Mechanisms of action of recombinant activated factor VII in the context of tissue factor concentration and distribution

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Supraphysiological concentrations of recombinant activated factor VII (rVIIa, NovoSeven) are used to control bleeding in hemophilia. Current experimental evidence suggests that rVIIa may increase thrombin generation via two pathways: one being tissue factor (TF)-dependent and another being activated platelet-dependent. Contribution of TF to the rVIIa action may justify different administration profiles of rVIIa. In the present study, thrombin and fibrin generation and spatial clot formation assays in platelet-free hemophilia A and normal plasma were used to investigate this contribution. By varying the concentration of TF and the way it becomes available to plasma, we obtained the following results. Activation of clotting with less than 5 pmol/l of TF facilitates thrombin and fibrin generation at low, but not at supraphysiological rVIIa concentrations. Activation with more than 13 pmol/l of TF saturates thrombin and fibrin generation kinetics, making it insensitive to rVIIa. rVIIa minimally modulates clot growth on the surface of TF-expressing fibroblasts. On the contrary, rVIIa produces spontaneous clot formation in nonflowing platelet-free plasma far away from fibroblasts via plasma lipid particles. Therefore, both the concentration and the distribution of TF determine relevance of a particular experimental system for the studies of rVIIa action. The results indicate that 300–1600 nmol/l (megadoses) of rVIIa may deliver coagulation outside of the TF-rich areas of blood vessel damage via the platelet-derived microparticles. Therefore, rate and extent of platelet-derived microparticles generation might be important with regard to rVIIa treatment safety. *Blood Coagul Fibrinolysis* 19:743–755 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Recombinant activated factor VII (rVIIa or NovoSeven; Novo Nordisk, Copenhagen, Denmark) administered at high doses (90–120 μg/kg each 2–3 h, which is approximately equivalent to a peak rVIIa concentration in blood of 50 nmol/l [1]), has been successfully used for the treatment and prevention of bleeding in hemophilia A and B patients with inhibitors [2–5]. In recent years, use of rVIIa was extended to a number of inherited and acquired bleeding disorders [6] but the main scope of rVIIa application remains to be the bleeding arrest in patients with fVIII or fIX deficiencies. Clinical trials demonstrated high efficacy of rVIIa treatment as well as its safety as confirmed by a very limited number of documented thrombotic complications [6]. However, there is presently an ongoing debate on the rVIIa mechanism of action in hemophiliacs [7–9].

The enzyme fVIIa is virtually inactive and can activate fX only in the presence of a cofactor, either tissue factor (TF) or phospholipid surface [10,11]. Accordingly, there are two possible mechanisms explaining the effect of rVIIa on thrombin generation. The TF-independent mechanism [12,13] is based on the finding that rVIIa can bind to activated platelets and activate fX directly on their surface. The hypothesis was put forward that this action of the platelet-bound rVIIa mediates the predominant therapeutic mechanism of rVIIa in hemophilia [10,14,15]. The alternative mechanism [16,17] suggests that hemostatic effect of rVIIa is explained by its action in a complex with TF. In addition to the

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dramatic increase of rVIIa catalytic activity toward fX, formation of the rVIIa–TF complex is thought to overcome inhibitory action of zymogen fVII [17,18], although the latter mechanism was questioned [9].

Although the TF-dependent and TF-independent mechanisms are not mutually exclusive, the experiments, sought to verify their relevance to rVIIa action, led different groups to contradictory conclusions. In a reconstituted system composed of coagulation factors, platelets and monocyte monolayer, thrombin generation was not inhibited by zymogen fVII [19]. In addition, rVIIa increased thrombin generation in a dose-dependent manner even in the absence of monocytes [20]. The above results favor the TF-independent hypothesis. However, a similar reconstituted model utilizing lipidated TF (i.e., TF protein reconstituted into phospholipid vesicles) instead of TF-expressing cells demonstrated the inhibition of thrombin generation by zymogen fVII and that rVIIa overcomes this inhibition [17]. Further, lipidated TF was essential for rVIIa to induce a considerable increase in thrombin generation of whole blood [21,22]. The latter data support the hypothesis of the TF-dependent rVIIa action. It is of interest that the two experimental models described above were composed of coagulation proteins and platelets and, therefore, were essentially similar in composition [9]. It was suggested, therefore, that the contradictory results might be related to differences in the sources of TF [7,8] or phospholipids [9].

Taking into consideration that TF concentration is a potent modulator of thrombin generation, we speculated that the efficiency of rVIIa *in vitro* depends on the concentration and/or physical distribution of TF, rather than on specific properties of different TF preparations. Computer simulations of the relative contributions of these mechanisms to thrombin generation supported this suggestion [15]. Here, we present experimental evidence that the relative potentiation of thrombin formation by rVIIa is decreased with the increase of TF concentration. Further, this potentiation was higher in the experiments with localized TF than in those with uniformly distributed TF. Summarizing, our results suggest the rVIIa action is not confined to the surface of the damaged vessel wall with TF and is preferentially determined by the TF-independent mechanism. Thus, TF-independent action of rVIIa ensures rapid clot formation around the site of injury. Consequently, rVIIa administration safety might be considered with respect to the presence of PMPs in patient's blood.

Methods

Materials

Human thrombin, D-Phe-Pro-Arg-chloromethylketone, lactic acid, and 7-Amino-4-methylcoumarin (AMC) were from Sigma (St Louis, Missouri, USA). Fluorogenic thrombin substrate Z-GGR-AMC was from Bachem (Torrance, California, USA). Bovine brain phosphatidylserine and phosphatidylcholine were from Avanti Polar Lipids (Alabaster, Alabama, USA). PerCP-conjugated antibody against CD61 (CD61, clone RUU-PL7F12) was from Becton Dickenson (San Jose, California, USA). Fluorescein (FITC)-conjugated Annexin V was from Molecular Probes (Eugene, Oregon, USA). Human recombinant TF (Innovin; Dade Behring, Mannheim, Germany) was from Baxter (Deerfield, Illinois, USA); functional activity (16 nmol/l) of frozen diluted stocks of TF was determined by Actichrome TF chromogenic activity kit (American Diagnostica Inc., Stamford, Connecticut, USA). rVIIa (NovoSeven) was a generous gift of Ulla Hedner (Novo Nordisk). Phospholipid vesicles (0.1 μm diameter, phosphatidylserine : phosphatidylcholine ratio 25 : 75) were prepared by extrusion and stored as described earlier [23].

Plasma donors

fVIII-deficient plasma was either from George King Bio-Medical Inc. (Overland Park, Kansas, USA) (four lots of pooled fresh frozen fVIII-deficient plasma) or was collected from seven severe hemophilia A patients. There was no difference in the results obtained using plasma from either of these two sources. fVIII activity in all samples was less than 1% of normal, as determined by one-stage fVIII : C assay. All patients had a history of frequent bleeding episodes. In accordance with the International Society on Thrombosis and Haemostasis (ISTH) recommendations [24], blood (13.5 ml) was drawn no earlier than 4–5 days, usually 7 days or more, after the last administration of fVIII-containing blood products. All patients were assayed for the presence of fVIII inhibitors (the Bethesda method [25]) in the year preceding the experiment. One patient had a high fVIII antibody titre [20 Bethesda units (BU)], one had a low titre (5 BU), and other patients exhibited no inhibitor (less than 0.5 BU). Normal plasma pools were either obtained from random healthy donors with no history of coagulopathy or purchased as frozen aliquots from George King Bio-Medical Inc. Their activated partial thromboplastin time (APTT) and prothrombin time (PT) values were within normal range.

