

Rapid and Short Communication

Injection of recombinant activated factor VII can induce transient increase in circulating procoagulant microparticles

Valérie Proulle^{1,2}, Bénédicte Hugel^{1,3}, Benoit Guillet², Catherine Trichet², Anne Rafowicz², Thierry Lambert², Jean-Marie Freyssinet^{1,3}, Marie Dreyfus^{1,2}

¹INSERM Unit 143, Hôpital Bicêtre, Le Kremlin Bicêtre, ²Laboratoire d'Hématologie, AP-HP, Faculté de Médecine, Paris XI, Hôpital Bicêtre, Le Kremlin Bicêtre, ³Institut d'Hématologie et d'Immunologie, Faculté de Médecine, Université Louis Pasteur, Strasbourg, France

Summary

Recombinant activated factor VII (rFVIIa) is an effective haemostatic treatment in haemophiliacs with inhibitors. *In vitro*, FVIIa concentrations corresponding to those obtained with therapeutic doses of rFVIIa have been shown to induce normal thrombin generation and platelet activation in the absence of factors VIII or IX. To further study the *in vivo* haemostatic changes induced by rFVIIa, circulating procoagulant microparticles (MP) were measured in patients treated with discontinuous injections of Novoseven[®]. In 6 out of 15 patients, a tran-

sient peak of procoagulant MP was observed after injection, occurring 15 min to 2 h after infusion. It was composed primarily of platelet-derived MP and was of very short duration. This peak was not observed in haemophiliacs without inhibitor, who were treated with conventional replacement therapies. Our results provide further *in vivo* evidence that rFVIIa specifically activates platelets, either directly or as a consequence of a burst of thrombin generation that could account for its haemostatic efficacy.

Keywords

Microparticle, platelet activation, haemophilia therapy

Thromb Haemost 2004; 91: 873–8

Introduction

The mechanistic basis for the haemostatic efficiency of recombinant activated factor VII (rFVIIa) is poorly understood. Much of our knowledge is based on results obtained in *in vitro* systems using purified reagents where tissue factor (TF) plays a controversial role (1–4). In such systems, FVIIa, when used at concentrations corresponding to those obtained with therapeutic doses, has been shown to induce normal thrombin generation and platelet activation in the absence of factors VIII or IX (FVIII or FIX). Cell activation during procoagulant, inflammatory or apoptotic processes is associated with the shedding of membrane-derived microparticles (MP). MP exhibit accessible phosphatidylserine (PS), which is the major procoagulant phospho-

lipid, as well as cell-surface antigens characteristic of their cellular origin. Several studies have shown that high circulating procoagulant MP levels correlate with coagulation activation in prothrombotic disorders such as paroxysmal nocturnal haemoglobinuria (5), type II heparin-induced thrombocytopenia (6) and acute coronary syndromes (7). To assess cell activation induced by rFVIIa treatment, we measured circulating procoagulant MP levels in 15 patients with FVIII or FIX < 0.01 IU/ml and inhibitor, receiving injections of Novoseven[®] and compared the results to those determined in 12 patients without inhibitor, receiving replacement therapies or DDAVP.

Correspondence to:

