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Thromboplastin Immobilized on Polystyrene Surface Exhibits Kinetic Characteristics Close to Those for the Native Protein and Activates *in vitro* Blood Coagulation Similarly to Thromboplastin on Fibroblasts

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Abstract—A method for transmembrane protein thromboplastin (tissue factor) immobilization on polystyrene surface is described. Tissue factor is the main activating factor launching the blood coagulation process. It is a cofactor of factor VIIa, the first protease in the cascade of coagulation reactions. The proposed method preserves kinetic characteristics specific for native tissue factor on the fibroblast surface. The kinetics of binding to factor VIIa and enzymic activity of the formed complex follow Michaelis–Menten kinetics, which is also characteristic of native complex. A small difference is that dissociation constant for tissue factor immobilized on polystyrene surface exceeds 2.7-fold that for native factor. The proposed technique of immobilization provides for protein density on the activating surface corresponding to the tissue factor density on the fibroblast surface. The immobilized tissue factor can be used to activate blood coagulation in methods simulating spatial dynamics of *in vitro* clot growth. Investigation in this direction will make it possible to register both hypo- and hypercoagulation states of the system. This approach is advantageous over traditional methods of estimation of the coagulation system conditions, which mainly register only hypocoagulation. Investigation of the storage time has shown that activators containing immobilized tissue factor can be stored and used during for at least 100 days in the method studying spatial dynamics of fibrin clot formation.

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Disorders of blood coagulation are among leading lethality factors in the modern world because they inevitably emerge in sepsis, trauma, many kinds of cancer, severe hemorrhages, any substantial surgery, etc. [1]. Diagnosis of these disorders is still rather inadequate and by no means makes it possible to reveal all disturbances in the homeostasis system. The main ideological disadvantage of most existing tests is that coagulation is simultaneous in the whole volume of the sample under investigation, and this is principally different from conditions of clot formation in a living organism. Now there is no doubt that results of such tests are not very sensitive to a number of stages of the process.

The hemostasis system is a complex cascade of enzymic reactions [2]. In this cascade a series of serine proteases activate each other. Precursors of these proteases are inactive in blood plasma and can be activated by site-specific proteolysis. The work of this cascade of successive activations of coagulation factors results in proteolytic activation of fibrinogen, which makes up 4% of all blood plasma proteins. Fibrinogen is converted to fibrin, forming a gel-like clot after polymerization.

Coagulation begins with blood contact with the surface of cells present in the damage zone of the blood vessel wall. Cells localized under endothelium have on their

Abbreviations: CTI, corn trypsin inhibitor; TF, tissue factor.
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surface a protein that is a signal for launching coagulation. This main activator of coagulation is the integral membrane protein thromboplastin (tissue factor, TF) [3]. It is located on the surface of all cells of our body not involved directly in contact with blood. Tissue factor does not exhibit any enzymic activity, but it is a cofactor of coagulation factor VIIa, a serine protease present in blood and exhibiting very low protease activity. This protease specifically cleaves the cascade factor X and thus activates the latter. Formation of the VIIa factor complex with tissue factor increases the VIIa activity by seven orders of magnitude, which launches the whole cascade and results in clot formation [2].

The process is rather complicated not only from the point of view of biochemical transformations. It begins locally in the zone of blood vessel damage. Tissue factor as an integral protein of cell membranes is not carried away by the blood flow but remains in the damage zone. Its complex with factor VIIa is localized together with it. However, the other active coagulation factors diffuse deep into the blood flow, thus causing spreading of the clot border in the form of a solid phase front [4]. The coagulation factor precursors are present in the whole plasma, and there are multiple feedbacks due to which the last enzyme of the cascade, thrombin, can activate its own precursor. This results in complicated dynamics of the process when certain biochemical reactions happen in different parts of the clot and at different moments in time. Spatial dynamics of coagulation can be conventionally divided into several stages.

1. Activation: in the damage zone the blood establishes contacts with cells having the immobilized tissue factor on the cell membranes; in this case VIIa–TF complex is formed, which launches the coagulation process by activation of factor X on the damaged surface;

2. The enlargement of the clot determined by diffusion and formation of new active factors, already not associated with activity of VIIa–TF complex, but rather caused by special reactions of growth maintenance.

3. Cessation of growth of the clot at a rather long distance from the activation zone is caused by a special network of biochemical reactions inactivating active coagulation factors.

In traditional coagulation tests, activators are added to the plasma volume and the whole mixture is actively stirred. As a result, only the first stage reactions become dominating. Disturbances in other stages are very poorly detected by such methods.

Investigation of the blood coagulation spatial dynamics makes possible the most complete modeling the space in which the blood is coagulated immediately in the blood vessel. In the human circulatory system the clot is formed in detail only in a small damage zone of the blood vessel wall. In this case such important characteristics can be measured as the clot enlargement rate, size, formation of spontaneous clots; this information cannot be obtained by homogeneous methods [5]. This allows one to register simultaneously and independently distortions at all stages of the process. Thus, it was shown that in classic hemophilia, when one coagulation factor is genetically defective, distortions take place at the phase of clot growth rather than during activation of coagulation, which appeared to be rather unexpected. There are high clinical perspectives for investigation of spatial dynamics of coagulation, but its introduction into clinical practice is retarded by the fact that in the published variant of the method a monolayer of fibroblasts, obtained in primary cell culture, was used to activate coagulation [5]. Such technology is expensive and labor-consuming and can hardly become routine.

Different forms of thromboplastin, including those obtained by gene engineering, are now produced by a number of companies. Therefore, the following aims of this investigation should be considered: (i) elaboration of a surface carrying immobilized thromboplastin; (ii) studying the ability of this surface to activate coagulation; (iii) comparison of different thromboplastin preparations and choosing the technology suitable for production of an activator that could be a good mimic of the contact zone between the damaged blood vessel wall and blood.