Blood collection and plasma preparation

Patient or donor plasma was obtained and treated in accordance with hospital and institutional protocols as described elsewhere [26]. Briefly, blood was collected into 3.8% sodium citrate (pH 5.5) at a 9 : 1 volume ratio. Blood was centrifuged at 1500 *g* for 25 min. Platelet-free plasma (PFP) was obtained by centrifuging the supernatant at 10 000 *g* for 5 min. In some experiments, plasma depleted from remaining platelets and cell-derived microparticles (microparticle-free plasma) was prepared by ultracentrifugation at 100 000 *g* for 1 h at 21°C [27]. To prevent pH shift during experiments, the pH value of the plasma was stabilized at 7.2–7.6 by lactic acid treatment

[28]. Commercial frozen plasma was subjected to pH stabilizing procedure after thawing and treated similarly. Experiments were performed within 8 h of blood collection; cooling was avoided to prevent fVIIa-dependent cold-promoted spontaneous clotting [29].

Preparation of platelet-derived microparticles

Platelet-derived microparticles (PMPs) were prepared from washed platelets as described [27]. Whole blood was centrifuged at 180 *g* for 10 min. Platelet-rich plasma was collected as supernatant, and its pH was subsequently maintained at 6.5 with 3.8% sodium citrate (pH 5.0). Platelets were sedimented by centrifugation at 350 *g* for 15 min. After removal of supernatant, platelets were washed twice in the buffer containing 145 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% BSA, pH 6.5 by centrifugation at 350 *g* for 15 min. Following washing, they were resuspended in the same buffer, pH 7.4. Platelet concentration was adjusted to 200 000–450 000 μl^{-1} as determined by blood cell counter (Beckman Coulter, Fullerton, California, USA). Platelets were activated by incubation with 10 μmol/l of calcium ionophore A23187 for 15 min at 37°C and then sedimented by centrifugation at 350 *g* for 15 min. Supernatant was collected and additionally centrifuged at 16 000 *g* for 30 min. Sedimented microparticles were resuspended in the same buffer, pH 7.4. PMP concentration was measured by flow cytometry (below).

Flow cytometry

Flow cytometry analysis was performed on a BD FACS Calibur flow cytometer (Becton Dickinson, Mountain View, California, USA). Ten-fold diluted suspension of PMPs (5 μl) was mixed with 40 μl of the buffer containing NaCl 145 mmol/l, KCl 2.7 mmol/l, HEPES 10 mmol/l, $CaCl_2$ 2.5 mmol/l, pH 7.4 and incubated for 20 min at room temperature with CD61 PerCP and Annexin V-FITC. This mixture was supplemented with 500 μl of buffer and then analyzed by flow cytometry. Microparticle concentration was calculated using calibration beads [Level II (Med-low); Epics Division of Coulter Health, Miami, Florida, USA) added to every sample (10 μl of 10^6 ml^{-1}). Background gates were set against the same mixture of antibodies and buffer without PMPs. All measured data were corrected using isotype and autofluorescense controls.

Fibroblast culture

Human fetal lung fibroblast line was from the Ivanovskii Research Institute of Virology (Russian Academy of Medical Sciences, Moscow, Russia). Cells (10^3 cells/ml) were grown for 2 days on polyethylene terephthalate film slips (Joint Institute for Nuclear Research, Dubna, Moscow Region, Russia) as described previously [26].

Thrombin and fibrin generation assays

Thrombin generation and fibrin generation were measured in parallel on fluorometric and photometric channels of the Genious Pro microplate reader (Tecan Corp, El Paso Texas, USA) as described previously [30]. Plasma (90% v/v) was supplemented with fluorogenic substrate Z-GGR-AMC (1.25% v/v, 800 μmol/l) and different concentrations of rVIIa in PBS buffer (6.25% v/v), and transferred to a 2.2-ml well 96-well plate (Qiagen Inc., Valencia, California, USA). After 10 min incubation at 37°C, the reaction was started by adding TF (Innovin) in 0.8 mol/l $CaCl_2$ (2.5% v/v). Hundred microliters of plasma samples were rapidly (within 30 s) transferred to a clear 96-well flat bottom plate (Falcon; BD Biosciences, San Jose, California, USA). The time between recalcification and start of measurements was recorded for each well and used to adjust the onset of recordings. The fluorometric (λ_{ex} 380 nm, λ_{em} 440 nm, each 30 s) and photometric measurements (410 nm) were conducted continuously for 1 h at 37°C without further shaking. Thrombin concentration was calculated from the rate of substrate cleavage using a calibration curve. The rate of substrate cleavage was linearly proportional to the dilution of active site titrated thrombin in the range of 0.1–1200 nmol/l. Fluorescent data were corrected for fluorescent quenching in plasma sample, and for α_2-macroglobulin-thrombin activity as described previously [31]. Lag time of fibrin generation was defined as the time interval required for the solution turbidity to reach the half-maximal value. Peak thrombin concentration and fibrin generation lag time were calculated for each well independently, and the mean values for replicates under each condition were calculated. All of the above data processing steps were performed semiautomatically using Lab Talk scripts of the Microcal Origin 6.2 program (Origin Labs Inc., Northampton, Massachusetts, USA). The software is available from our laboratory upon request.

Spatial clot growth

Spatial parameters of clot formation were studied using a light-scattering video microscopy system [26]. A microchamber was assembled in a 35-mm polystyrene Petri dish (Corning, New York, New York, USA) around a 1-mm-thick microscope glass slide (Fisher, Pittsburgh, Pennsylvania, USA) fixed to its bottom. The glass slide edge, which formed a vertical wall of the chamber, was wrapped around with a cell-coated polyethylene terephtalate slip. Then, it was covered with a piece of black polystyrene that formed the upper surface of the microchamber. Recalcified ($CaCl_2$, 20 mmol/l final concentration) human plasma was transferred into the assembled chamber (time $t = 0$), 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 (peak wavelength 660 nm) and the light-scattering image from a 7.2 mm × 5.4 mm microchamber area was recorded every 30 s by OS-75D camera (Mintron Enterprise, Taipei Hsien, Taiwan ROC)

coupled to EZ98 framegrabber (Lifeview Inc., Fremont, California, USA) and processed as described below.

Image processing

Parameters of spatial clot formation were determined from the experimental image series as described in [26,30]. Briefly, clotting in the microchamber was considered as a combination of two processes: TF-bearing cell monolayer-initiated clot formation, which appeared as a propagation of clot from cells to plasma; cell monolayer-independent spontaneous clotting, which appeared as clots scattered across the microchamber. The TF-induced clot growth was characterized by the rate of clotting propagation from the cell monolayer. For every frame, the clot size was determined as a distance between the activator 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 [26]. Subsequently, the initial rate of clot growth was derived from the clot size versus time curve as a mean rate in the range of 1–10 min following the onset of clotting. These measurements were performed in three different parts of image for each set of experimental images. The spontaneous clotting was characterized by the kinetics of fibrin formation in the areas of microchamber that were sufficiently far from the activator. Coagulation in these regions occurred independently of the initiating TF-bearing cells. A 3 mm × 3 mm square area separated by at least 1.5 mm distance from the cell surface was selected on the first frame, and the mean light-scattering intensity within this square area was calculated and plotted as a function of time. The lag time of spontaneous clot formation was defined as the time interval required for the mean light scattering to reach the half-maximal value.