Valérie Proulle

CHU Bicêtre

Laboratoire d'Hématologie

78 rue du Général Leclerc

94275 Le Kremlin Bicêtre Cedex

France

Tel.: 33 1 49 59 56 08, Fax: 33 1 46 71 94 72

E-mail: proulle@kb.inserm.fr

Received May 19, 2003

Accepted after resubmission February 12, 2004

Prepublished online March 3, 2004 DOI: 10.1160/TH03-05-0301

Patients	Treatment and dosage	Age (years)	Platelet count ($\times 10^9/L$)	Inhibitor level (BU)	Circumstances of treatment	Time elapsed since the last perfusion	Sampling time points
<u>rFVIIa ($\mu\text{g/kg}$)</u>							
A-1	100	21	180	2.4	preoperative	> 8 days	T0, 15 min, 1, 2, 3 h
A-2	120	30	192	9.5	pharmacokinetic study	> 8 days	T0, 30 min, 1, 2 h
A-3	230	63	236	0.6	preoperative	> 8 days	T0, 30 min, 1, 2 h
A-4	100	26	291	0.6	hematoma	> 8 days	T0, 30 min, 1, 2 h
A-5	125	35	381	32	hematoma	> 8 days	T0, 15 min, 1, 2, 6 h
A-6	100	30	643	19	hematoma, hemarthrosis	4 h	T0, 30 min, 1, 2 h
A-7	100	50	344	1.8	post surgical	6 h	T0, 15, 30 min, 1, 2 h
A-8	150	3	289	0.7	acute hematoma	> 8 days	T0, 30 min, 1, 2 h
A-9	100	2	485	0.6	acute hemarthrosis	72 h	T0, 30 min, 1, 2 h
A-10	138	7	262	2	acute hemarthrosis	20 h	T0, 30 min, 1, 2 h
A-11	130	5	364	7	acute hematoma	24 h	T0, 30 min, 1, 2 h
A-12	130	67	174	21	post surgical	> 8 days	T0, 15, 30 min, 1, 2, 20 h
A-13	100	58	204	40	hematoma	6 h	T0, 15, 30 min, 1, 2, 6 h
A-14	100	89	233	1126	acute hematoma	> 8 days	T0, 30 min, 1, 2 h
A-15	200	20	259	1	preoperative	20 h	T0, 30 min, 1, 2 h
<u>rFVIII (IU/kg)</u>							
B-1	117	4	305	0.5	acute hemarthrosis	24 h	T0, 30 min, 1, 2 h
B-2	30	23	240	-	pharmacokinetic study	48 h	T0, 15, 30 min, 1, 2, 3 h
B-3	55	9	357	0.4	prophylactic	48 h	T0, 15, 30 min, 1, 2, 3 h
B-4	43	10	332	-	hemarthrosis	48 h	T0, 30 min, 1, 2, 3, 4, 6 h
B-5	25	23	222	-	prophylactic	48 h	T0, 30 min, 1, 2, 4 h
B-6	30	42	124	-	pharmacokinetic study	> 8 days	T0, 30 min, 1, 2, 4 h
B-7	30	41	140	0.5	pharmacokinetic study	> 8 days	T0, 30 min, 1, 2, 4, 8 h
<u>rFIX (IU/kg)</u>							
B-8	30	40	135	-	pharmacokinetic study	> 8 days	T0, 30 min, 1, 2 h
<u>DDAVP ($\mu\text{g/kg}$)</u>							
B-9	0.2-0.4	3	305	-	pharmacokinetic study	> 8 days	T0, 30 min, 1, 2, 4, 6 h
B-10	0.2-0.4	5	364	-	pharmacokinetic study	> 8 days	T0, 30 min, 1, 2, 4, 6 h
B-11	0.4	39	266	-	prophylactic	> 8 days	T0, 30 min, 1, 2, 4, 6 h
B-12	0.2-0.4	10	458	-	prophylactic	> 8 days	T0, 30 min, 1, 2, 4, 6 h

Table 1: Biological and clinical parameters of the 27 patients. The 15 patients of group A received recombinant activated factor VII (rFVIIa, Novoseven®). The 12 patients of group B received either recombinant factor VIII (rFVIII), recombinant factor IX (rFIX) or DDAVP. All patients had inherited haemophilia A or B except patients A-12 and A-14 who had an acquired haemophilia A. Inhibitor levels are expressed using Bethesda units (BU) according to the method described by Kasper et al. Patients A highlighted in grey exhibited a peak of microparticles after rFVIIa injection.