There are two main methods of enzyme immobilization: physical (sorption) and chemical (covalent cross-linking). We have chosen the chemical method of immobilization because in this case more firm protein fixation on the surface is achieved. Besides, immobilized proteins are as a rule much more stable.

Numerous methods for protein immobilization on surfaces of different nature are known. One of the most widespread approaches is chemical modification of the surface. For example, treatment of glass surface with (3-aminopropyl)triethoxysilane results in binding to the glass surface of molecules incorporating primary amino groups. Later these groups can covalently bind to proteins [6]. A method is described for nitration of polystyrene surface by nitrotetrafluoroborate in tetramethylene sulfone with subsequent reduction of nitro groups by stannous (II) chloride, also resulting in formation of primary amino groups on the surface [7].

A different approach consists in formation on the surface of a polymer film incorporating active groups, for example, amino groups. The method for preparation of a carrier for enzyme mobilization is described which includes precipitation of polyamine on inorganic oxide type carrier (porous glass, silica), and the polymer-covered carrier is treated with bifunctional reagent (glutaraldehyde), forming cross-links on polyamine to fix it on the carrier in the form of a homogeneous film [8]. A disadvantage of this method is that a good film is formed only on fine macroporous carriers with a highly developed surface. Such a film does not stick to smooth surfaces and cracks. We have taken this method as the basis and modified it to obtain an activator with the required properties.

This resulted in elaboration of a method for obtaining a surface covered by immobilized tissue factor whose density is approximately equal to that of the protein on fibroblasts, cells that are most active in activation of coagulation. Immobilized TF exhibits good activity and is characterized by functional dissociation constant K_s of VIIa–TF complex of 0.94 ± 0.28 nM. The surface activates coagulation as well as fibroblasts do. The immobilized factor is inactivated during a characteristic time of ~100 days.

MATERIALS AND METHODS

Reagents. Polyethyleneimine (ICN Biomedicals, Inc, USA), glutaraldehyde (Serva Feinbiochemica, Germany), glycine (Panreac Quimica, Spain), soluble thromboplastin (Renam, Russia), Innovin (soluble recombinant human thromboplastin) (Dade Behring, Marburg GmbH, Germany), factor VIIa (Novo Nordisk, Denmark), factor X (Enzyme Research Laboratories, USA), fluorogenic substrate S-2765 for factor Xa (Chromogenix, USA), and corn trypsin inhibitor (CTI) (Institute of Protein Research, Russian Academy of Sciences, Pushchino, Russia) were used in this work. Albumin, sodium citrate, lactic acid, $CaCl_2$, EDTA, NaCl, PEG 6000, Tris(hydroxymethyl)aminomethane hydrochloride, and Tris(hydroxymethyl)aminomethane were from Sigma (USA).

Immobilization of thromboplastin on polystyrene surface. The polystyrene surface was covered by a thin layer of polyethyleneimine incorporating primary amino groups [8]. To obtain firmly fixed film, polyethyleneimine (2%), glutaraldehyde (1%), albumin (4 g/liter) were mixed at ratio of 2 : 1 : 1 and applied onto the polystyrene surface in a thin layer at the rate of 13 μl/cm^2; in this case a ~10 μm thick layer is formed after drying. The film was dried for 24 h at room temperature. Thromboplastin was covalently attached to the aminated surface using glutaraldehyde. The polystyrene surface with dried film was incubated in 0.5% glutaraldehyde solution (0.2 ml/cm^2) for 1 h at room temperature. Specimens were washed free of unbound glutaraldehyde during 1 h by four 10 ml changes of distilled water. The resulting surface was incubated in 12 nM (0.2 ml/cm^2) thromboplastin solution for 1 h at room temperature. Unbound thromboplastin was washed off during 30 min by two 10 ml changes of water. Remaining aldehyde groups were blocked by glycine; to do that, the surface was incubated for 1 h in 1 M glycine solution (0.2 ml/cm^2). Then the surface was washed during 1 h in four 10 ml changes of water. After that the polystyrene surface with cross-linked tissue factor was dried for 30 min at 37°C, sealed in polyethylene, and stored in a refrigerator at 4-8°C. The change in the immobilized TF activity and ability to activate blood plasma coagulation were observed in time.

Methods for investigation of kinetics of immobilized tissue factor binding to factor VIIa. 1. Determination of immobilized TF density on polystyrene surface. *Principle of the method*. TF immobilized on a preset size surface was treated with an excess of VIIa coagulation factor to form the VIIa–TF active complex, and then a certain amount of factor X was added. The latter was converted into active form, factor Xa, in response to the VIIa–TF complex. The rate of factor X activation under chosen conditions is proportional to the amount of TF. The reaction was stopped by addition of EDTA, because the complex is inactive in the absence of calcium ions. The concentration of factor Xa was determined photometrically by the kinetics of the cleavage of chromogenic substrate [9].

A plate with immobilized TF (1 × 4 mm) was placed in a well of a 96-well plate and 0.02 ml buffer A (1 M Tris buffer, 150 mM NaCl, 0.1% PEG 6000, pH 8.7) was added. To plot the calibration curve, thromboplastin with concentrations from 0 to 40 pM with step of 4 pM was introduced into the wells. Then 0.02 ml of factor VIIa solution (0.3 μM) containing calcium chloride (15 mM) in buffer A was introduced. Then 0.02 ml of factor X solution (1.5 μM) in buffer A was added. After 30 min incubation at 37°C the activators were taken away and 0.04 ml of solution containing EDTA (25 mM) and substrate S-2765 (3.76 mM) was added to factor Xa in buffer A.