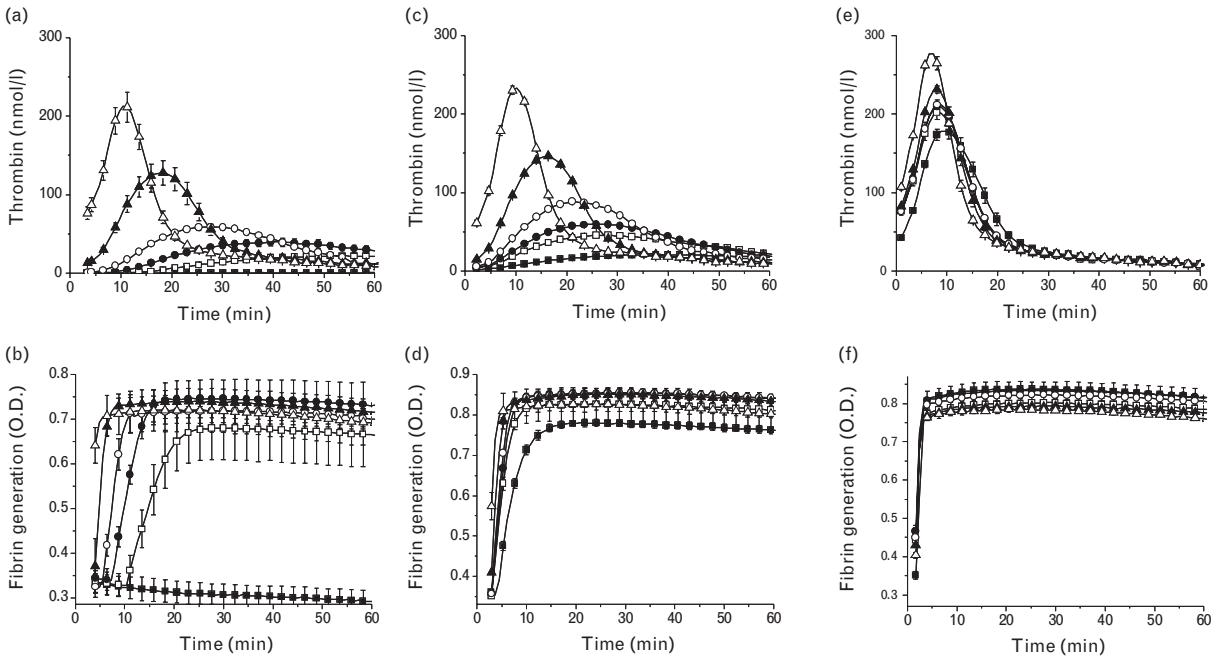
Results

Relative efficiency of recombinant activated factor VII is decreased in the presence of uniformly distributed tissue factor

It was reported that high concentrations of TF mask the effect of rVIIa addition in plasma-based clotting assays [32,33]. Therefore, we first determined the optimal range of TF concentrations for studies of rVIIa action in thrombin generation and fibrin generation assays. In the experiments with no TF added (Fig. 1a), rVIIa alone was able to stimulate thrombin generation in a dose-dependent manner. Clot formation, which was monitored in parallel with thrombin generation, was also in a good correlation with rVIIa concentration (Fig. 1b). In agreement with the reports by other authors [34–36], the clotting process was completed rapidly, before thrombin concentration achieved its peak value (compare Fig. 1a and b).

The experimentally determined minimal TF concentration (0.5 pmol/l, Fig. 1c) did not change the effect of rVIIa on thrombin formation substantially. However, the

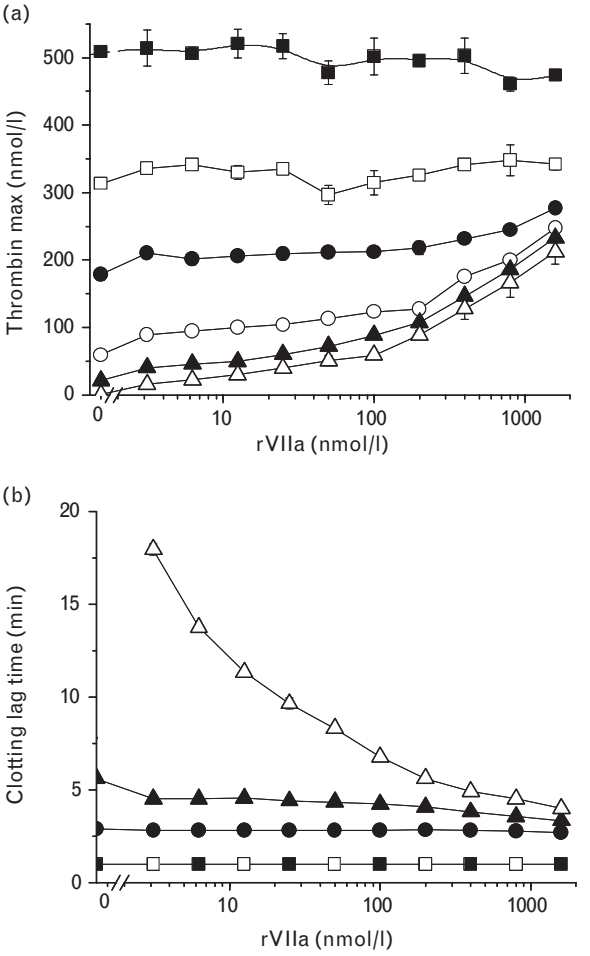
Fig. 1



Effect of recombinant activated factor VII on thrombin and fibrin generation in fVIII-deficient plasma in the absence (a and b) and in the presence of tissue factor at 0.5 pmol/l (c and d) or 4.3 pmol/l (e and f). rVIIa concentration: 1600 nmol/l (open triangles), 400 nmol/l (filled triangles), 100 nmol/l (open circles), 25 nmol/l (filled circles), 6.25 nmol/l (open squares), and 0 nmol/l (filled squares). A representative experiment is shown here. Each data point is a mean value ± SD, obtained from two wells. rVIIa, recombinant activated factor VII.

thrombin peak achieved in each of these experiments was 20–50 nmol/l higher than in respective experiments without TF added (compare Fig. 1a and c). To quantitatively describe the effect of rVIIa addition, we calculated the relative extent of the thrombin peak increase with the raise of rVIIa concentration from 3 to 400 nmol/l. This analysis demonstrated that addition of TF to plasma does not increase the relative efficiency of rVIIa. On the contrary, rVIIa efficiency was decreased approximately two-fold. Although in the absence of TF the relative increase of peak thrombin was 700%, addition of 0.5 pmol/l TF resulted in only 270% increase in peak between 3 and 400 nmol/l of rVIIa. When TF concentration was further increased by nine-fold (4.3 pmol/l, Fig. 1e), 400 nmol/l of rVIIa increased peak thrombin by only 16%. Finally, thrombin generation did not depend on rVIIa at TF higher than 13 pmol/l (see Fig. 2a). These experiments indicate that high concentrations of TF are not adequate for studies of rVIIa action as they 'mask' the effect of rVIIa on thrombin generation making thrombin parameters insensitive to rVIIa. Figure 2b demonstrates that, in consistence with previous studies [32,33], clot formation time was even less sensitive to rVIIa, because it did not depend on rVIIa at TF concentrations higher than 4.3 pmol/l. Similar experiments on TF/rVIIa titrations in normal pooled plasma demonstrated identical disappearance of the rVIIa effect, when high TF was present in the reaction (data not shown). This confirms that the observed effect was not specific to hemophilia plasma. The maximal peak value in these experiments was approximately 500 nmol/l thrombin (approximately 80 NIH units/ml [37,38] or 70 IU/ml [39]), at TF concentration of 118 pmol/l (approximately 3.5 ng/ml, assuming molecular weight of Innovin to be 30 kDa [40]).