Patients and methods

Patients

After obtaining informed consent, 15 patients (group A) with FVIII or FIX < 0.01 IU/ml and inhibitor were studied sequentially before and after the administration of 100 to 230 $\mu\text{g/kg}$ rFVIIa (Novoseven®, NovoNordisk, Bagsvaerd, Denmark). They had either severe haemophilia A with inhibitor (patients A-1-11 and A-13), acquired haemophilia A (patients A-12 and A-14) or severe haemophilia B with inhibitor (patient A-15). Eight patients were studied after their first injection, while 7 had already received an injection 4 to 72 h before. Five patients were treated at the onset of an acute bleeding event, 6 to prevent the relapse of a bleeding episode or having undergone surgery more than 2 days before, 3 prior to surgery, and 1 to perform a pharmacokinetic study. Platelet count ranged from 174 to 643 $\times 10^9/l$ and fibrinogen levels ranged from 2.3 to 6.8 g/l before treatment; both parameters remained stable during the treatment course. An additional 12 patients (group B) with FVIII or FIX ranging from 0.01 to 0.25 IU/ml were studied sequentially before and at various times after FVIII or FIX levels increased secondary to other therapies, such as recombinant FVIII or FIX (30 to 117 IU/kg) or DDAVP (0.2 to 0.4 $\mu\text{g/kg}$). They had either

severe haemophilia A with inhibitor ≤ 0.5 Bethesda units (patients B-1-7), severe haemophilia B (patient B-8) or mild haemophilia A (patients B-9-12). Seven patients were studied during their first injection, whereas 5 had already received a rFVIII injection 24 to 48 h before. One patient was treated at the onset of an acute bleeding event, 5 to prevent the relapse of a bleeding episode, which had occurred more than 2 days before, and 5 to perform a pharmacokinetic study. Platelet count ranged from 124 to 458 $\times 10^9/l$ and fibrinogen level ranged from 2 to 3.6 g/l before treatment, and both parameters remained stable during the treatment course (Table 1).

Blood sampling

Blood was collected in vacutainer tubes (Becton Dickinson, San Jose, CA) with either EDTA for platelet count or sodium citrate for coagulation studies and for MP determination.

Platelet count and coagulation studies

Platelet count was performed using a H3 Bayer™ automated blood analyzer (Bayer Diagnostics, Tarrytown, NY). Coagulation studies were performed on platelet poor plasma (PPP) obtained after centrifugation at 2,500 g for 15 min either immediately following blood sampling or after storage at -20°C . The

fibrinogen level was assessed using an automated coagulometer STA™ and Fibriprest® from Diagnostica Stago (Asnières, France). Factor VIII and IX activities were measured using a one-stage clotting assay and PTTA® and Factor VIII and IX deficient plasmas® from Diagnostica Stago on a fully automated Sysmex CA6000™ coagulometer from Dade Behring (Marburg, Germany). Factor VIII and IX inhibitor neutralizing activity titres were determined according to the method described by Kasper et al. (8), and expressed as Bethesda units (BU). The assessment of FVIIa circulating activity was performed using Factor VII one-stage clotting assay using Innovin® from Dade Behring, Factor VII deficient plasma® from Diagnostica Stago and an automated STA™ coagulometer. FVII was determined in patients' PPP diluted 1/20 and 1/40 vol/vol in FVII deficient plasma and times before clotting was measured after the addition of fixed quantity of recombinant human tissue factor and phospholipids (Innovin®) and calcium. Results were expressed in IU/ml using a calibration curve obtained with several dilutions of a starting solution of Novoseven® diluted 1/10 vol/vol in FVII deficient plasma, which FVIIa level was determined using the 1st FVIIa international standard from the National Institute for Biological Standards and Controls® (NIBSC) (Hertfordshire, United Kingdom).