The rate of cleavage of the chromogenic substrate S-2765 was determined at 405 nm using a Thermomax microplate reader (Molecular Devices) in kinetic regime. The TF concentration was determined from a calibration curve using the Origin program for data analysis (version 6.0, MicroCal Software, Inc.).

2. Determination of functional dissociation constant (K_s) of VIIa–TF complex. *Principle of the method*. The surface-immobilized tissue factor was titrated by factor VIIa at different concentrations. The amount of formed VIIa–TF complex was determined by the rate of activation of factor X. K_s was supposed to be equal to factor VIIa concentration at which the half-maximal rate of factor X activation is achieved [10, 11]. For comparison, K_s values of fibroblast monolayer and soluble thromboplastin forms of Renam or Dade Behring were studied. The surface density of tissue factor on films with fibroblasts and tissue factor was determined in advance by immunofluorescence and maximal activity in factor X activation in the presence of excess factor VIIa.

A film with immobilized TF (1 × 4 mm) and another film with fibroblasts (2 × 2 mm) were placed in a well and 0.02 ml buffer A was added. Thromboplastin solution (40 pM, 0.02 ml) was introduced into other wells. Factor VIIa solution (0.02 ml) within concentration interval from 0 to 8 nM in buffer A containing calcium chloride (15 mM) was added. Then 0.02 ml factor X solution (1.5 μM) in buffer A was added. After incubation for

30 min at 37°C, the insoluble activators were taken out from the wells and 0.04 ml of solution containing EDTA (25 mM) and substrate S-2765 (3.76 mM) was added to factor Xa in buffer A. In the case of soluble factor the reaction was stopped by addition of 0.04 ml solution containing EDTA (25 mM) and substrate S-2765 (3.76 mM). The rate of the chromogenic substrate S-2765 cleavage was estimated according to the method of immobilized TF density determination. Functional dissociation constant of factor VIIa–TF complex (K_s) was determined by data approximation using the equation of reaction with a single binding center with the Origin program.

Investigation of spatial dynamics of fibrin clot growth in blood plasma upon coagulation activation by immobilized tissue factor. 1. Blood plasma preparation. Donor blood was stored in 3.8% citrate buffer, pH 5.5, at blood/citrate ratio 9 : 1. After preliminary pelleting of the erythrocytes at 1600*g* for 15 min, the plasma was centrifuged for 5 min at 10,000*g* to remove thrombocytes. Then for pH stabilization at the level of 7.2-7.4, plasma was incubated for 1 h with 10% lactic acid (14 µl acid for 1 ml plasma) [5]. The plasma was stored at −80°C and thawed 2 h before experiment.

Before experiment, plasma was incubated for 10 min with corn trypsin inhibitor (CTI) (0.2 mg/ml plasma) for inhibition of factor XII responsible for the contact phase of coagulation. The plasma was recalcified, i.e. 20 µl 1 M calcium chloride solution was added per ml plasma, and in this case the concentration of free calcium ions reached physiological level. Concentration was measured on an AVL 988-3 device (Vital Scientific N. V., Netherlands).

2. Spatial dynamics of thromb formation in blood plasma was investigated using the Thromboimager-09 device. The principle of the device is based on registration of clot growth by light scattering using the dark field technique described in [5]. The recalcified specimen of blood plasma was placed in a thin flat cell and brought into contact with the surface covered by immobilized tissue factor (activator). In response to activator, the clot began to grow, and its image was continuously registered using the high-sensitivity digital camera of the device. The resulting series of images was analyzed using special software. The result of experiment is a series of photographs of the growing clot. An experiment in which activation of coagulation by the fibroblast monolayer was carried out in the same plasma served as control (Fig. 1).

To obtain quantitative characteristics of clot growth, software of Thromboimager-09 allows choosing a region on the growing clot image and measuring light scattering profiles in the direction perpendicular to the activator surface at different time after activator contact with plas-