Fig. 2



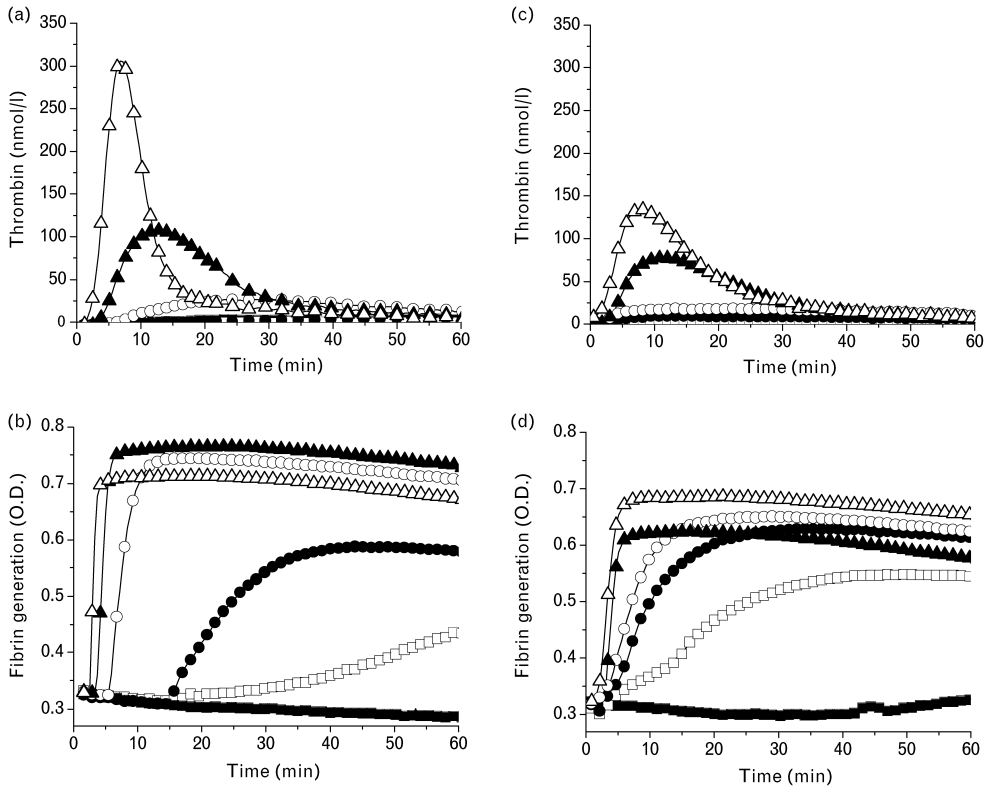
Combined effect of recombinant activated factor VII and tissue factor on the maximum of generated thrombin concentration (a) and plasma clotting lag time (b) in fVIII-deficient plasma. TF concentration: no TF (open triangles) and 0.5 pmol/l (filled triangles), 1.5 pmol/l (open circles), 4.3 pmol/l (filled circles), 13 pmol/l (open squares), 118 pmol/l (filled squares). Mean ± SD, $n = 2$. rVIIa, recombinant activated factor VII; TF, tissue factor.

Relative efficiency of recombinant activated factor VII is increased when tissue factor is distributed nonuniformly

In vivo, TF is mainly localized to the surface of damaged vessel wall. Only small quantities of TF of controversial origin were detected in blood [41]. In contrast, *in vitro* experiments are usually conducted under nonphysiological conditions, with lipidated TF uniformly mixed with plasma samples [31,34]. In view of this observation, and because the rVIIa effect was observed in the limited range of TF concentrations, the distribution of TF can also be an important regulator of rVIIa action. To imitate the effect of nonuniform TF distribution, in our *in vitro* experiments we placed 1.5 μl drops of TF at different concentrations in the centers of microplate wells, followed by addition of recalcified plasma. This approach was chosen because it allows comparison of the uniform versus limited exposures of TF to plasma keeping the concentration and source of TF defined. For example, preliminary experiment revealed that TF quickly and irreversibly loses activity after drying out of TF drops. Under 1.5 μl drop conditions, mixing of TF and plasma is minimal, and only a small portion of plasma is exposed to TF. In control experiments conducted in parallel, plasma samples were mixed with the drops of TF by repeated pipetting. Figure 3a and c shows that at high TF (>2.2 pmol/l), extensive mixing increased the thrombin peak, as well as the rate of fibrin formation. In contrast, clot formation was initiated earlier in nonmixed plasma than in mixed samples when only low TF concentrations (<2.2 pmol/l, Fig. 3b and d). This experiment was reproduced on several occasions with different plasma donors (mean data are shown in Fig. 4a and b).

To study the effect of rVIIa in the case of nonuniform TF distribution, we chose 2.2 pmol/l of TF, because at this TF concentration both thrombin peaks and clot formation times were similar in mixed and nonmixed samples (see Fig. 4a and b). As can be seen in subparts

Fig. 3



Generation of thrombin and fibrin in plasma activated with increasing concentrations of mixed (a and b) or nonmixed (c and d) tissue factor. TF drops (1.5 μl) were placed on the bottom of microtitre well, fVIII-deficient plasma was recalcified and 98 μl of plasma was transferred into each well. Thereafter, half of the wells were mixed with several strokes of 12-channel pipette ('mixed' TF). TF concentration: 20 (open triangles), 6.7 (filled triangles), 2.2 (open circles), 0.75 (filled circles), 0.25 (open squares), and 0 pmol/l (filled squares). TF, tissue factor.

