British Journal of Haematology, 2001, **114**, 114–120

High-dose factor VIIa increases initial thrombin generation and mediates faster platelet activation in thrombocytopenia-like conditions in a cell-based model system

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Received 4 December 2000; accepted for publication 2 March 2001

Summary. Clinical experience has shown that high doses of recombinant factor VIIa (rFVIIa) may ensure haemostasis in thrombocytopenic patients. We have used a cell-based model system to mimic thrombocytopenia and analyse the effect of rFVIIa. Lowering the platelet density from 200×10^9/l (reflecting normal conditions) to 100, 50, 20 and 10×10^9/l revealed a platelet density-dependent decrease in the maximal rate of thrombin generation, a prolongation in the time to maximal thrombin activity and a lower maximal level of thrombin formed. The platelet activation, measured as the time to half-maximal P-selectin (CD62) exposure, was not significantly dependent on the platelet density in the range of 200×10^9/l to 10×10^9/l, although there was a tendency for slower platelet activation at 20×10^9 and 10×10^9 platelets/l than at the higher platelet densities. Addition of 50–500 nmol/l rFVIIa to samples with 20×10^9 or 10×10^9 platelets/l shortened the lag phase of thrombin generation as well as the time to half-maximal platelet activation. Our data indicate that high doses of rFVIIa may help to provide haemostasis in thrombocytopenic patients by increasing the initial thrombin generation, resulting in faster platelet activation and thereby compensating for the lower number of platelets present.

Keywords: factor VIIa, thrombin, thrombocytopenia, platelets, bleeding.

Activated factor VII (FVIIa) circulates in plasma in concentrations of around 1% of the zymogen form of factor VII (Wildgoose *et al*, 1992; Morrissey *et al*, 1993). Upon binding to its receptor and cofactor tissue factor, FVIIa gains full catalytic activity and activates its substrates factor X and factor IX, thereby initiating coagulation. High doses of recombinant FVIIa (rFVIIa, NovoSeven) are used to control bleeding episodes in haemophilia A or B patients who have inhibitory antibodies against factor VIII or factor IX respectively.

Potential conflicts of interest of the authors: Ulla Hedner and Marianne Kjalke are employed by Novo Nordisk. Mirella Ezban has been employed by Novo Nordisk (until November 2000) but is now employed by another company, namely M & E Biotech. Harold Roberts is a member of an advisory commitee for the use of recombinant factor VIIa (NovoSeven), for which he receives an honorarium. Dougald Mac Monroe and Maureane Hoffman do not have any conflicts of interest.

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rFVIIa, at pharmacological doses, is able to bind activated platelets, exposing negatively charged phospholipids, and activate factor X, leading to thrombin generation (Monroe *et al*, 1997). The binding requires activation of platelets, which explains the localization of activity to the site of injury. The binding to activated platelets is much weaker than binding to tissue factor, which explains the requirement for high levels of rFVIIa. An alternative hypothesis is that zymogen factor VII competes with FVIIa in binding to tissue factor and that rFVIIa at pharmacological doses overcomes this inhibition (van't Veer *et al*, 2000). The two models for the mechanism of action of rFVIIa are not necessarily mutually exclusive.

In normal individuals, the platelet density in peripheral blood is between 200×10^9/l and 300×10^9/l. Individuals with platelet densities of 100×10^9/l and otherwise normal coagulation factors rarely suffer from bleeding problems. However, the risk of bleeding increases with lower platelet density and major haemorrhage can be observed in patients with platelet counts in the range of $20–5 \times 10^9$/l (Murphy, 1998). In cases of platelet densities of $20–10 \times 10^9$/l and

lower, prophylactic platelet transfusions are recommended. High doses of rFVIIa shortened the bleeding time in 55 out of 105 thrombocytopenic patients (Kristensen *et al*, 1996). Eight thrombocytopenic patients with bleeding in spite of platelet transfusions have been treated with high doses of rFVIIa (Kristensen *et al*, 1996). Bleeding decreased in all patients and stopped in six patients. This indicates that rFVIIa may help control bleeding episodes in thrombocytopenic patients.

