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A possible mechanism of action of activated factor VII independent of tissue factor

D. M. Monroe, M. Hoffman, J. A. Oliver and H. R. Roberts

We have used a cell-based model system to examine some aspects of coagulation. Unactivated platelets and tissue factor (TF)-bearing cells were mixed with plasma levels of zymogen factors IX (FIX), FVIII, FX, FV, and prothrombin, as well as coagulation inhibitors antithrombin III and TF pathway inhibitor. Reactions were initiated with plasma levels (0.2 nmol/l) of activated factor VII (FVIIa). We were able to measure platelet activation and subsequent thrombin generation in this system and have established parameters for the normal amount of thrombin generation and the range of values seen with different individuals. If FIX or FVIII were not added to this system, platelet activation but not thrombin generation was seen. We have used this system to examine the mechanism of action of high-dose FVIIa. If platelets were activated with the thrombin receptor agonist peptide SFLLRN and incubated with inhibitors and zymogen factors X, V, and prothrombin, no thrombin generation was observed. Addition of increasing amounts of FVIIa gave increasing amounts of thrombin generation. At the FVIIa concentrations present in the plasma of patients given 60 μg/kg recombinant FVIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark), 10–40 nmol/l, thrombin generation in the model system approached the normal amount seen in the TF-initiated model system. When FIX and FVIII were included in the above reaction, FVIIa could initiate thrombin generation at levels three to four times the amount seen in the TF-initiated model system. We speculate that this platelet-localized thrombin generation may, in part, account for the clinical efficacy of high-dose FVIIa. *Blood Coag Fibrinol* 9 (suppl 1):S15–S20 © 1998 Lippincott-Raven Publishers

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Introduction

One of the fundamental mechanisms for regulating coagulation is the formation of protein complexes that consist of an activated coagulation protease with a membrane-associated cofactor protein [1]: activated factor VII (FVIIa) with tissue factor (TF); FIXa with FVIIIa; and FXa with FVa [2–6]. The activities of coagulation proteases are enhanced up to three orders of magnitude when the proteases are associated with their appropriate cofactors [1]. While coagulation proteases can cleave their protein substrates in the absence of cofactor, this process is inefficient. At plasma concentrations of coagulation factors, there is essentially no activity of the coagulation proteases in the absence of their cofactors. For example, the absence of FVIII results in a haemophilic state, even though physiological mechanisms to generate FIXa are still functional. Hedner *et al.* [7] observed that if FVIIa were given at sufficiently high doses, it was possible to provide therapy in a haemophilia A patient without replacement of FVIII. Subsequent studies established that effective therapy for bleeding in patients with haemophilia A or B required doses of FVIIa greater than 60 μg/kg given every 2–4 h [8]. This generated plasma concentrations of FVIIa [8,9] that were up to 40 nmol/l

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and therefore 500 times greater than physiological levels of FVIIa (0.075 nmol/l) [10]. These levels were sufficiently high to raise the possibility that FVIIa at these concentrations had significant activity in the absence of cofactor. Previous studies have shown that, at the concentrations present in the plasma of patients given high-dose FVIIa, FVIIa could activate FX directly on a phospholipid surface [2,11,12]. However, the amounts of phospholipid required and the composition of the lipid vesicles used in these studies were non-physiological. We speculated that activated platelets might provide a suitable physiological surface for the action of FVIIa independent of TF. We have examined the ability of FVIIa to bind to activated platelets and subsequently to activate FX and catalyse thrombin generation.

Materials and methods

Materials

TenStop and Spectrozyme FXa were purchased from American Diagnostica (Greenwich, CT, USA). Chromozyme Th was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). Ser–Phe–Leu–Leu–Arg–Asn (SFLLRN) was purchased from Multiple Peptide Systems (San Diego, CA, USA). All other reagents were of a high commercial grade.