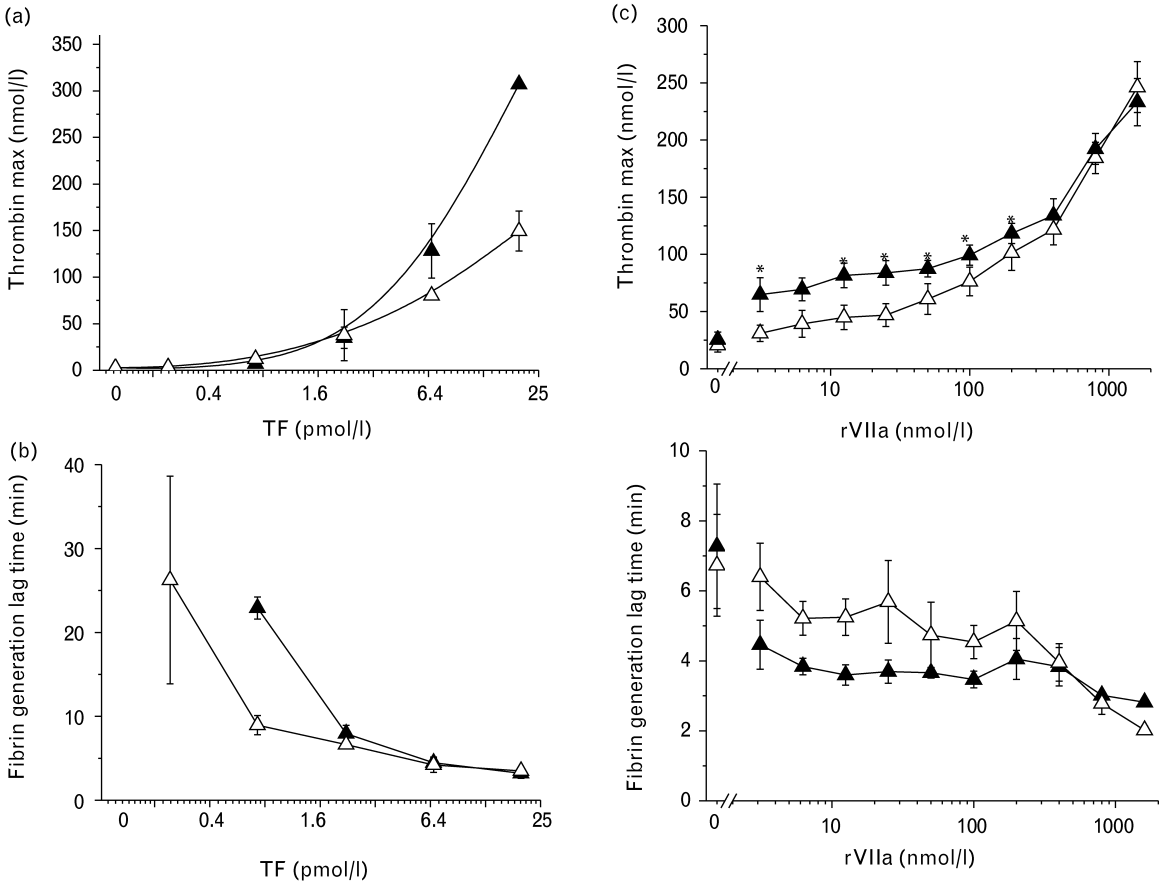
(c) and (d) of Fig. 4, mixing did not change significantly the overall effect of rVIIa on thrombin and fibrin generation except at low rVIIa concentrations. However, this difference is essential for the analysis of TF-dependent and TF-independent mechanisms of rVIIa action. First, the TF-dependent mechanism of rVIIa action is often described as the increase in thrombin generation after addition of rVIIa above the baseline (rVIIa = 0 nmol/l). After the addition of moderate concentrations of rVIIa (0.2–100 nmol/l, Fig. 4c), the peak of thrombin increased by two-fold to three-fold in plasma samples uniformly mixed with TF and only by approximately 1.5-fold without mixing. This supports the importance of the TF-dependent mechanism of rVIIa for experiments with uniform but not with nonuniform distribution of TF. Furthermore, the ability of rVIIa to act independently of TF is often implied as the ability of incremental increase of 'megadoses' of rVIIa to result in higher thrombin generation. The relative increase of thrombin generation after the increase of the rVIIa dose from 5 to 500 nmol/l was only approximately 1.3-fold in mixed samples as compared with approximately 3.5-fold increase in nonmixed samples (Fig. 4c), thus indicating the relative efficiency of megadoses of rVIIa in nonuniform experiments but not in mixed experiments.

Overall, mixing of TF with plasma highlighted the TF-dependent mechanism of rVIIa action while keeping TF distribution nonuniform revealed the importance of the TF-independent one. Analysis of the clotting curves (Fig. 4d) revealed the same effect: mixing of TF was required for small rVIIa doses to induce coagulation, whereas the clotting times were sensitive to the incremental increase in rVIIa concentrations up to 1000 nmol/l only without mixing. Therefore, the sensitivity of thrombin generation and clot formation tests to the action of high rVIIa concentrations was critically regulated by mixing, that is, by the way through which the plasma came in contact with TF. If smaller portion of plasma was in contact with TF, the rVIIa action appeared to be less TF-dependent. The uniform TF distribution led to the opposite conclusion.

Effect of recombinant activated factor VII in the spatial model of blood coagulation

Because of the nonuniformity of TF distribution *in vivo*, physiological coagulation involves two spatial phases: initiation of coagulation on the surface of exposed TF-expressing subendothelial cells and clot growth into the

Fig. 4

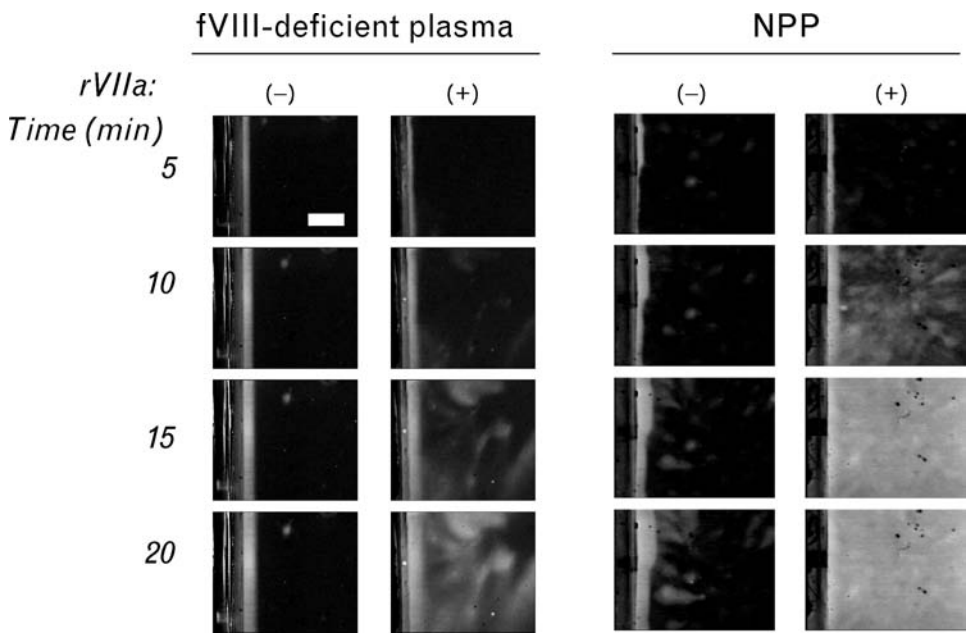


Effect of VIIa concentration and tissue factor distribution on thrombin generation. Activation of fVIII-deficient plasma was established as described in legend to Fig. 3. (a and b) Effect of TF concentration on parameters of thrombin and fibrin generation in mixed (filled triangles) or nonmixed (open triangles) plasma. No clotting was observed without TF and at 0.25 pmol/l of mixed TF. Results are mean ± SD of two separate experiments performed with different plasma donors. (c and d) Effect of recombinant activated factor VII concentration on parameters of thrombin and fibrin generation in mixed (filled triangles) or nonmixed (open triangles) plasma, TF concentration is 2.2 pmol/l. Results are mean ± SD of five separate experiments performed with plasma from different donors. Asterisks show significant ($P < 0.05$, paired *t*-test) difference between mixed and nonmixed experiments. rVIIa, recombinant activated factor VII; TF, tissue factor.