The present study was carried out to provide a possible explanation for the haemostatic effect of high-dose rFVIIa in thrombocytopenia. We used a cell-based model system (Monroe *et al*, 1994) to mimic thrombocytopenic conditions and evaluate the effect of adding high doses of rFVIIa. In this model, platelet activation and thrombin generation are measured at timed intervals after mixing tissue factor-expressing monocytes, unactivated platelets, isolated coagulation proteins and calcium. Thrombocytopenic conditions were mimicked by lowering the platelet density from 200×10^9/l to 20×10^9/l and 10×10^9/l. Addition of 50–500 nmol/l rFVIIa did not alter the overall thrombin generation time-courses, but did shorten the lag phase of thrombin generation, thereby increasing the initial amount of thrombin formed and shortening the time to half-maximal platelet activation.

MATERIALS AND METHODS

Proteins. Human coagulation factors V, XI, prothrombin and anti-thrombin III (ATIII) were purchased from Haematologic Technologies and human factor X from Enzyme Research Laboratories. Factor VIII in complex with von Willebrand factor (MelATE, New York Blood Center) and factor IX (Mononine, Armour Pharmaceutical) were from the hospital pharmacy at the University of North Carolina at Chapel Hill. rFVIIa and tissue factor pathway inhibitor (TFPI) were prepared as described (Thim *et al*, 1988; Pedersen *et al*, 1990). Factor X and prothrombin were treated with a protease inhibitor mix to neutralize traces of active enzymes as described (Monroe *et al*, 1994). Factor VIII was gel filtered on Sepharose CL2B equilibrated with 20 mmol/l HEPES pH 7·4 containing 150 mmol/l NaCl (HEPES buffered saline; HBS) to remove traces of fibrinogen and other coagulation factors.

Cell isolation. Peripheral blood samples of 10–30 ml were taken from healthy volunteers who had not taken acetylsalicylic acid within the last 10 d or other non-steroidal anti-inflammatory drugs within the last 2 d. All donors had signed an informed consent form. Mononuclear cells and platelets were isolated by centrifugation on Lymphoprep (Nycomed, Life Technologies) as described (Kjalke *et al*, 1997). Prostaglandin E_1 (Sigma) was added to the blood to a final concentration of 5 μg/ml throughout the isolation procedure when platelets were isolated in order to prevent activation.

The mononuclear cells containing 5–15% monocytes, determined as strongly CD14-positive cells by flow cytometry (see below), were resuspended in macrophage serum-free media (Life Technologies) and plated at a density of 5000 monocytes per well in two rows in a 96-well tissue culture plate (Nunclon, Nunc). Bacterial lipopolysaccharide (LPS, *Escherichia coli* serotype O128:B12, Sigma L2755) was added to 0·5 μg/ml. The cells were allowed to adhere for 1 h at 37°C in 5% CO_2. Non-adherent cells, mainly lymphocytes and remaining platelets, were removed by washing in media. The cells were incubated overnight with 0·5 μg/ml LPS at 37°C in 5% CO_2. Before carrying out the assay, the wells were washed with calcium-free Tyrodes buffer (15 mmol/l HEPES, 3·3 mmol/l Na_2PO_4, pH 7·4, 138 mmol/l NaCl, 2·7 mmol/l KCl, 1 mmol/l $MgCl_2$, 5·5 mmol/l dextrose) containing 1 mg/ml bovine serum albumin (BSA).

The platelets were gel filtered on Sepharose CL2B equilibrated with the Tyrodes buffer immediately before use to remove plasma proteins and added prostaglandin E_1. The platelet density was determined using flow cytometry (see below) and the volumes adjusted with the Tyrodes buffer to give final densities of 200×10^9, 100×10^9, 50×10^9, 20×10^9 and 10×10^9 platelets/l.

Cell-based assay. The assay was carried out in principle as described previously (Hoffman *et al*, 1995). Briefly, rFVIIa (final concentrations of 0·2 nmol/l, 50 nmol/l, 100 nmol/l and 500 nmol/l) was mixed with factor X (8 μg/ml), factor IX (5 μg/ml), factor XI (5 μg/ml), prothrombin (86 μg/ml), TFPI (0·1 μg/ml), ATIII (120 μg/ml), factor V (7 μg/ml), factor VIII (1 U/ml) and calcium chloride (3 mmol/l) in microtitre wells. The assay was initiated by mixing the proteins and unactivated platelets with a multichannel pipette and transferring proteins plus platelets to the tissue factor-expressing monocytes. Aliquots of 10 μl were withdrawn at timed intervals and analysed for platelet activation (time points between 0 and 10 min, see below) and thrombin amidolytic activity (time points between 0 and 3 h). The thrombin activity was determined by adding the aliquots to 90 μl of HBS containing 1 mg/ml BSA, 1 mmol/l EDTA, 50 μmol/l Pefabloc Xa (Pentapharm) and 0·5 mmol/l Chromozyme TH (Boehringer Mannheim). The colour development was stopped after 19 min by adding 100 μl of 2 mol/l acetic acid and A405 was measured. The absorbance values were transformed into thrombin concentration by comparison with a standard curve made from human α-thrombin (Boehringer Mannheim). The area under the thrombin generation curve was determined using GRAPHPAD PRISM (GraphPad Software).