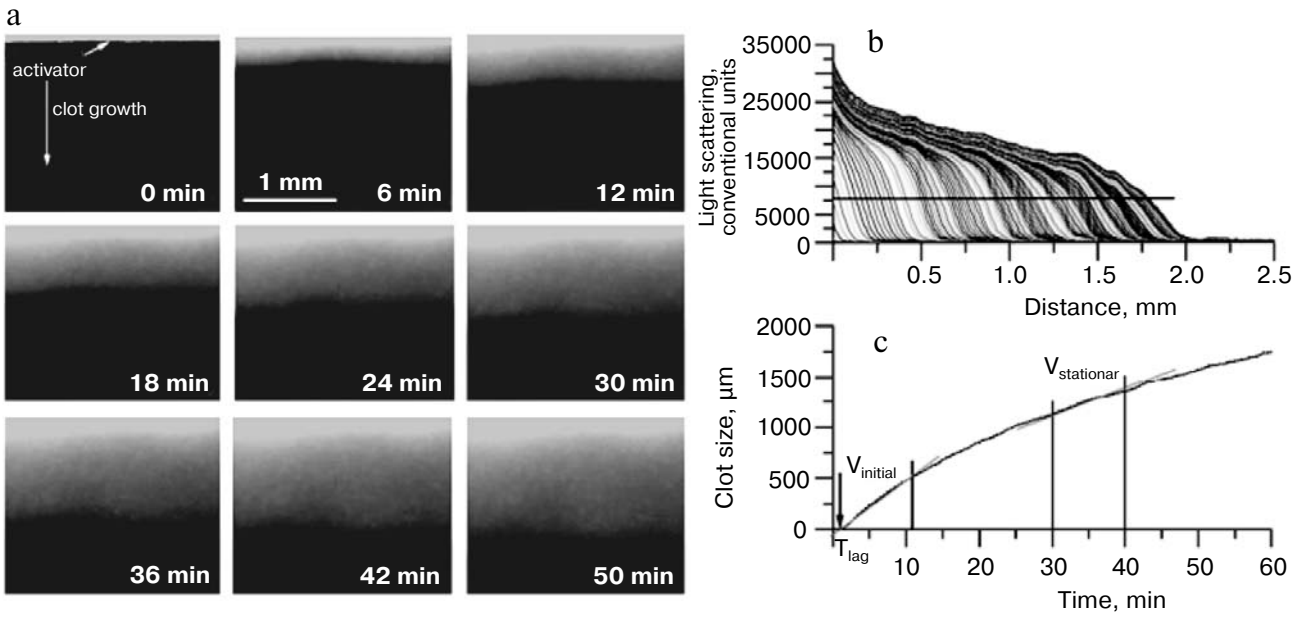

Fig. 1. Investigation of spatial dynamics of thromb formation in blood plasma upon activation by fibroblast monolayer like in [5]. a) A successive series of the growing clot photographs (the result of a typical experiment is shown). b) Clot light scattering profiles plotted on the basis of results of experiment shown in (a). The high peak near the activator is due to the light scattering from the plastic surface covered by tissue factor. The horizontal straight line is at the level of half-maximal light scattering at the distance of 0.5 mm from the activator. It is assumed to consider the intersection of this line with each profile as the clot size at the moment of its measurement. c) Time dependence of the clot size obtained for experiment shown in (a) and (b). Quantitative characteristics of the clot growth were calculated on the basis of this graph: lag time T_{lag} (the clot growth retardation time) is defined as time interval during which the clot size achieves 50 µm (in this experiment T_{lag} = 1.5 min); initial rate of the clot growth V_{in} is the mean rate of the clot growth during 10 min after the lag time (in this experiment it was calculated in the time interval from 1.5 to 11.5 min and is equal to 52 µm/min); stationary rate of the clot growth V_{st} is mean rate of the clot growth within the time interval from 30 to 40 min (in this experiment V_{st} is 22 µm/min).

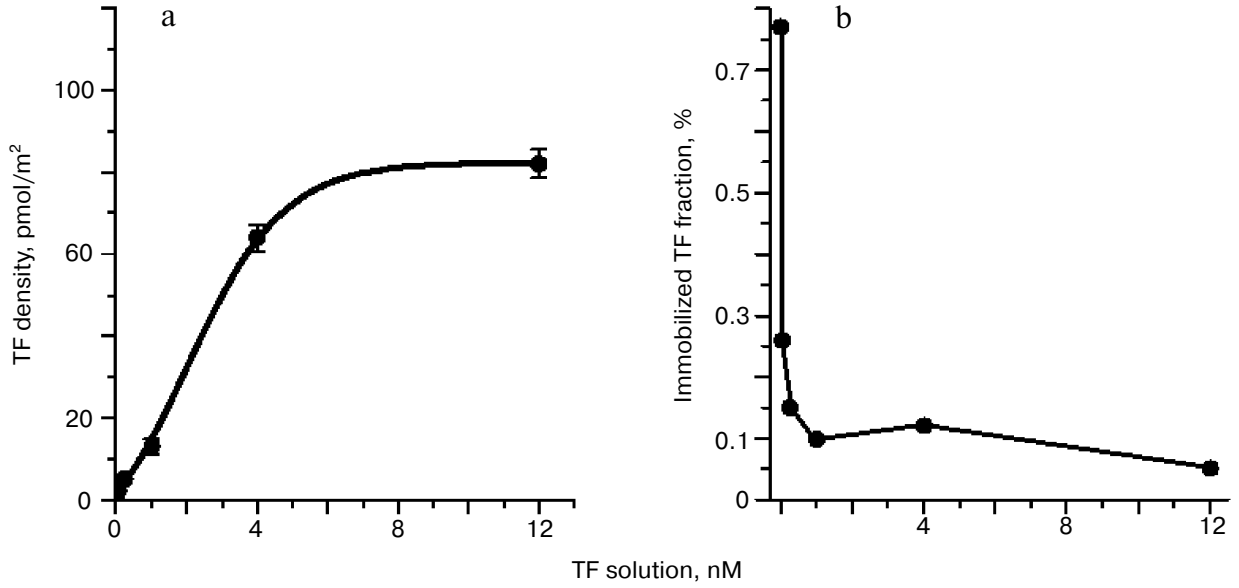

Fig. 2. a) Dependence of TF density on the activator surface on concentration of TF solution used for TF binding to the surface. The density was determined by the TF functional activity (see "Materials and Methods" section) ($N = 3$). b) Dependence of the immobilized TF fraction on concentration of TF solution used for TF binding to the surface.

ma (Fig. 1b). After choosing the light scattering level corresponding to the formed fibrin clot (horizontal line in Fig. 1b), the time dependence of clot size can be plotted (Fig. 1c). This level is usually considered as equal to the half maximal level of the plateau of the fibrin clot front spreading. As seen in Fig. 1c, there is no growth for some time, then it begins and two regions with different growth rates can be distinguished. This gives three main parameters characterizing the process: coagulation lag time, initial rate of clot growth, and fixed rate of clot growth.

RESULTS

Figure 2a shows the relationship between density of TF cross-linked to polystyrene surface and TF concentration in solution used for factor binding. At TF concentration ~4 nM and lower, the density of bound tissue factor grows linearly along with increase in factor concentration in solution. In the case of plate incubation in solution with TF concentration above 5-6 nM, the cessation in TF density increase on the surface is observed and the graph reaches a plateau. In this case maximal density of TF on the surface reaches 98 ± 12.5 pmol/m^2, $N = 2$ (N is the number of experiments). TF density on the surface of fibroblasts in continuous monolayer was insignificantly higher: 113 ± 12.3 pmol/m^2, $N = 3$.