bulk of flowing blood. Thrombin activity and fibrin formation *in vitro* are usually determined as the mean values with respect to the reaction volume. Therefore, this method cannot resolve the initiation of coagulation and growth phases of fibrin clot formation. To study the effect of rVIIa onto each of these phases, we used a videomicroscopy system, which allows monitoring of clot growth initiated by contact of plasma with TF-expressing fibroblast monolayer [26]. In Fig. 5, representative video sequences of clot growth in normal and fVIII-deficient plasmas are shown. In the absence of rVIIa, a nascent clot localized to the surface of initiating cells appeared within 1 min of contact of plasma with fibroblasts. After 20 min of experiment, clot size was 0.8 mm for normal and 0.5 mm for fVIII-deficient plasmas. Addition of rVIIa at 50 nmol/l had no apparent effect on fibroblast-initiated clot growth in both normal and fVIII-deficient plasmas (Fig. 5). However, it caused the beginning of coagulation far from the TF-bearing monolayer, which appeared as 'spontaneously' scattered clots throughout the microchamber [42]. These spontaneous clots with time led to the clotting of total reaction volume in the microchamber.

Additional analysis of the clot light-scattering density along the line perpendicular to initiating cells demonstrated that rVIIa, in the concentration from 5 to 500 nmol/l, had no effect on the clot formation kinetics near fibroblasts (i.e., within the first 2–3 min of experiment, see Fig. 6e). On the contrary, the rate of fibrin clot propagation was decreasing with time in the fVIII-deficient plasma in the absence of rVIIa (Fig. 6a), but remained constant at 50 nmol/l of rVIIa (Fig. 6c and d). As we had shown previously, this termination of propagation of clot size is caused by deficient intrinsic Xase (fIXa : fVIIIa) in hemophilia plasma and it is not observed in normal plasma [26,43]. Therefore, in our

Fig. 5



Effect of recombinant activated factor VII on clot growth initiated by fibroblast monolayer studied by light-scattering video microscopy. Factor VIII-deficient plasma or normal pooled plasma (NPP) were supplemented with rVIIa (50 nmol/l) and placed in contact with monolayers of human fibroblasts grown on polyethylene terephtalate slips. 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 (au) of digital brightness] were linearly extended to full grayscale range (256 au) uniformly for each image in set. Bar: 1 mm. Time-lapse images shown are representative of seven independent experiments. rVIIa, recombinant activated factor VII.

experiments rVIIa was able to normalize the growth phase of clotting. At high rVIIa concentrations plasma started clotting not only near fibroblast surface but also in the whole volume of microchamber. Therefore, it was not possible to determine the rate of clot growth quantitatively after 10 min of the experiment. The effect of rVIIa on spatial coagulation was analyzed using two parameters: initial clot growth rate near the monolayer of TF-expressing cells (Fig. 6e) and lag time of spontaneous clotting of plasma far from the activator (Fig. 6f). As shown in subparts (a) and (b) of Fig. 7, increase of rVIIa concentration from 5 to 500 nmol/l in seven samples of plasma from patients with severe hemophilia A caused a modest increase in the initial clot growth rate ($30 \pm 3\%$, $n = 7$) from TF-bearing cells. Further, rVIIa caused shortening of spontaneous clotting lag time (lag time was reduced by the factor of 4.9 ± 2.3).

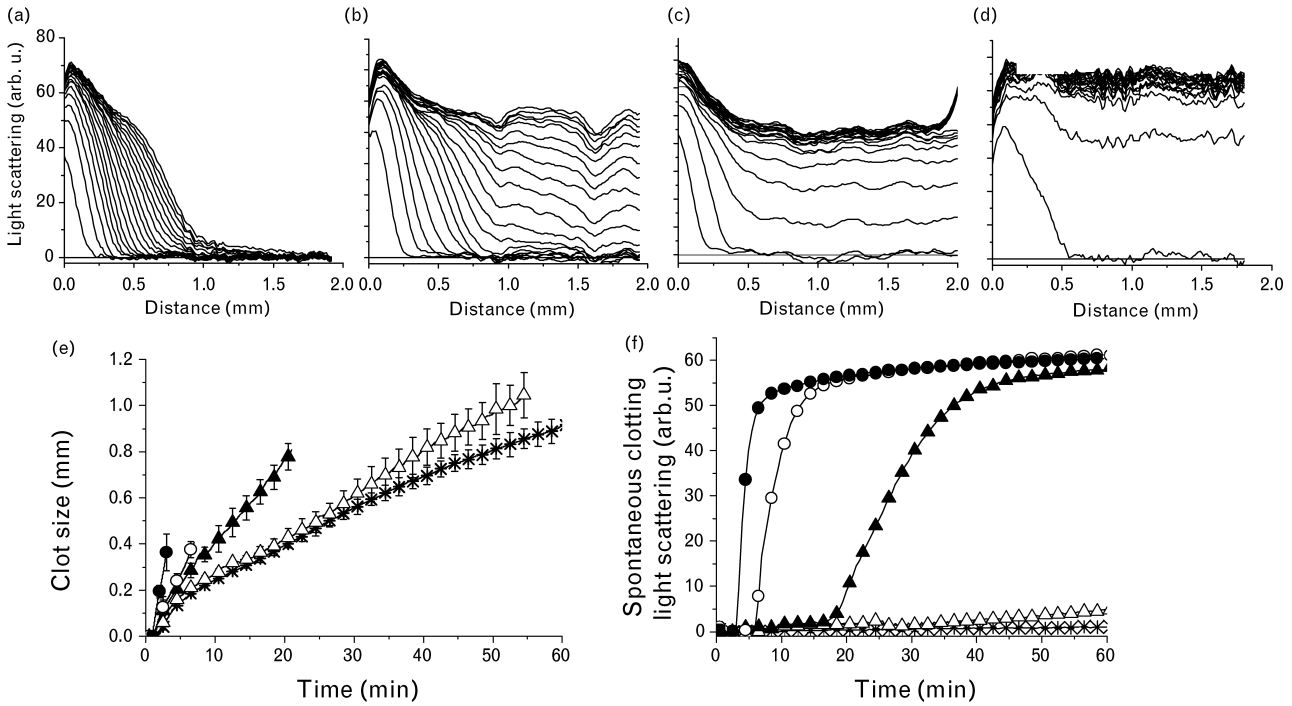
To confirm that rVIIa effect on spontaneous clotting in microchamber was independent of cell surface TF, we carried out similar experiments for normal plasma with activation by either fibroblast-covered or cell-free glass slide. Addition of rVIIa led to a dose-dependent decrease of plasma coagulation time both in the absence and presence of fibroblasts (data not shown). Clot time at 500 nmol/l rVIIa was independent of the activation pathway confirming independence of the spontaneous clotting caused by rVIIa of fibroblasts.