Flow cytometry. The percentage of monocytes in the mononuclear cell preparations were determined as strongly CD14-positive cells. An aliquot of the cell preparation containing 10^6 cells was labelled with phycoerythrin-conjugated anti-CD14 IgG (Becton Dickinson). The samples were analysed on a FACScan flow cytometer (Becton Dickinson). The forward- and side-scatter light channels were set on linear scale and the fluorescence channel 2 amplifier set on log. A gate was set on the mononuclear cells on a forward/sideward light scatter dot-plot and 10 000 events within this gate were analysed for CD14 fluorescence intensity.

The platelet density was determined using TruCount

[no notes on this page]

Table I. Platelet activation and thrombin generation at various platelet densities and 0·2 nmol/l rFVIIa.

Platelets ($\times 10^9$/l)	Time to half-maximal platelet activation* (min)	Parameters of thrombin generation			
		Maximal rate† (nmol/l/min)	Time to maximal activity (min)	Maximal activity (nmol/l)	Total activity (area under the curve) (nmol/l × min)
200	6·0 (3·9–8·2)	16·3 (7·8–28·3)	21·7 (15–25)	142 (102–195)	4155 (2790–6166)
100	5·9 (3·7–6·9)	8·7 (3·2–15·9)	24·3 (20–40)	94 (52–128)	4041 (2425–6086)
50	6·4 (3·6–8·6)‘	3·8 (1·2–6·0)	46·4 (25–70)	‘59 (32–94)	4048 (2378–6248)
20	6·6 (3·5–12·5)	1·8 (0·5–3·6)	n.d	n.d	n.d
10	7·2 (4·1–12·0)	1·2 (0·5–2·1)	n.d	n.d	n.d

*The values for platelet activation are the times at which half the platelets were positive for P-selectin (CD62).
†The maximal rate corresponds to the maximal slope of the thrombin generation curve.
The values show the mean and range of data (in parentheses) from a total of seven donors. n.d., not detectable.

Tubes (Becton Dickinson). Two aliquots of 10 µl were diluted 150-fold in 2% paraformaldehyde in HBS, and the platelets were quantified relative to the fluorescent beads in the TruCount tubes, as described by the manufacturers. The forward- and side-scatter light channels as well as the fluorescent channels were set on log on the flow cytometer and 10 000 total events were counted.

Platelet activation was determined by measuring the exposure of the α-granule marker P-selectin (CD62) on the surface of the platelets after labelling with phycoerythrin-conjugated anti-CD62 IgG (Becton Dickinson) as described previously (Kjalke *et al*, 1997). Unactivated platelets were used as a negative control, samples with 200×10^9 platelets/l taken at the 10-min time point were used as a positive control, and the percentage of CD62-positive cells were measured relative to these. The time to half-maximal platelet activation was determined by fitting the data to the equation for IC50 using GRAFIT (Erithacus Software).

RESULTS

Thrombin generation and platelet activation at various platelet densities

A cell-based model system (Monroe *et al*, 1994) comprising tissue factor-expressing monocytes, unactivated platelets and purified coagulation proteins was used to mimic thrombocytopenia. The platelet density was varied beginning with 200×10^9/l and lowered to 100, 50, 20 and 10×10^9/l. The platelets and proteins were added to the monocytes at the start of the assay, and aliquots were removed at timed intervals and analysed for platelet activation, i.e. exposure of P-selectin (CD62) on the cell surface by flow cytometry, and thrombin amidolytic activity. The initial platelet activation proceeds as a consequence of factor VIIa binding to tissue factor generating an initial small amount of factor Xa and, subsequently, thrombin (Monroe *et al*, 1994). This thrombin binds to and activates platelets, thereby altering the surface into a procoagulant state including exposure of negatively charged phospholipids. This makes the platelets an efficient surface for assembly of the coagulation complexes factor VIIIa/IXa