Microparticle determination

Factor V was from Diagnostica Stago. Recombinant human annexin V was purchased from Euromedex (Souffelweyersheim, France). High binding capacity streptavidin-coated microtitration plates, 1-O-n-octyl-β-D-glucopyranoside, biotin-X-OSu, and Chromozym TH were from Roche Diagnostics (Mannheim, Germany). PPP for MP determination was obtained by double centrifugation. First, blood samples were centrifuged at 1,500 g for 15 min at room temperature. Second, the collected supernatant was centrifuged at 13,000 g for 2 min at room temperature. The last supernatant containing MP was stored at -80°C for further analysis. The capture of MP by immobilized annexin V, and the prothrombinase assay used to measure their amount, were carried out as described in detail by Aupeix et al. (9). In brief, annexin V was biotinylated (annexinV^{Bi}) and insolubilized onto streptavidin-coated microtitration plates. After incubation for 30 min at room temperature, plates were washed 3 times. PPP samples were then added and left in contact with insolubilized annexinV^{Bi} for 30 min at room temperature in the presence of 30 mM Ca²⁺. The plates were then washed 3 times and the anionic phospholipid content was determined using a prothrombinase assay. In the assay, purified clotting factors (activated Factor X, Factor V, Factor II) and calcium concentrations were determined to ensure that PS is the rate limiting parameter of the reaction. After 2 h incubation at 37°C, the reaction was stopped by addition of EDTA. Linear absorbance changes were recorded at 405 nm after addition of Chromozym

TH using a microtitration plate reader equipped with kinetics software (Powerwave X340 Biotek instruments™). Results were expressed as nanomolar phosphatidylserine equivalent (nM PS eq) by reference to a standard curve constructed by using liposomes of defined composition. Variations in MP levels induced by the treatment were calculated as the difference between the maximal value in MP level observed 15 min to 2 h following infusion, and the baseline level before infusion. They were expressed as deltas (Δ nM PS eq).

Antigenic capture and characterization of released microparticles

The anti-platelet glycoprotein Ib (GPIb) monoclonal antibody (MoAb) was kindly provided by Dr F. Lanza (INSERM Unit 311, Strasbourg, France). The anti-leukocyte anti-CD11a MoAb and the irrelevant biotinylated IgG (IgG1) were obtained from Leinco Technologies (Ballwin, MO). Biotinylated antibodies were insolubilized onto streptavidin-coated microtitration plates. After incubation for 30 min at room temperature, plates were washed 3 times. PPP samples were then added and left in contact with insolubilized antibodies for 2 h at room temperature. The plates were then washed 3 times and the anionic phospholipid content was determined using a prothrombinase assay as described above.

Soluble glycoprotein V determination

Soluble glycoprotein V (GPV) determination was assessed by a 2 step ELISA and Asserachrom Soluble GPV from Diagnostica Stago, according to the manufacturer's instructions. In brief, residual PPP samples available after MP determinations were diluted 1/20 vol/vol and incubated for 2 h at room temperature in microtitration plates precoated with the first specific anti-soluble GPV monoclonal antibody. After 5 washing steps, immobilized soluble GPV was revealed after a 2 h incubation period with the second monoclonal antibody coupled with peroxidase, and directed to a distinct epitope of soluble GPV. After 5 washing steps, level of bound antibody was revealed by the addition of ortho-phenylenediamine in the presence of hydrogen peroxide. The reaction was stopped after 8 min by adding 3 M H₂SO₄ and absorbance at 492 nm was read in a microtitration plate reader equipped with kinetics software (Powerwave X340 Biotek instruments™). Results were expressed in ng/ml using a calibration curve obtained with known concentrations of human soluble GPV.

Two-stage clotting assay for tissue factor (TF)

TF bound to MP was measured using a two-stage clotting assay as described by Key et al. (10). Supernatants containing MP were centrifuged at 200,000 g for 30 min and washed twice using Hanks' balanced salt solution (HBSS, Sigma-Aldrich, St Louis, MO). Fifty μl of the sample was mixed with 5 nM human FVIIa (Novoseven®), 250 nM human FX (Kordia Life