Studying the problem whether saturation is defined by TF deficiency in solution used for incubation has shown that no more than 1% protein is bound even at minimal concentrations (Fig. 2b). Thus, at TF concentration in solution above 1 nM, the portion of bound protein does not exceed 0.1%.

Kinetics of immobilized tissue factor binding to factor VIIa. To study changes in kinetic characteristics of immobilized tissue factor, we began with investigation of kinetics of the formation of factor VIIa complex with thromboplastins of different origin in solution. The kinetics of factor X activation by soluble TF–VIIa com-

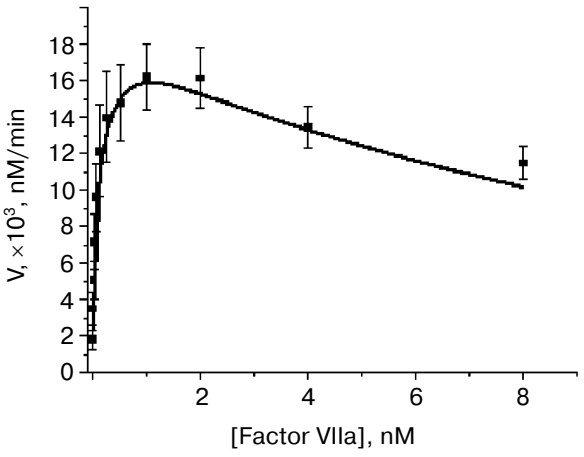

Fig. 3. Dependence of factor Xa activation rate on factor VIIa concentration in the complex with gene-engineered water-soluble thromboplastin (Dade Behring). TF concentration, 40 pM; V is the rate of chromogenic substrate formation in the test for determination of the TF–VIIa complex activity (see "Materials and Methods").

plex for gene-engineered thromboplastin Innovin (Dade Behring) is shown in Fig. 3. The curve has a clear maximum and beginning from VIIa concentrations above 1 nM factor Innovin inhibits activation of factor X. The phospholipid component is always present in tissue factor isolated from natural sources because TF is an integral protein of cell membrane [12]. Synthetic phospholipids incorporated in the gene-engineered TF of Dade Behring apparently influence the conformation of the formed complex.

For rabbit thromboplastin from Renam, the data on kinetics shown in Fig. 4a are well described by a hyperbola. In double inverse coordinates some regular deviations from a straight line, pointing to slight negative coopera-