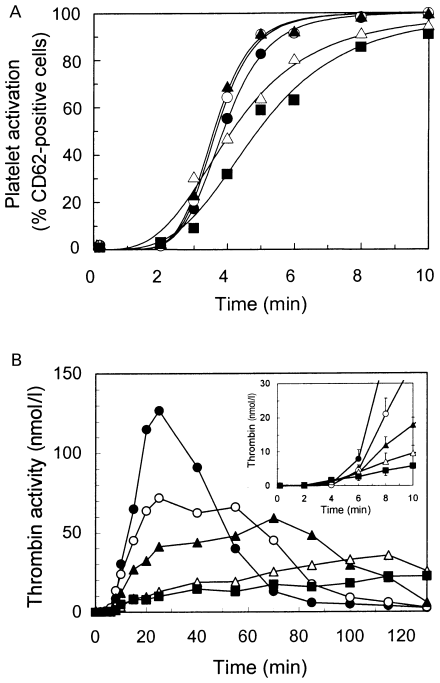

Fig 1. Platelet activation and thrombin generation at various platelet densities. Unactivated platelets at final densities of 200×10^9/l (●), 100×10^9/l (○), 50×10^9/l (▲), 20×10^9/l (△) and 10×10^9/l (■) were mixed with factors V, VIII, X, IX and XI, prothrombin, ATIII, TFPI, calcium and 0·2 nmol/l rFVIIa and added to tissue factor-expressing monocytes. Aliquots were removed and analysed for platelet activation, measured as P-selectin (CD62)-positive cells by flow cytometry (A) and thrombin amidolytic activity (B). A representative example of a total of seven experiments is shown. For four of the platelet donors similar time-courses of thrombin generation were obtained, and the insert shows the mean and SEM of the thrombin generation within the first 8 min for these four donors.

1. **The initial platelet activation proceeds as a consequence of factor VIIa binding to tissue factor g...**
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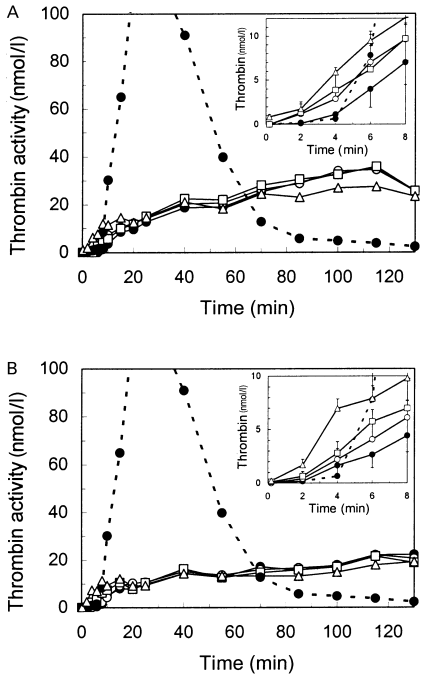

Fig 2. The effect of high-dose rFVIIa on thrombin generation at low platelet densities. rFVIIa at 0·2 nmol/l (●), 50 nmol/l ○, 100 nmol/l □ and 500 nmol/l (△) was added to samples containing platelets, factors V, VIII, X, IX and XI, prothrombin, ATIII, TFPI, calcium and tissue factor-expressing monocytes, and thrombin generation was analysed. Representative examples of the thrombin generation curves are shown for platelet densities of 20×10^9/l (A) and 10×10^9/l (B). The dotted curves show the thrombin generation in samples with 200×10^9 platelets/l and 0·2 nmol/l rFVIIa representing normal conditions. A total of seven experiments were carried out. The inserts show the mean and SEM for values for thrombin generation within the first 8 min of the assay for four donors with similar time-courses of thrombin generation.

and factor Va/Xa which are responsible for the physiologically important large-scale thrombin generation.

