments did not create diffusion constraints for thrombin, because the clot growth pattern observed was quantitatively consistent with the assumption of constant thrombin input into the system containing the substrate of the reaction and lacking its inhibitors.

Thrombin that diffuses into the plasma forms 1:1 complexes with inhibitors. Nevertheless, the concentration of its active free form in the plasma compartment continuously increases, because equilibrium between free thrombin and thrombin–inhibitor complexes exists. Therefore, we expected that the differences between fibrinogen solutions and plasma in the clot growth patterns would be only quantitative: antithrombin III and other thrombin inhibitors abundant in plasma decrease the rate of fibrin clot growth, but do not change its dynamics qualitatively. Under conditions of constant input rates of thrombin, the region where its concentration is sufficient to form fibrin should increase continuously. Actually, this pattern was observed only for thrombin concentrations below 40 nM (Fig. 3). At higher thrombin concentrations, the pattern of clot growth changed qualitatively. The clot increased in weight for only 1–2 h and then stopped growing (Fig. 5). The clot weight that had stopped growing depended on the thrombin concentration only slightly in a broad range. Several hours later, the clot that had stopped growing could resume its growth, but again stopped 1–2 h after the second start (Fig. 6).

It is unlikely that the presence of antithrombin III and other plasma inhibitors of thrombin could explain why larger clots could be formed at lower concentrations of thrombin in the same plasma (see Fig. 7) or why a clot that had stopped growing resumed

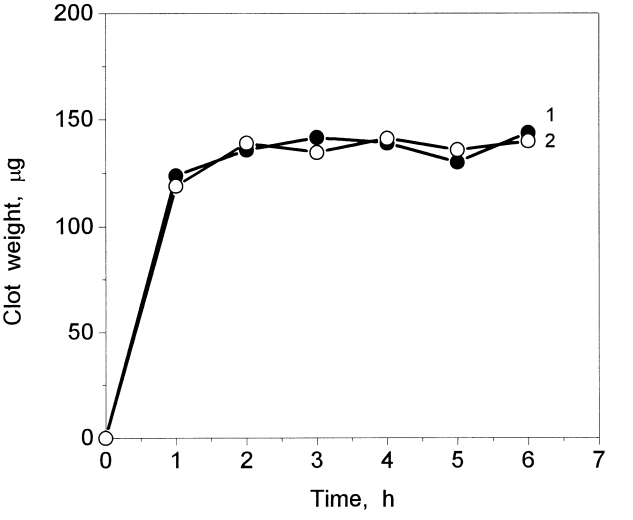

Fig. 12. Clot growth induced by thrombin diffusing into plasma with (1) or without (2) an inhibitor of fibrinolysis ε-aminocaproic acid (1 mM). Thrombin concentration in the thrombin compartment was 325 nM.

its growth and then stopped growing again (Fig. 6). If we assume that plasma inhibitors caught all thrombin diffusing into plasma and thus terminated the clot growth (first plateau), then the second growth phase of a clot should be interpreted as the depletion of inhibitors. Evidently, the clot growth in this phase would proceed until substrate depletion. The second plateau in the curves of clot weight against time is inconsistent with this interpretation (because the clot size at this plateau is not maximum).

Thus, it is difficult to explain the unusual clot growth patterns observed in plasma by interaction of thrombin with preexisting plasma inhibitors. Other factors should be implicated.

The variability in experimental conditions (temperature and plasma pH) could be excluded, because they were carefully controlled. The activity of thrombin in the thrombin compartment was kept constant (see Section 2). Oncotic and hydrodynamic pressures on both sides of the membrane were equalized.

Thrombin is known to activate protein C giving activated protein C (APC), which enzymatically breaks positive feedbacks in the cascade of thrombin generation. However, free calcium concentration in the citrate plasma used ranged from 30 to 50 μM (data not shown); hence, in these plasmas, thrombin could not be generated. Thus, the contribution of APC into the dynamics of clot growth was unlikely.