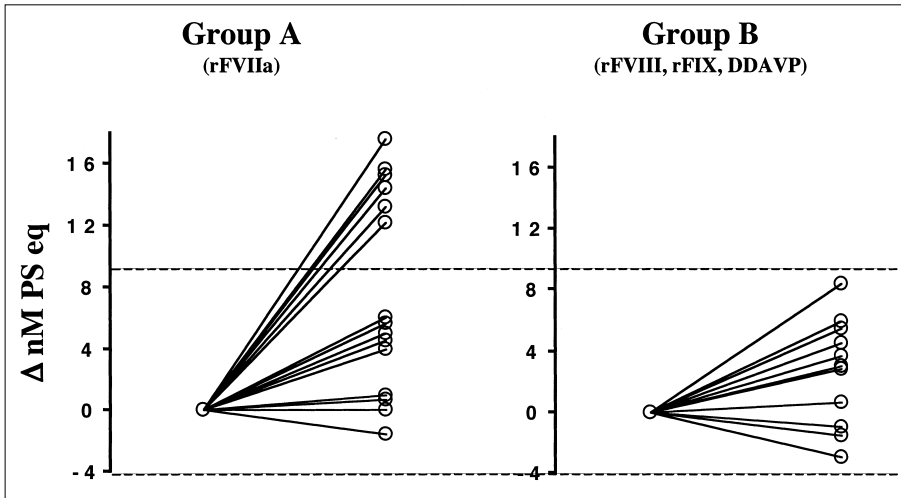


Figure 1: Variation in microparticle levels in 27 patients. The 15 patients of group A received recombinant activated factor VII (rFVIIa, Novoseven®). The 12 patients of group B received either recombinant factor VIII (rFVIII), recombinant factor IX (rFIX) or DDAVP (see Table 1). Results are expressed as delta nM phosphatidylserine equivalent (Δ nM PS eq), defined as the difference between the maximal values in microparticle levels observed 15 min to 2 h following infusion and the baseline level before infusion. Normal range was determined as mean \pm 2 SD (-----).

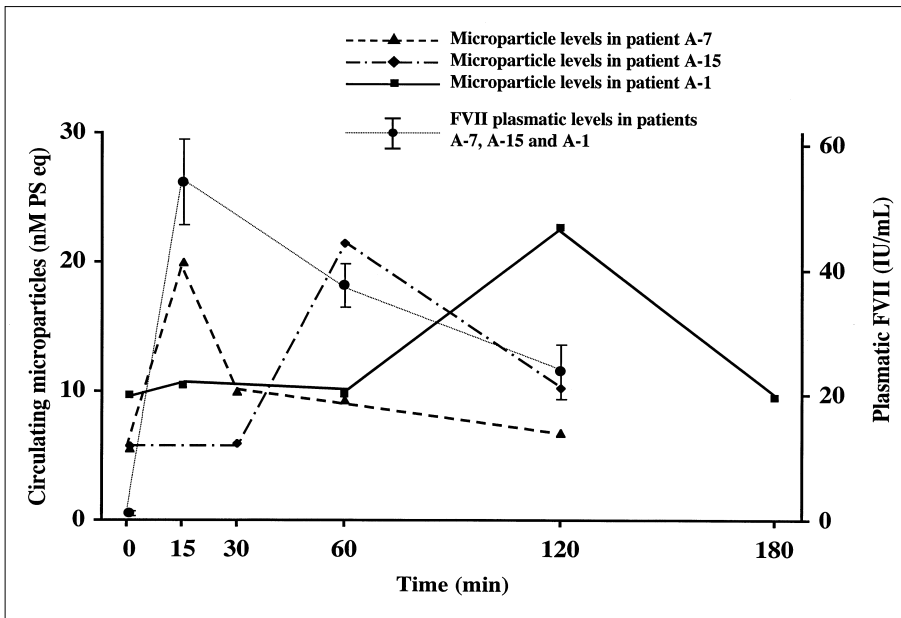


Figure 2: Variations in microparticle and FVII plasmatic levels. Variations in MP levels are represented for patient A-7, A-15 and A-1 exhibiting a peak at 15 min, 1 h and 2 h respectively. Corresponding mean \pm SD of FVII plasmatic levels in these 3 patients are represented.