Role of plasma lipids in recombinant activated factor VII effect

A requirement for the expression of TF-independent catalytic activity of rVIIa is the formation of a complex between rVIIa and procoagulant lipids [10,11,13], which could be provided *in vivo* by activated platelets or TF-expressing cells. The experiments of this study were carried out in plasma without platelets. However, the possibility of some activated platelet membrane presence remained, because centrifugation at 3000–10 000 *g* does not deplete plasma from platelets and PMPs completely. Therefore, we subjected plasma to additional ultracentrifugation [27], which removed remaining platelets and decreased concentration of CD62p-positive microvesicles as was confirmed by flow cytometry (data not shown), in accordance with previous reports [44]. The absence of changes in protein composition of plasma was confirmed by normal values of APTT and PT of ultracentrifuged plasma [27].

In the ultracentrifuged microparticle-free normal donor plasma, the clot growth from TF-bearing fibroblasts was reduced by 30–50% in comparison with PFP [30], and spontaneous clotting was significantly suppressed (Fig. 8). Addition of 50 nmol/l of rVIIa into microparticle-free plasma did not change clot growth rate or spontaneous clot formation (filled columns in Fig. 8a and b). When the microparticle-free plasma was supplemented

Fig. 6



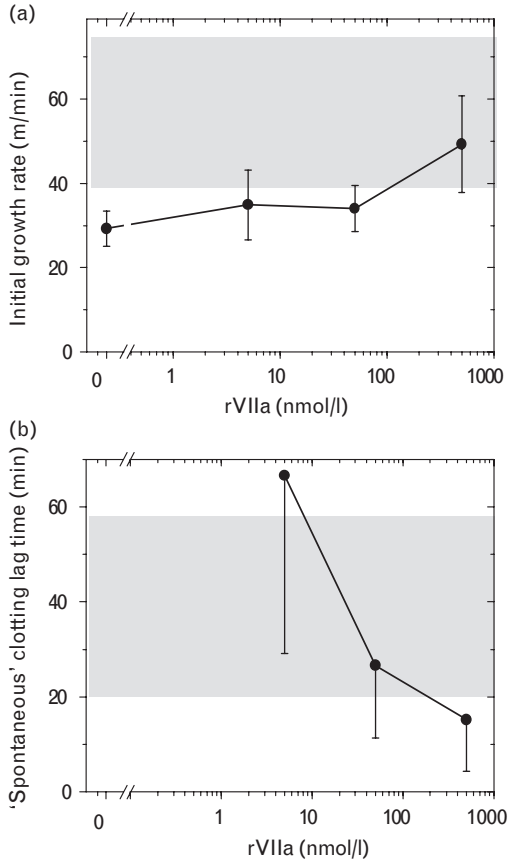
Effect of recombinant activated factor VII on clot growth initiated by fibroblast monolayer. Factor VIII deficient plasma was supplemented with increasing concentrations of rVIIa and placed in contact with monolayers of fibroblasts. (a)–(d): Series of light-scattering profiles measured along a normal to fibroblast monolayers as described in Materials and Methods. Profiles are separated by 2 min intervals, total of 40 min is shown starting from the first min. rVIIa concentration 0 (a), 5 (b), 50 (c), and 500 nmol/l (d). Kinetics of initial clot growth (e) and mean spontaneous clotting within 2–5 mm area away from fibroblasts (f) were studied in plasma supplemented with the following concentrations of rVIIa: 0 (×), 0.5 (open triangles), 5 (filled triangles), 50 (open circles), and 500 nmol/l (filled circles). The data are representative of seven independent experiments. Symbols correspond to each fourth point from kinetic data (mean ± SD, $n = 2$ for two determinations within one experiment). rVIIa, recombinant activated factor VII.

with washed PMPs ($7 \times 10^3\,\mu l^{-1}$), clot growth rate (Fig. 8a) and spontaneous clotting (Fig. 8b) were restored back to normal values. Addition of rVIIa into microparticle-free plasma supplemented with PMPs increased initial clot growth rate and reduced the time of total plasma clotting by two-fold. To exclude possible contribution of microparticle-associated TF to the effect of rVIIa [45], we supplemented microparticle-free plasma with synthetic phospholipid vesicles. A concentration of 1.25 μg/ml was chosen from titration experiments as the concentration that normalizes clot lag times and thrombin peaks in microplate assays (not shown). Synthetic phospholipids restored clot growth rate, spontaneous clotting, and rVIIa effect (Fig. 8a and b). These data indicate that negatively charged phospholipids provided by platelet microparticles are likely to contribute to the experimental hemostatic effect of rVIIa.

Discussion

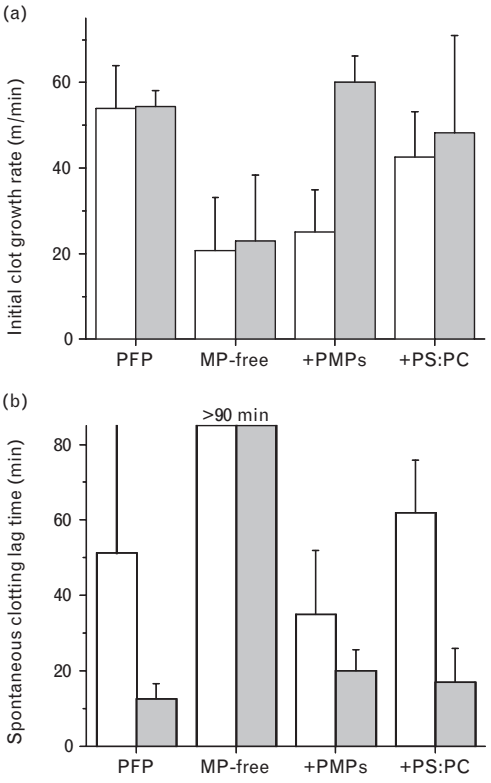
In this work, we varied the quantity and the method of exposure of TF to plasma and found out that activity and localization of TF regulate the observed effect of added rVIIa. Although rVIIa has been widely used for treatment of hemophilia and other bleeding disorders, there is no general agreement about its mechanism of action *in vivo*. Our data suggest that this can be at least partially explained by different conditions of TF delivery into experimental systems. When relipidated TF is uniformly distributed in the reaction volume, absolute values of achieved thrombin peaks are determined by TF concentration. At each rVIIa concentration, they are higher in the presence of TF than in its absence. However, results of this study (Fig. 2a) show that the relative effect of rVIIa is much higher at lower TF concentration. From a physiological point of view, this can mean that rVIIa has only minor effect on coagulation on the TF-expressing surface of the vessel wall, but significantly accelerates thrombin formation far from it. Physiologically, TF-independent action of rVIIa can be potentiated by activated platelets. As compared with 7–15 min required for the formation of fibrin clot, formation of the initial platelet plug occurs within several minutes after the damage, and platelet activation in hemophilia remains normal [34]. Therefore, platelet-dependent activation of fX by rVIIa could efficiently contribute to thrombin generation within this plug and could allow to avoid formation of flabby,

Fig. 7



Effect of recombinant activated factor VII on clot growth rate and lag time. FVIII-deficient plasma was supplemented with rVIIa at indicated concentrations and placed in contact with fibroblast monolayer. Gray area in (a) and (b) represents normal values of parameters observed for $n = 20$ healthy donors, mean ± SD. Initial rates of clot growth from fibroblasts to plasma (a) and lag times of spontaneous clot formation (b) were calculated as described in Materials and Methods; mean ± SD ($n = 7$). rVIIa, recombinant activated factor VII.