Figure 1A shows an example of platelet activation at various platelet densities in the presence of 0·2 nmol/l rFVIIa. After a lag phase of 2–4 min (depending on the platelet donor), the rate of platelet activation increased and full activation (usually more than 95% CD62-positive platelets) was obtained within 8–10 min. The time at which half the platelets were positive for CD62 was used as a measurement for platelet activation and the values are shown in Table I. At platelet densities of 20×10^9 and 10×10^9 platelets/l, a tendency of slower platelet activation was observed compared with samples with higher platelet densities. However, the time to half-maximal platelet activation was not significantly prolonged at the lower platelet densities.

An example of time-courses of thrombin generation at 0·2 nmol/l rFVIIa and various platelet densities is shown in Fig 1B. At normal platelet density (200×10^9/l), the thrombin generation curve showed a lag phase of around 4–7 min (depending on the platelet donor) followed by a steep increase in the thrombin generation. The thrombin generation reached a maximal level followed by a decline. The values for different parameters for the thrombin generation curves are shown in Table I. These include the maximal rate of thrombin generation calculated as the maximal slope of thrombin generation, the time to maximal thrombin activity, the maximal level of thrombin activity, and the total thrombin activity calculated as the area under the thrombin generation curve. When the platelet density was lowered, the maximal rate of thrombin generation was significantly decreased in a platelet density-dependent manner ($P < 0{\cdot}01$ for comparison of samples with 200×10^9 and 100×10^9 platelets/l, 100×10^9 and 50×10^9 platelets/l, or 50×10^9 and 20×10^9 platelets/l, and $P < 0{\cdot}05$ for comparison of samples with 20×10^9 and 10×10^9 platelets/l, two-tailed paired *t*-test). The lowered maximal rate of thrombin generation at the lower platelet densities led to a longer time to maximal thrombin generation ($P < 0{\cdot}01$ for comparison of samples with 200×10^9 and 50×10^9 platelets/l, or with 100×10^9 and 50×10^9 platelets/l. Samples with 200×10^9 and 100×10^9 platelets/l were not statistically different). Also, the maximal level of thrombin generation was significantly decreased at lower platelet densities ($P < 0{\cdot}01$ for comparison of samples with 200×10^9 and 100×10^9 platelets/l or with 100×10^9 and 50×10^9 platelets/l). The total thrombin activity (measured as the area under the thrombin generation curve) did not show a significant decrease by lowering the platelet density from 200×10^9/l to 100×10^9 or 50×10^9/l. For samples with 20×10^9 and 10×10^9 platelets/l, a full time-course of thrombin generation was not obtained within the time of the assay and, therefore, the time to maximal thrombin generation, the maximal level of thrombin activity and the total thrombin activity could not be determined.

Addition of high-dose rFVIIa to samples with 20×10^9 and 10×10^9 platelets/l

Examples of thrombin generation curves at 20×10^9 and 10×10^9 platelets/l in the presence of physiologically relevant (0·2 nmol/l) or high doses (50, 100 and 500 nmol/l) of rFVIIa are shown in Fig 2, together with the thrombin generation curve at 200×10^9 platelets/l and 0·2 nmol/l rFVIIa, reflecting normal conditions. The maximal rate of thrombin generation at 20×10^9 or 10×10^9 platelets/l was not significantly affected by the addition of 50, 100 or 500 nmol/l rFVIIa compared with samples with 0·2 nmol/l rFVIIa and the same platelet density (Table II). In contrast, rFVIIa at 50–500 nmol/l shortened the lag phase of thrombin generation significantly compared with samples with 0·2 nmol/l rFVIIa. This gave more thrombin formed within the initial minutes of the assay (see inserts in Fig 2). The time to half-maximal platelet activation was shortened by addition of 50, 100 or 500 nmol/l rFVIIa at

Table II. The effect of high-dose rFVIIa on platelet activation and thrombin generation at low platelet density.