Sciences, Leiden RG, The Netherlands) and 8.3 mM Ca^{2+} in HBSS containing 0.1% bovine serum albumin, final volume 60 μL . After 3 min incubation at 37°C, 100 μL of normal human plasma containing vesicles (20 μM phosphatidylserine 30: phosphatidylcholine 70) was added together with 100 μL 25 mM Ca^{2+} . The clotting time was measured using a STARt™ from Diagnostica Stago. Results were expressed in arbitrary units (AU) using a calibration curve constructed with serial dilutions of a starting solution of Innovin® containing recombinant human TF and phospholipids. The anti-human TF MoAb was from American Diagnostica (Stamford, CT).

Results

Patients of both groups were male with inherited haemophilia, except 2 patients from group A, who had acquired haemophilia

A. They were comparable with respect to age (mean \pm SD: 33.73 \pm 26.46 years in group A and 20.75 \pm 15.92 years in group B, $p = 0.097$, F test) and sampling numbers (mean \pm SD: 4.47 \pm 0.74 for group A and 5.58 \pm 0.9 for group B, $p = 0.57$, F test). In 12 patients receiving replacement therapies or DDAVP, in whom the expected FVIII or FIX increase was achieved, and in 9 out of 15 patients receiving rFVIIa injection, the difference between the maximal value in MP level observed 15 min to 2 h following infusion and the baseline level before infusion expressed as deltas (Δ) varied between -4.2 to 9.2 nM PS eq (mean \pm 2 SD: 2.5 \pm 6.72 nM PS eq). In contrast, 6 out of the 15 patients receiving rFVIIa displayed Δ beyond the upper limit of this range, testifying to a transient and dramatic increase of procoagulant MP (Δ ranging between 12 and 18 nM PS eq) (Fig. 1). The peak increase in procoagulant MP was noted at different intervals following rFVIIa infusion: at 15 min in 1 patient

(A-7), at 1 h in 3 patients (A-4, A-8 and A-15) and at 2 h in 2 patients (A-1 and A-12) and did not correlate with either high platelet count or the highest doses of rFVIIa infused (Table 1). Statistical analysis could include 13 out of 15 patients in group A and the 12 patients in group B who had strictly comparable sampling time points (before, 30 min, 1 and 2 h after treatment, Table 1). Five out of 13 patients in group A exhibited a MP peak while 8 out of 13 patients in group A and the 12 patients B did not, which was significantly different ($p = 0.05$, Fisher test). There was no time concordance between increase in procoagulant MP and the measured plasmatic peak of FVII, which occurred at 15 min following injection in all cases (Fig. 2). The increase in MP was transient, lasting less than 30 min as observed in 1 patient (A-7) (Fig. 2). Using annexin V and MoAbs specific of platelets (anti-GPIb), leukocytes (anti-CD11a), and an irrelevant IgG1, the cellular origin of MP was examined in 3 patients who exhibited a peak after rFVIIa injection (A-1, A-12 and A-15). The differences between the levels of MP measured using annexin V and MoAbs are mainly due to different affinities of MP for these ligands. However, it should be noted that the data obtained with annexin V and anti-GPIb MoAb were indeed comparable, showing a concomitant marked increase at respectively 60 min (patient A-15) and 120 min (patient A-1 and A-12) (Fig. 3), whereas no detectable variation was observed using anti-CD11a or control IgG1 antibodies. This indicates that platelet-derived MP account for most of the increase of circulating MP.

No rise of soluble GPV was observed in 2 of the 5 patients where it could be assessed (patients A-5 and B-11). In contrast, a rise of soluble GPV was observed in 3 patients (patients A-6, A-7 and B-6), among whom one from group A exhibited a concordant MP peak (patient A-7) (data not shown).