In an attempt to explain the results obtained, we considered the following possibilities: (1) a plasma clot growing on the membrane decreases its permeability to thrombin; (2) a concentration gradient in thrombin between the compartments rapidly decreases, decreasing the thrombin input rate; (3) active thrombin diffusing into plasma adsorbs to the plasma clot; and (4) fibrinolysis is activated and dissolves the clot until a dynamic equilibrium between the clot growth and lysis is achieved.

The capacity of fibrin to adsorb thrombin is known [12]. The possibility that adsorption of thrombin to a clot affects the dynamics of its growth was excluded by direct measurements of the amount of clot-bound FITC–thrombin (Fig. 11), which appeared very low (5–10% of the amount detected in the plasma compartment). This percentage remained unchanged throughout the experiment, suggesting that in clots that had stopped growing all binding sites were already occupied.

Thrombin freely permeated fibrin clots formed in fibrinogen solutions and the reaction proceeded until substrate depletion. However, plasma clots are known to be much denser, due to the presence of factor $XIII_a$, antithrombin III, etc. [13]. If plasma clots became impermeable to thrombin with time, their growth would stop. However, in experiments with FITC thrombin, we found that the rate of thrombin input into the plasma compartment did not change throughout the experiment (Fig. 10a). Fig. 10a also shows that, despite the fact that the average concentration of thrombin in the plasma compartment increased with time, it remained much lower than that in the thrombin compartment. The rate at which thrombin permeated plasma clots was about half the rate of FITC–thrombin. Hence, the estimation made with the use of FITC–thrombin are valid for thrombin with a correction factor of 2.0. Therefore, it can be concluded that the gradient in thrombin concentrations between the compartments remained high throughout the experiment, and the termination of clot growth could not be accounted for by the termination of thrombin diffusion caused by the clot formation or disappearance of a thrombin gradient.

An inhibitor of fibrinolysis ε-aminocaproic acid was used to assess the contribution of fibrinolysis into the clot growth dynamics in plasma under nonstirring conditions. The absence of calcium ions disables the coagulation cascade, but does not prevent contact activation and, consequently, factor XII_a-dependent fibrinolysis [14]. However, the pattern of clot growth did not change in the presence of 10^{-3} M ε-aminocaproic acid (Fig. 12). It is likely that inhibitors of fibrinolysis naturally occurring in plasma provided effective protection against plasmin formation caused by contact activation.

Thus, our results provided evidence that none of the factors studied is responsible for the termination of clot growth induced by thrombin diffusing into nonstirred citrate plasma. Therefore, we have to suggest that an as yet unknown mechanism is involved in this process, e.g., the formation of an inhibitor of thrombin activity induced by the thrombin itself, as it was suggested in our previous study [4]. This supposed inhibitor is not formed until thrombin concen-

tration is below some threshold. A possible variant of such an inhibitor can be conceived from the known fact that thrombin has two states in one of which it is active towards fibrinogen and in the other its major substrate is protein C. The effectors switching thrombin to the state with low activity towards fibrinogen and high activity towards protein C that have been found thus far include thrombomodulin, sodium ions, and synthetic organic compound LY254603 [15–17]. However, the substrates of thrombin in plasma are not limited to fibrinogen and protein C. It is conceivable that one of the products (or byproducts) of thrombin interaction with its plasma substrates can act as an effector of thrombin activity. This effector is not formed at low thrombin concentrations. Therefore, a continuously growing clot was observed when thrombin concentration in the thrombin compartment was low. At higher concentrations, the effector is rapidly accumulated and terminates clot growth by switching the thrombin state. The amount of the effector at each point where 'procoagulant' thrombin concentration is high is limited by the amount of its precursor in plasma. Continuous diffusion of 'procoagulant' thrombin results eventually in a new wave of clot growth at a father distance from the membrane. Again, the effector is formed and switches off the procoagulant thrombin. Indirectly, this suggestion can be tested by microscopic observations of the spatial organization of clots growing in plasma where thrombin diffuses according to its concentration gradient.

The suggestion by which we tried to account for the results obtained in this study is highly speculative. Nevertheless, we believe that further analysis of this suggestion and its experimental testing can give new insights into the mechanism operating to terminate clot growth.

Acknowledgements

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