Platelets ($\times 10^9$/l)	rFVIIa (nmol/l)	Time to half-maximal platelet activation (min)	Parameters of thrombin generation	
			Lag phase† (min)	Maximal rate (nmol/l/min)
200	0·2	6·0 (3·9–8·2)	5·6 (3·7–6·9)	16·3 (7·8–28·3)
20	0·2	6·6 (3·5–12·5)	3·6 (0·4–5·9)	1·8 (0·5–3·6)
	50	4·7 (3·5–7·6)*	1·8 (0·0–4·6)**	1·9 (0·5–4·2)
	100	5·1 (3·2–10·1)*	1·9 (0·0–3·8)*	1·9 (0·7–4·2)
	500	3·9 (2·2–5·2)*	0·7 (0·0–2·1)**	1·9 (0·7–3·6)
10	0·2	7·2 (4·1–12·0)	3·3 (0·7–5·4)	1·2 (0·5–2·1)
	50	5·5 (3·5–11·9)*	2·1 (0·0–4·8)*	1·2 (0·4–1·9)
	100	4·7 (3·4–8·2)**	1·1 (0·0–2·3)*	1·4 (0·5–3·1)
	500	4·3 (1·4–6·8)**	0·5 (0·0–1·5)**	1·5 (0·5–2·5)

†The lag phase of thrombin generation was calculated as the time at which the maximal slope crossed the baseline. The apparently shorter lag phase at low platelet density compared with high platelet densities reflects the lower maximal rate of thrombin generation. Therefore, the lag phases should only be compared for samples with similar maximal rates of thrombin generation.

*Statistically different ($P < 0·05$) from samples with 0·2 nmol/l rFVIIa and the same platelet density. A two-tailed paired *t*-test was used for the analyses.

**Statistically different ($P < 0·01$) from samples with 0·2 nmol/l rFVIIa and the same platelet density.

The values show the mean and range of data (in parentheses) from the same seven donors as in Table I. Time to half-maximal platelet activation and the maximal rate of thrombin generation are defined in Table I.

20×10^9 or 10×10^9 platelets/l, resulting in faster platelet activation than in samples with 0·2 nmol/l rFVIIa and 200×10^9, 20×10^9 or 10×10^9 platelets/l (Table II).

rFVIIa at platelet densities of 5×10^9 and 1×10^9/l

The thrombin generation at platelet densities of 5×10^9/l or 1×10^9/l was very low, and the detection level in the model system did not allow the calculation of the various parameters for thrombin generation. However, the thrombin generation curves indicated that addition of 50–500 nmol/l rFVIIa caused an increased initial thrombin generation at 5×10^9 and 1×10^9 platelets/l (not shown). Also the lag phase of platelet activation was decreased upon addition of 50–500 nmol/l rFVIIa.

DISCUSSION

Thrombin generation in thrombocytopenia-like conditions in a cell-based model system of tissue factor-initiated coagulation was characterized by a lower maximal rate of thrombin generation, an increased time to maximal thrombin activity and a lower peak level of thrombin activity (Fig 1 and Table I). A platelet density-dependent decrease in maximal rate of thrombin generation has also been seen in model systems comprising defibrinated plasma and gel-filtered platelets (Reverter *et al*, 1996), platelet-rich plasma (Peyrou *et al*, 1999) and a minimally altered whole-blood system (Cawthern *et al*, 1998a). An increased time to maximal thrombin activity at low platelet densities has also been seen in model systems comprising plasma as the source of the coagulation proteins (Reverter *et al*, 1996; Peyrou *et al*, 1999), but the prolongation seems to be relatively more pronounced in our reconstituted model system. The total thrombin activity (measured as the area under the thrombin generation curve) was not significantly affected by lowering the platelet density from 200×10^9/l to 50×10^9/l. In plasma-based systems, the total thrombin activity, i.e. the endogenous thrombin potential, was strongly dependent on the platelet density (Reverter *et al*, 1996; Peyrou *et al*, 1999). The relatively prolonged time to maximal thrombin generation at low platelet densities and similar total thrombin activity at platelet densities between 200×10^9 and 50×10^9 platelets/l in our experiments are probably the results of less efficient inhibition of the thrombin formed and could be a consequence of the lack of plasma inhibitors such as α_2-macroglobulin in our model system. The initial part of the thrombin curve, i.e. until maximal thrombin activity was achieved, was similarly affected in our system and in the plasma-based model systems.

The unique contribution of this work is to show that addition of rFVIIa (50, 100 and 500 nmol/l) to samples with low platelet densities (10×10^9/l and 20×10^9/l) shortened the lag phase of thrombin generation leading to an increased initial amount of thrombin formed (Fig 2 and Table II). The maximal rate of thrombin generation was not significantly affected by the addition of 50–500 nmol/l rFVIIa to samples with 10×10^9 and 20×10^9 platelets/l. A shortening of the time to half-maximal platelet activation was observed after addition of 50–500 nmol/l rFVIIa, probably as a consequence of the increase in initial thrombin generation. Clinical experience (Kristensen *et al*, 1996) has shown a beneficial effect of rFVIIa in controlling bleeding episodes in eight patients with low platelet density.