Proteins

Prothrombin was purified using barium citrate, DEAE-cellulose and a copper chelate column. FIX was purified as described by McCord *et al.* [13]. FX was purchased from Enzyme Research Labs (South Bend, IN, USA). All zymogen coagulation factors were treated with an inhibitor mixture (tosyl-lysyl chloromethyl ketone, tosyl-phenylalanine chloromethyl ketone, phenylmethyl sulphonyl fluoride, Phe–Pro–Arg chloromethyl ketone and dansyl Glu–Gly–Arg chloromethyl ketone) for 1 h, then repurified on Q Sepharose fast flow using calcium chloride elution, essentially as described by Yan *et al.* [14]. FV was purchased from Calbiochem (San Diego, CA, USA). FVIII that contained von Willebrand factor (vWF) was repurified from Profilate by gel filtration on Sepharose CL-2B. FVIII without vWF (Recombinate) was obtained from the Comprehensive Hemophilia Treatment Center at the University of North Carolina, USA. Antithrombin III (ATIII) was prepared as described by Church *et al.* [15]. FVIIa and full-length tissue factor pathway inhibitor (TFPI) were the generous gift of Dr Ulla Hedner (Novo Nordisk, Bagsvaerd, Denmark). Anti-tissue factor antibody (neutralizing antibody catalogue number 4509) was purchased from American Diagnostica (Greenwich, CT, USA).

Platelet purification

Monocytes and platelets were prepared as described previously using Accu-prep Lymphocyte separation medium (Accurate Chemicals, Westbury, NY, USA) [16]. Monocytes were isolated by plating as described by Hoffman *et al.* [16]. Platelets were further isolated by gel filtration on Sepharose CL-2B [17,18]. Analysis by flow cytometry did not detect any cells except platelets present in the preparation ($<1 \times 10^6$ contaminating cells).

Activity assays

Monocytes (20 per µl) were obtained 18–24 h prior to initiating the reactions and were cultured overnight with lipopolysaccharide to induce maximal expression of TF. Unactivated platelets (200 000 per µl) were added to a concentrated solution of calcium and proteins to give 3 mmol/l calcium and plasma concentrations of: prothrombin (100 µg/ml), FX (8 µg/ml), FV (7 µg/ml), FVIII (0.2 µg/ml), ATIII (150 µg/ml), and TFPI (0.1 µg/ml). FIX was included in selected experiments at 4 µg/ml. The zymogen proteins had been preincubated with 20-fold plasma concentrations of ATIII and TFPI for at least 12 h to ensure that there were no activated proteases contaminating the zymogens. To initiate the reactions, FVIIa (0.2 nmol/l) was added, and the platelets and proteins put in wells containing monocytes. At timed intervals, 10 µl samples were removed and assayed for thrombin generation by addition to 90 µl of a solution of 5 mmol/l ethylenediamine tetraacetic acid (EDTA), 0.5 mmol/l Chromozyme Th and 50 µmol/l TenStop (sufficient to block any FXa activity in these experiments). EDTA served to chelate the calcium and stop further activation of coagulation proteins. After 13 min, cleavage of the synthetic substrate Chromozyme Th was stopped by addition of 100 µl of 50% acetic acid. The amount of thrombin was determined by measuring the absorbance at 405 nm and comparing the values obtained with a standard curve.

Experiments looking at the effect of high-dose FVIIa on thrombin generation were performed as described above, except that monocytes were not included in the reactions, and platelets were activated by incubation with 50 µg/ml of the thrombin receptor agonist peptide SFLLRN for 15 min at 37°C [19]. FVIIa was mixed with the other proteins immediately prior to addition to platelets.

To measure activation of FX, activated platelets (200 000 per µl) were added to a concentrated solution of calcium and proteins to give 3 mmol/l calcium and plasma concentrations of FX with varying concentrations of FVIIa. FIX and FVIII (free from vWF) were included in selected experiments. FVIIa was mixed