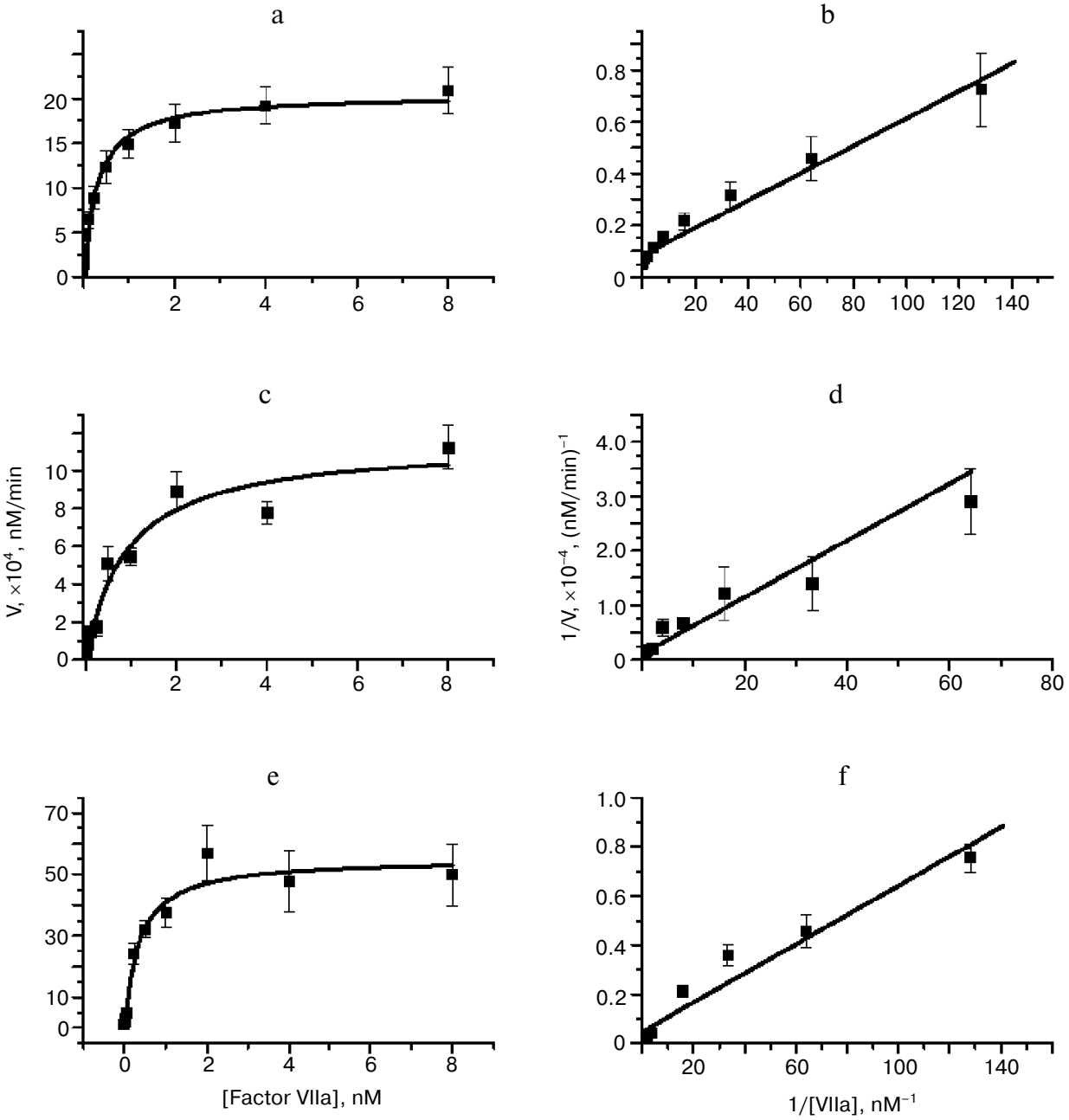

Fig. 4. Dependence of factor Xa activation rate on factor VIIa concentration in complex with tissue factor of different origin in conventional (a, c, e) and double reciprocal coordinates (b, d, f). a, b) Source of tissue factor, water-soluble thromboplastin (Renam). TF concentration, 40 pM. c, d) Source of tissue factor, tissue factor (Renam) immobilized on polystyrene surface. Size of activator, 1 × 4 mm; TF density, 90 pmol/m^2. e, f) Source of tissue factor is that on the surface of fibroblast monolayer. Size of film with fibroblast monolayer, 2 × 2 mm; TF density on surface measured by functional test, 89 pmol/m^2. *V* is the rate of chromogenic substrate formation in the test for determination of TF–VIIa complex activity (see "Materials and Methods").

Table 1. Functional dissociation constant of factor VIIa complex with tissue factors of different origin

Source of TF	Mean K_s, nM
Soluble TF (Renam) ($N = 3$)	0.31 ± 0.05
Soluble TF (Innovin)* ($N = 3$)	0.03 ± 0.005
Immobilized TF ($N = 3$)	0.90 ± 0.28
Film with fibroblast monolayer ($N = 5$)	0.35 ± 0.05

* Kinetics strongly differ from hyperbolic.

tivity, are observed (Fig. 4b). However, these deviations are within the limits of measuring error. Therefore, we believe that factor VIIa binding to TF follows simple bimolecular kinetics. The functional dissociation constant K_s for this complex is 0.31 ± 0.05 nM (Table 1).

Immobilization of the tissue factor from Renam on the surface has no qualitative effect on the kinetics of TF interaction with factor VIIa (Fig. 4c). In inverse coordinates, the dots also fit well in the straight line (Fig. 4d). However, in this case the dissociation constant was three times higher than for soluble TF, namely 0.90 ± 0.28 nM (Table 1).

The kinetics of native tissue factor complex formation with factor VIIa on the surface of fibroblast monolayer is also described by a hyperbola (Fig. 4e). This is confirmed by analysis of a graph plotted in reciprocal coordinates (Fig. 4f). Dissociation constants for tissue factor on the cell surface and for soluble thromboplastin (Renam) were very close to each other (Table 1).

Thus, immobilization of tissue factor using the method described in this work preserves its cofactor activity but changes to some extent its kinetic characteristics compared to TF on the cell surface and to its soluble form. Most likely, this points to a certain distortion of the normal conformation of the complex upon immobilization. We have studied in experiments on spatial dynamics to what extent these differences are important for coagulation activation and normal clot growth.

Spatial dynamics of fibrin clot growth in blood plasma upon coagulation activation by immobilized tissue factor. The ability of immobilized TF to activate coagulation in donor plasma was studied. Figure 5 shows a successive series of photographs of the growing clot taken after activation of fibrin clot formation by tissue factor immobilized on polystyrene surface.

The activator stimulates initiation and growth of normal clot in the blood plasma. Qualitative and quantitative parameters of all stages of this process are similar to those upon activation by fibroblasts (Figs. 1 and 5). Quantitative analysis of growth kinetics confirmed the similarity between artificial and natural activators. The clot light scattering profiles in the case of coagulation activation by tissue factor immobilized on the polystyrene surface (Fig. 5b) are very similar to those for fibroblasts.