Fig. 8



Combined effect of procoagulant phospholipid vesicles and recombinant activated factor VII on tissue factor initiated and 'spontaneous' clot formation. Plasma, depleted from microparticles (microparticle-free) by ultracentrifugation, was supplemented with 7×10^3 vesicles/μl of platelet-derived microparticles (PMPs) or with 1.25 μg/ml of synthetic phosphatidylserine : phosphatidylcholine 25 : 75 vesicles and placed in contact with fibroblast monolayers. Control experiments were also performed on the same platelet-free plasma (PFP) samples before ultracentrifugation. rVIIa concentration: 0 (white bars) and 50 nmol/l (gray bars); mean ± SD ($n = 4$). MP-free, microparticle-free plasma; PFP, platelet-free plasma; PMP, platelet-derived microparticle, PS : PC, phosphatidylserine : phosphatidylcholine vesicles.

lysis-susceptible fibrin clots observed at fVIII deficiency [46].

Elucidation of the rVIIa mechanism of action is necessary to form the basis for the therapeutic strategy, especially in the case of megadosage (the doses that result in plasma concentrations exceeding 300 nmol/l) [47]. According to the TF-independent mechanism, the increase of these doses should lead to a dose-dependent increase of rVIIa hemostatic effect. On the contrary, according to the TF-dependent mechanism, there is no reason to increase rVIIa concentration significantly above the concentration of zymogen (10 nmol/l) competing with rVIIa for TF. For the majority of patients, therapeutically established concentration is 90–120 μg/kg, which corresponds to approximately 50 nmol/l of rVIIa in blood. In the experiments of this study, thrombin generation was dose-dependently increased with the increase of rVIIa up to 1600 nmol/l under all conditions, with the exception of the highest TF concentration (Figs 1 and 2). Therefore, rVIIa seems to work at concentrations high above the zymogen concentration, which has no explanation in terms of TF-dependent mechanism. This is in agreement with clinical reports that, for some patients, therapeutic doses of rVIIa were ineffective, whereas megadoses (more than 900 μg/kg) were able to stop bleeding [47,48].

Another result of our study is comparison of thrombin generation and clot time assays by their sensitivity to hemostatic effect of rVIIa. Even in the presence of trace TF concentration of 4.3 pmol/l, clot time was nearly independent of rVIIa. However, this does not mean saturation of rVIIa action (in contrast to the conclusion of previous studies [21]), as maximal thrombin concentration in the same samples was increased proportionally

with the increase of rVIIa concentration from 0 up to 1600 nmol/l (Fig. 4). The independence of clot time of rVIIa is likely caused by the fact that very small quantities of thrombin are sufficient for the total cleavage of plasma fibrinogen. In agreement with the reports of other authors [34,35], we observed clot formation when only initial thrombin (~5 nmol/l, i.e., ~5% of maximal value) was formed. We suggest the remaining 95% thrombin is important *in vivo* for the formation of hemostatic plug, but its effect cannot be observed *in vitro* in an experimental setting with uniformly distributed TF. Another possible contribution of this additional thrombin could be activation of thrombin-activatable fibrinolysis inhibitor and prevention of clot lysis [49]. According to our concept, normal clots *in vivo* are spatially localized, at least partially, because of localization of the plasma contact with TF. At the site of vascular damage, TF concentration is maximal, and TF acts there as a potent activator of coagulation. It was reported that, upon the damage of tissues with high TF content, hemostasis in hemophilia patients occurred normally [50]. Therefore, on the surface of damaged vessel walls, with excess of TF, only extrinsic tenase and prothrombinase are required for coagulation. However, in large vessels, further clot growth occurs in the bulk of the vessel in the absence of TF. Under these conditions, thrombin generation depends on additional pathways of coagulation system: on fX activation by the intrinsic tenase complex, and on fIX activation by fXIa, which in turn is produced by thrombin. Activation of the components of intrinsic tenase and of fXI requires significant thrombin to be already present. These reactions begin to contribute to thrombin formation only at later stages, that is, when vessel wall is already covered with adhered platelets and initial layer of fibrin. However, at this moment thrombin diffusion into plasma and its production on the surface of activated platelets allow for the beginning of fibrinogen cleavage far from the site of activation. Therefore, localization of TF leads to appearance of two phases of clot formation: initiation, during which initial clot is formed on the surface of vessel wall (this clot can be created by initial traces of thrombin); and spatial clot growth supported by continuous thrombin generation at some distance from the activator. Catalytic efficiency of rVIIa in the absence of TF is small, leading to a delayed response of plasma clotting to it; and therefore, the rVIIa hemostatic effect should play a role mostly during the second phase of clot formation. In our experiments, when relipidated TF contacted plasma, thrombin generation was lower than in thoroughly mixed samples, but rVIIa effect was twofold more significant (Fig. 4). Most probably, this was because rVIIa was able to work at the locations where no TF was present. This is in agreement with the results observed in videomicroscopy experiments: initial clot near the activator was formed quickly and independently of rVIIa concentration, whereas coagulation far from the activator required 15–40 min, and was significantly accelerated by rVIIa (Fig. 5).

In vivo, coagulation far from the activator should be initiated by VIIa bound to activated platelets, which were absent in our experiments. However, procoagulant surface in plasma can also be provided by circulating cell-derived (especially by platelet-derived) microparticles [30,44]. We believe that the hemostatic effect of rVIIa in our experiments is caused by its binding to these microparticles, because rVIIa had no effect on coagulation in the absence of microparticles (Fig. 8). Interestingly, our preliminary conclusion about the role of microparticles in the rVIIa effect [51] has been recently confirmed in the work of Tonda *et al.* [52] in a flow model. It is unlikely that microvesicle-associated TF contributes to the rVIIa effect in our experiments, because not only platelet microvesicles but also synthetic phosphatidylserine-containing vesicles were able to recover rVIIa effect in the microparticle-free ultracentrifuged plasma (Fig. 8). Procoagulant activity of microparticles is very high and comparable with that of activated platelets [27]. It is possible that efficiency of rVIIa *in vivo* depends on the concentration of circulating microparticles in different patients, which varies significantly [53], and/or on the ability of platelets to form these microparticles. The latter possibility is supported by the recent finding that rVIIa infusion is accompanied by the generation of PMPs [53]. From a methodological point of view, the concentration of platelet microparticles should probably be one of the parameters to characterize reference plasmas for the functional monitoring of infused rVIIa in clinical practice. It could be also necessary to avoid platelet activation in the process of sample preparation. Off note, although our study was not designed to model safety issues of rVIIa action, it is plausible to conclude that high plasma concentration of microparticles might be considered a risk factor for rVIIa administration.

Summarizing, we have found that *in vitro* the mechanism of action of rVIIa depends on the concentration and distribution of TF in plasma. Our data support the importance of the TF-independent mechanism for the expression of rVIIa hemostatic activity *in vivo*. In particular, our results favor the use of megadoses of rVIIa (plasma levels of rVIIa exceeding 200 nmol/l, which is equivalent to the dose of 400 μg/kg [1]) for the normalization of hemostasis and suggest an important role of microparticles in the hemostatic effect of rVIIa.

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