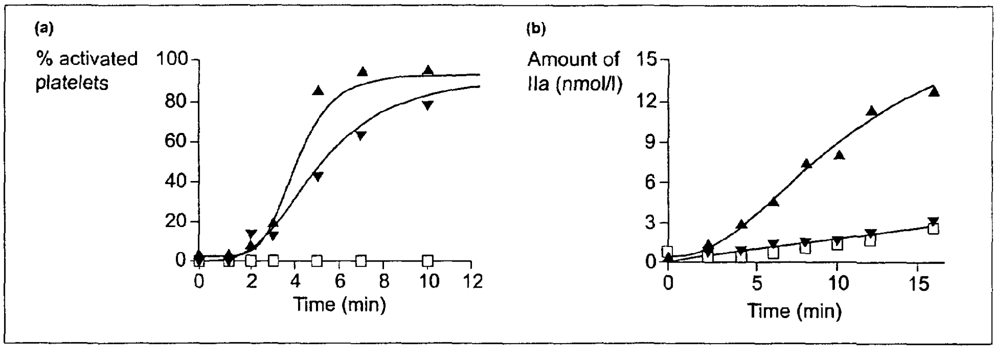

Fig. 1. Activity in the model system. Monocytes (stimulated to produce tissue factor), unactivated platelets and plasma concentrations of coagulation factors and inhibitors were incubated as described in Materials and methods. At the indicated times, samples were removed and assayed for **(a)** platelet activation and **(b)** thrombin (IIa) generation, as described in Materials and methods. ▲, Data when all of the components were included in the model system; ▼, data when factor IX (FIX) was omitted from an otherwise complete system; □, data when monocytes were omitted from the system.

with the other proteins immediately prior to addition to platelets. At timed intervals, 15 µl samples were removed and added to 35 µl of EDTA to stop further activation of coagulation proteins. Once all the samples had been taken, 50 µl of 0.2 mmol/l Spectrozyme FXa was added. The amount of FXa was determined from a kinetic measurement of the absorbance at 405 nm and compared with a standard curve.

Results and discussion

The model

We have been studying a model of coagulation that incorporates cells as an essential feature of the model [20–23]. In studies to date, we have used two types of cell: monocytes and unactivated platelets. Monocytes are obtained by separating mononuclear cells from other blood cells by centrifugation on density gradient media, then monocytes are separated from other white cells by allowing the monocytes to attach to the wells of a microtitre plate [16]. These monocytes can be stimulated to express TF by culturing the cells overnight in media containing bacterial lipopolysaccharide.

The other cells we have used are unactivated platelets. Platelets are separated from plasma proteins by centrifugation on density gradient media followed by a low speed spin to remove mononuclear cells. Platelets are then isolated by gel filtration [18]. These platelets are unactivated as judged by measuring expression of CD62 (P-selectin, GMP-140) by indirect immunofluorescence and flow cytometry. Platelets are used within an hour of isolation and are, in general, less than 2% activated at the start of our experiments.

In addition to cells, in this model of coagulation we have also used zymogen protein factors and inhibitors. We used coagulation factors at their plasma concentrations: prothrombin (100 µg/ml), FX (8 µg/ml) and FIX (4 µg/ml). We also used unactivated coagulation cofactors at their plasma concentrations: FV (7 µg/ml) and FVIII (0.2 µg/ml as defined in a clotting assay). The FVIII used in these experiments was bound to vWF. Finally, we used plasma concentrations of the coagulation inhibitors ATIII (150 µg/ml) and TFPI (0.1 µg/ml). All reactions were run in the presence of 3 mmol/l calcium. To initiate this model of coagulation, we added 0.2 nmol/l FVIIa (0.01 µg/ml), which is equivalent to 2% of the plasma concentration of zymogen FVII and is at the upper end of the range of the values reported for circulating levels of FVIIa in normal individuals.

Mixing unactivated platelets (200 000 per µl) with TF-bearing cells (20 per µl) and with the proteins described above leads to a rapid activation of platelets, as shown in Figure 1a. Subsequent to this platelet activation, we measured large-scale, nanomolar generation of thrombin as shown in Figure 1b. This series of events is dependent on a number of elements and neither platelet activation nor thrombin generation is observed if: calcium is omitted; TF-bearing cells are omitted (Fig. 1a) or TF is blocked by a neutralizing antibody; prothrombin is omitted; FX is omitted. A haemophilic condition can be mimicked by omitting FIX from the reaction mixture and including a neutralizing antibody towards FIX (since platelets contain and release small amounts of FIX). Under these