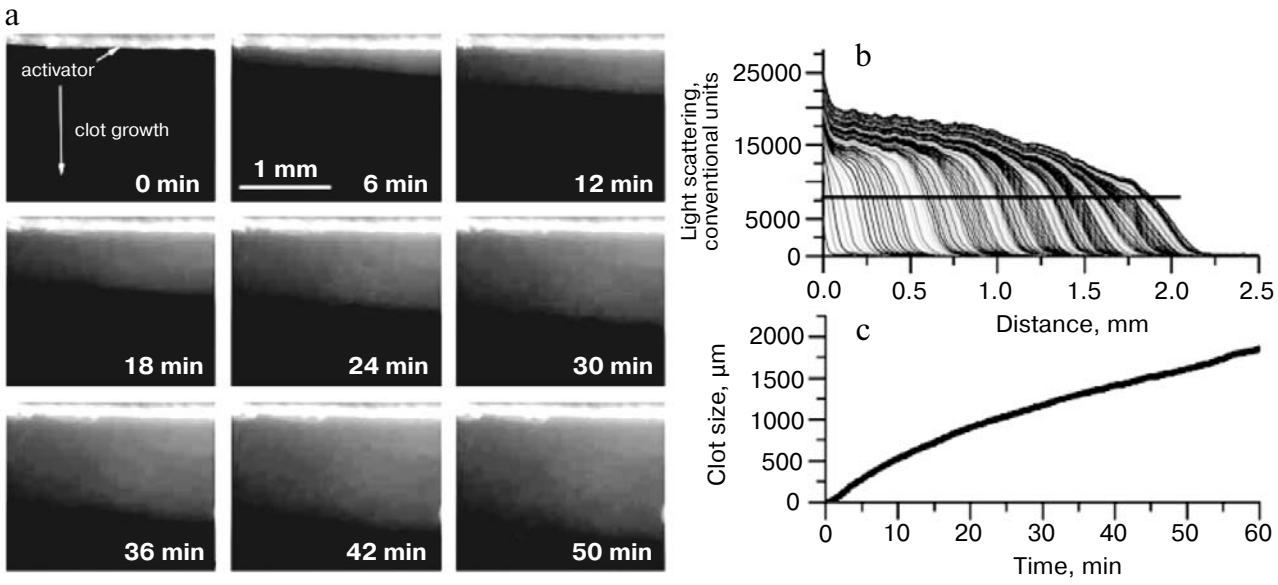

Fig. 5. Investigation of spatial dynamics of thromb formation in blood plasma after activation by tissue factor immobilized on polystyrene surface. a) Successive series of photographs of the growing clot. b) Clot light scattering profiles plotted on the basis of results of the experiment shown in (a). The horizontal straight line is at the level of half-maximal light scattering. c) Time dependence of the clot size obtained for the experiment shown in (a). Quantitative characteristics of the clot growth in this experiment are as follows: T_{lag} = 1.0 min; initial rate of clot growth, V_{in} = 54.7 μm/min; stationary rate of clot growth, V_{st} = 22 μm/min.

The artificial activator even appeared to be somewhat better, because the TF-covered polystyrene surface produces less parasitic scattering in the near-activator region than the support used for fibroblast growth. In this case the light scattering peak near the activator on the polystyrene support is significantly lower. The main quantitative parameters of the clot growth (the coagulation delay time and the initial and stationary rates of clot growth) for immobilized tissue factor and that on the surface of fibroblast monolayer are essentially identical (Fig. 5c and Table 2).

Investigation of dependence of the clot growth parameters on the tissue factor density on the surface has shown that the lag time begins to increase only when the density falls below 20 pmol/m^2 (Fig. 6a) and rather quickly increases as the density decreases. Both initial (Fig. 6b) and stationary (Fig. 6c) rates of the clot growth show practically no change in the case of TF density above 20 pmol/m^2.

Storage of activators with immobilized TF. Slow decrease in the immobilized tissue factor activity per activator area is observed upon storage of dried polystyrene plates covered by tissue factor at 4-8°C (Fig. 7a).

The characteristic time of activity decrease is 96 ± 13 days. Since the decrease in activity of the immobilized tissue factor begins to influence the clot growth parameters only after 4-5-fold lowering, the effect of this decrease is not immediate. Figure 7b shows how the lag time changes during activator storage. The first changes begin only after storage for 100 days. Similar data were also obtained for the clot growth rates (data not shown).

DISCUSSION

Tissue factor is an integral protein of most cell membranes of the human body. As mentioned above, it is absent only from endothelial cells of blood vessels and from blood cells. Such localization is necessary for the main physiological function of the blood coagulation system. The tissue factor complex with factor VIIa is formed at the site of tissue factor localization, and there it exhibits its enzymic activity by cleavage of factor X [5]. Owing to this, kinetic parameters of soluble forms of this protein can differ from those for native protein localized in the membrane.

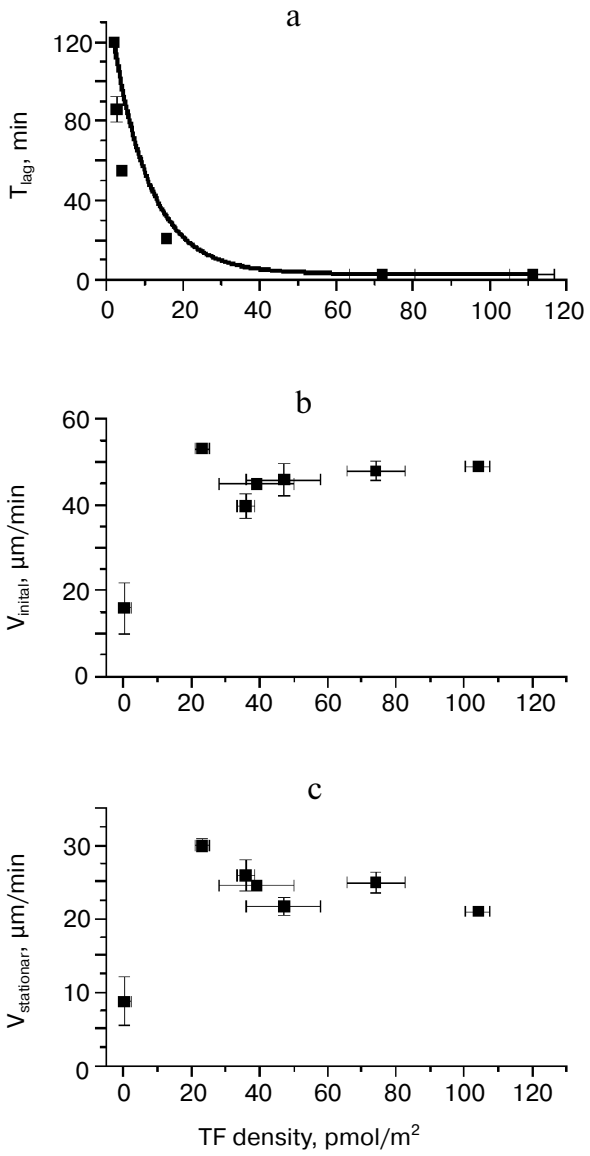

Fig. 6. Dependence of main clot growth parameters on tissue factor density on the surface of the activator. a) Dependence of coagulation retardation time on the tissue factor density on the coagulation activating surface. Data are approximated by the exponential $A = A_0\exp(-t/\tau)$. b) Dependence of initial rate of the clot growth on the density of tissue factor on the coagulation activating surface. c) Dependence of stationary rate of the clot growth on the density of tissue factor on the coagulation activating surface.