Our data suggest that rFVIIa may ensure haemostasis in patients with thrombocytopenia by enhancing the thrombin generation on the few platelets available. Also, at very low platelet densities (1×10^9 and 5×10^9/l), an increased initial thrombin generation was observed upon addition of 50–500 nmol/l rFVIIa, indicating that rFVIIa may help to provide haemostasis in patients with critically low platelet counts.

rFVIIa is registered for use in haemophilia patients with inhibitory antibodies to factor VIII or factor IX. The recommended dose is 90–120 μg/kg every 2 h. Thrombocytopenic patients normally have functional intrinsic coagulation factors, so the amount of rFVIIa required for therapeutic efficacy in these patients may be very different from the doses used in haemophilia patients with inhibitors. The doses of rFVIIa used to control bleeding in eight patients with thrombocytopenia varied between 50 and 100 μg/kg body weight (Kristensen *et al*, 1996); one patient needed more than one dose. The plasma level of rFVIIa after injection of 100 μg/kg would roughly correspond to 2·5 μg of rFVIIa/ml plasma (50 nmol/l rFVIIa) assuming 100% recovery. However, the recovery of vitamin K-dependent proteins shows wide variations between individuals. For 13 haemophilia patients given a total of 40 doses of rFVIIa, a range of 25–91% recovery was obtained (Glazer *et al*, 1995).

Platelets contain TFPI that will be released during platelet activation and TFPI in complex with factor Xa will inhibit tissue factor-bound rFVIIa. The platelet-associated TFPI comprises 10–30 ng/10^9 platelets (Novotny *et al*, 1988). In samples with 200×10^9 platelet/l, the platelet-associated TFPI would account for 2–6 ng/ml TFPI, which reduces to less than 0·6 ng/ml TFPI in samples with 20×10^9 or 10×10^9 platelets/l. In all cases, the amount of platelet-associated TFPI is small compared with the 100 ng/ml TFPI added in the assay. Therefore it is improbable that the 50–500 nmol/l rFVIIa added was particularly effective at low platelet densities in our experiments owing to lower amounts of TFPI in these conditions compared with the sample with 200×10^9 platelets/l. The plasma levels of TFPI were found to be markedly decreased in a majority of patients with thrombotic thrombocytopenic purpura (TTP) (Kobayashi *et al*, 1995). TFPI-mediated inhibition of coagulation might therefore be dampened in patients with TTP.

Previous data have provided evidence that the initial platelet activation is mediated by thrombin generated on the tissue factor-bearing cells (Monroe *et al*, 1994, 1996; Hoffman *et al*, 1995). The lack of statistically significant differences in the rate of platelet activation between samples with normal and lowered platelet density supports the hypothesis that the initial platelet activation is primarily dependent on the thrombin formed on the tissue factor-bearing cells. Using a minimally altered whole blood clotting system, Mann and co-workers (Rand *et al*, 1996; Cawthern *et al*, 1998b; Mann, 1999) have shown that clot formation occurs between the initiation and propagation phases of thrombin generation and that the thrombin generation proceeds after the clot is formed. Hemker & Béguin (1995) reported that clotting occurred when 10–20 nmol/l of thrombin were formed. Preliminary data comparing thrombin generation and fibrin polymerization (Wolberg *et al*, 1999) suggest that the initial thrombin generation and not the total amount or the maximal level of thrombin formed affects the clot formation and structure. In line with this is the observation that fibrin generation proceeds faster upon addition of exogenous thrombin to recalcified plasma, leading to an earlier clotting time and faster increase in clot turbidity (Blombäck *et al*, 1994). Taken together, this suggests that the initial thrombin generation is important for haemostatic efficacy. Our data, together with clinical data, are in agreement with this.

In summary, the data presented here show that high doses of rFVIIa (50–500 nmol/l) caused faster initial thrombin generation and platelet activation. Therefore, the few platelets available in thrombocytopenic conditions are being used more efficiently and may be able to compensate for the low number.

ACKNOWLEDGMENT

Lone Odborg, Novo Nordisk, is gratefully acknowledged for expert technical assistance.

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