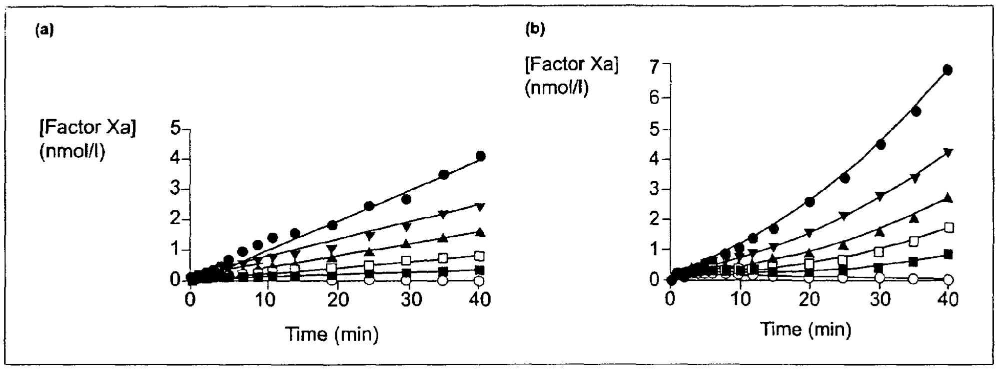

Fig. 2. Activated factor VII (FVIIa) activation of FX on platelets. Platelets were activated with the peptide Ser–Phe–Leu–Leu–Arg–Asn (SFLLRN), as described in Materials and methods. Activated platelets were incubated with calcium, plasma concentrations of FX, and varied concentrations of FVIIa either in **(a)** the absence or **(b)** the presence of plasma concentrations of FIX and FVIII (without von Willebrand factor). At the indicated times, samples were removed and assayed for FXa, as described in Materials and methods. Concentrations of FVIIa were: ○, none; ■, 2 nmol/l; □, 5 nmol/l; ▲, 10 nmol/l; ▼, 20 nmol/l; ●, 50 nmol/l.

circumstances, we see platelet activation (Fig. 1a) but not thrombin generation (Fig. 1b).

Coagulation signals in vivo

These studies and others have led us to suggest that *in vivo* haemostasis might involve two coagulation signals [20]. One signal is mediated by activation of FX by TF–FVIIa. The FXa generated on the TF-bearing cell remains associated with that cell, forms a complex with FVa, and converts a small amount of prothrombin to thrombin. This thrombin is critically important to activate platelets, release FVIII from vWF, and activate FV on the platelet surface.

The other signal required for coagulation is mediated by activation of FIX by TF–FVIIa. We believe that this FIXa can associate with the surface of activated platelets, form a complex with FVIIIa, and convert FX to FXa. Subsequently, this platelet surface FXa forms a complex with platelet-bound FVa and converts prothrombin to thrombin. We believe that this platelet surface-generated FXa is critical to large-scale thrombin generation. We do not believe that FXa generated on a cell other than platelets can efficiently transport to the platelet surface, possibly as a result of solution phase inhibition by ATIII or TFPI. A figure of this model is shown by Hoffman *et al.* (this supplement, pages S61–S65).

We have examined the ability of FVIIa to bind to activated platelets and, once bound, to activate FX. As described previously [24,25], FVIIa binding to platelets was measured by adding FVIIa to platelets in the presence of 3 mmol/l calcium. Under these conditions, we observed saturable binding of FVIIa to activated platelets. Assuming a single class of binding sites, the dissociation constant (K_d) for this binding is about 100 nmol/l, which is consistent with binding of γ-carboxyglutamic acid (Gla)-containing proteins to phospholipid surfaces [26]. This binding does not occur if calcium is omitted. Only minimal binding to unactivated platelets is observed, which suggests that binding might be mediated by phosphatidylserine exposed when platelets are activated. This binding is independent of TF. We have used sensitive immunofluorescent detection methods and have failed to find any evidence of TF antigen on platelets [24]. These results are in agreement with a previous study which showed that platelets contain no TF [27].

We have examined the ability of FVIIa to cleave FX on activated platelets. Platelets were activated by incubation with the agonist peptide for one of the thrombin receptors (SFLLRN). Activated platelets were incubated with plasma concentrations of FX in the presence of 3 mmol/l calcium. At timed intervals, samples were removed and assayed for FXa using a chromogenic substrate. In the absence of added FVIIa, no activation of FX was observed (Fig. 2a). Increasing concentrations of FVIIa up to 50 nmol/l gave increasing rates of FXa activation (Fig. 2a). FXa generation was linear with time, which implies that FVIIa directly activates FX. This FXa remained associated with the platelet surface. When platelets were spun through sucrose and extracted, FXa could be detected by Western blotting on those platelets that had been incubated with FVIIa but not on platelets to which FVIIa had not been added [24,25].

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We also examined the effect of adding plasma concentrations of FIX and FVIII (without vWF) to the reactions described above (Fig. 2b). As before, when no FVIIa was added, no FX activation was detected. Increasing concentrations of FVIIa gave increasing activation of FX (Fig. 2b). However, in this case, activation of FX was not linear with time but steadily increased with time. The data can be fitted to a second-order polynomial and are consistent with a mechanism by which FVIIa activates FIX. This FIXa, in conjunction with platelet-bound FVIIIa, could then activate FX more efficiently than FVIIa alone.