Table 2. Quantitative parameters of clot growth after coagulation activation by immobilized tissue factor and fibroblast monolayer (native tissue factor of cell membranes)

Coagulation activator	T_{lag}, min	$V_{initial}$, μm/min	$V_{stationar}$, μm/min
Immobilized TF (N = 26)	1.82 ± 0.33	38.1 ± 3.3	23.3 ± 1.56
TF on the surface of fibroblast monolayer (N = 6)	2.59 ± 0.74	45.5 ± 4.58	26.5 ± 2.57

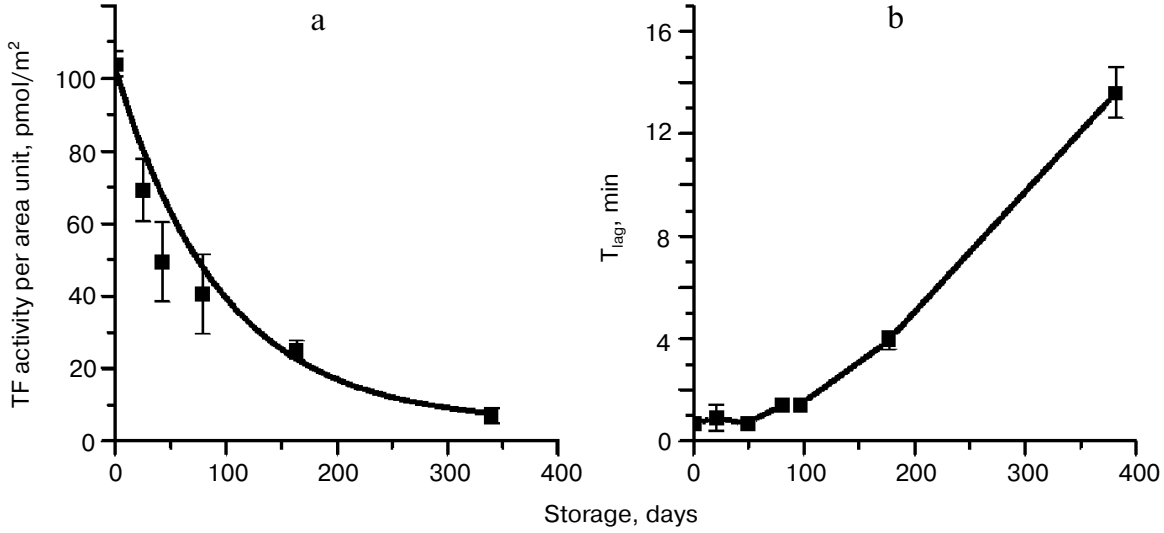

Fig. 7. Dependence of activator (polystyrene plates covered by tissue factor) parameters on the time of storage in hermetically packed conditions at 4-8°C. a) Decrease in TF activity per area unit during activator storage. Data are approximated by the exponent $A = A_0\exp(-t/\tau)$, where A_0 is 97.7 ± 4.3 pmol/m^2, $\tau = 96 \pm 13$ days. b) Dependence of coagulation retardation time (T_{lag}) on the time of activator storage.

Covalent immobilization of soluble protein forms is able to stimulate their subsequent changes. We have chosen the fibroblast monolayer grown in primary culture as a standard for comparison of different tissue factor modifications. These cells grow well and are sufficiently differentiated. Tissue factor on their surface has high density [13]. As shown by our experiments, this factor forms a highly efficient complex with factor VIIa. The kinetics of this complex follow the simple Michaelis–Menten form and have effective constant $K_s = 0.35 \pm 0.05$.

Unlike the gene-engineered factor Innovin (Dade Behring), kinetic characteristics of soluble thromboplastin isolated from rabbit brain membranes (Renam) were close to those of tissue factor from fibroblast membranes (Table 1). The gene-engineered soluble thromboplastin Innovine is characterized by kinetics much different from that of Michaelis–Menton and shows inhibition by substrate. This is most likely due to the synthetic lipid component. The membrane factor is isolated with a significantly large lipid component and evidently upon dissolving it forms lipid vesicles that allow the protein to acquire a conformation close to native.

To obtain coagulation activator with the required properties, a method for reactive film formation on polystyrene surface was chosen. Covalent cross-linking was carried out via protein amino groups, which is the most widespread and simple method of immobilization. Necessary mechanical properties of the film such as firmness of its fixation, elasticity, and homogeneous covering were achieved by addition into solution of polyethyleneimine, glutaraldehyde, and albumin.

The thromboplastin density obtained on the plastic surface was close to that of fibroblast membranes. Kinetic properties of thromboplastin somewhat changed upon immobilization but this was not dramatic. The process kinetics remained as that of Michaelis, but the K_s constant increased three-fold (Table 1). Despite differences in binding kinetics of factor VIIa and normal factor, the obtained activator quite well activates coagulation and clot growth on the fibroblast surface. Its ability to activate coagulation is close to that for fibroblasts. All parameters of the clot growth are similar for both types of activators (Table 2) and change little upon lowering the density (up to five times) of immobilized tissue factor. Immobilization sharply increases the stability of the activator on storage. In the case of storage at 4-8°C the tissue factor activity decreases no more than three-fold during 100 days.

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