Platelet surface FVIIa activity

To examine the role of platelet surface FVIIa activity on thrombin generation, we used a variation on the coagulation model system. In order to understand the activity of FVIIa in the absence of TF, we eliminated TF-bearing monocytes. Instead, platelets were activated with SFLLRN so that no proteases were added to the system. To the activated platelets, we added plasma concentrations of coagulation factors prothrombin, FX and FV, as well as the inhibitors ATIII and TFPI. This mimics a haemophilic state since no FIX or FVIII are present. In this system, no thrombin generation was observed. Addition of increasing concentrations of FVIIa gave increasing rates of thrombin generation in all cases tested (Fig. 3). When plasma concentrations of FIX and FVIII were included in the reaction, significantly more thrombin generation was catalysed than in the haemophilic model (Fig. 3). This increased activity in the presence of FIX and FVIII is consistent with the greater rates of FX activation seen in the presence of FIX and FVIII.

We have previously observed that the 'activity' of platelets from different individuals can vary significantly. If we take the same number of platelets from two people (A and B) and assay them at the same time in the TF-initiated model system using identical protein solutions, person A may make three times as much thrombin as person B. This result is quite reproducible, so that we can define a 'normal' amount of thrombin generation. The data shown in Figure 3 have been normalized so that the amount of thrombin that we expect a given individual to make has been defined as 1. The grey area in Figure 3 shows the anticipated concentration range for FVIIa in a person given 60 µg/kg recombinant FVIIa (rFVIIa; NovoSeven, Novo Nordisk, Bagsvaerd, Denmark) every 3 h [8,9]. In this model of the haemophilic condition, the highest concentration of FVIIa catalyses a rate of thrombin generation that approaches the thrombin generation we expect under TF-initiated

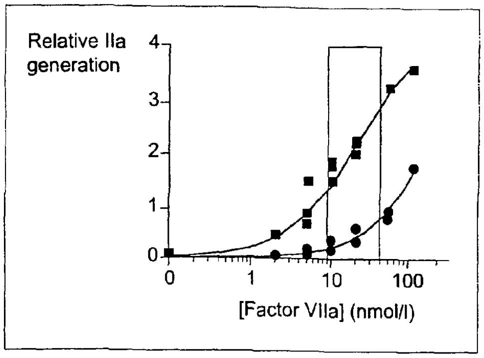

Fig. 3. Activated factor VII (VIIa) catalyses thrombin generation on activated platelets. Platelets were activated with the peptide Ser–Phe–Leu–Leu–Arg–Asn (SFLLRN), as described in Materials and methods. Activated platelets were incubated with plasma concentrations of prothrombin, FX, FV, ATIII and tissue factor pathway inhibitor (●). In some experiments, plasma concentrations of FIX and FVIII were included (■). The indicated concentrations of FVIIa were added and the rate of thrombin generation was measured as described in Materials and methods. This rate of thrombin generation was normalized to the rate of thrombin generation expected for the platelets used in this study (all the data from three individuals are shown). The shaded region shows the plasma concentration of FVIIa expected in an individual given 60 µg/kg recombinant FVIIa every 3 h.

conditions. When FIX and FVIII are included in the model, thrombin generation exceeds what we expect from TF-initiated coagulation and may reach three to four times the amount of thrombin generation we would normally expect.

Summary and conclusions

To the extent that data from this *in vitro* model system can be extrapolated *in vivo*, these studies indicate that FVIIa can directly activate FX on activated platelets even though platelets do not contain TF. This platelet surface FXa can complex with FVa and generate nearly normal levels of thrombin. This thrombin generation should be enough to provide for haemostasis in haemophilic patients. These studies also indicate that when all of the coagulation factors are present, thrombin generation can be increased to several times the normal levels, i.e. fewer platelets are required to reach a given level of thrombin generation. This observation may, in part, account for the effectiveness of FVIIa in treating some bleeding patients who have thrombocytopenia [28].

1. To the extent data from this model system can be extrapolated in these studies indicate that FVIIa ...
Anchor Name: Novoseven acts on the surface of the activated platelets only (site of vascular injury) [ZRMM (Zahra Bou Melhem)]

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