For TF assessment, due to sample shortage, 5 additional patients receiving rFVIIa were recruited. One of them showed a 3.6-fold increase of MP-borne TF 30 minutes after injection with respect to basal level before (29 μ AU), while the others did not exhibit noticeable changes 2 hours after injection. Patient A-5 presented a 4.8-fold increase of MP-borne TF 15 minutes after injection, but without significant change of MP levels (8.5 versus 5.6 nM PS eq). Four hours after injection, patient A-7 had no appreciable modification of MP-borne TF. It should be mentioned that in the two patients with significant changes 15 and 30 minutes after injection respectively, TF bound to MP levels returned to basal values 2 hours after injection.

Discussion

A peak of procoagulant MP was observed after injection of rFVIIa in 6 out of 15 patients of group A. The statistical difference found in patients suitable for analysis suggests the specificity of the phenomenon. Significant inter-individual variations of the time of occurrence and magnitude of the MP peak were

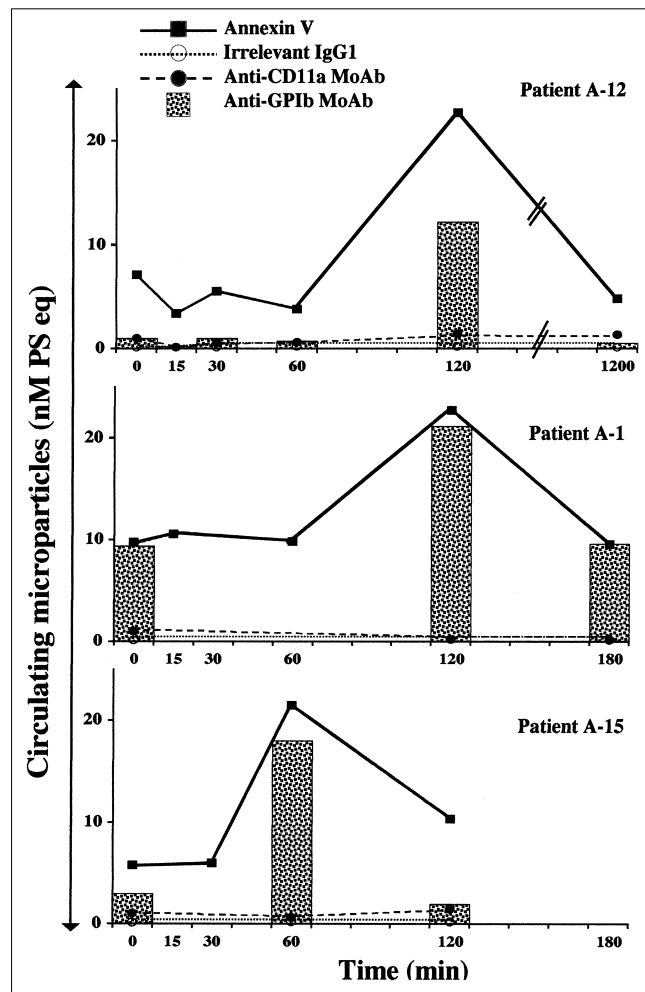


Figure 3: Characterization of the microparticles cellular origin. Variations in microparticle levels in patients A-1, A-12 and A-15 were assessed using either annexin V to evidence total circulating procoagulant microparticles, anti-GPIb monoclonal antibody (anti-GPIb MoAb) to evidence platelet-derived microparticles, anti-CD11a monoclonal antibody (anti-CD11a MoAb) to evidence leukocyte-derived microparticles, and irrelevant IgG1 for control.

noted. *In vitro* studies showed a significant difference regarding the amount of thrombin generated, as well as the time required to reach its maximal value when platelets from different healthy individuals were activated using thrombin (11). In contrast, little variation in thrombin generation was observed when human platelets were replaced by synthetic phospholipids even though levels of coagulation factors and inhibitors varied (12). It can thus be hypothesized that inter-individual variations may be due to the heterogeneity of platelet reactivity among patients. As such in this respect, it is possible that we failed to detect this peak in some of the 9 other patients due to sampling schedules and transience of the phenomenon. The mechanistic basis for the induction of the shedding of platelet-derived MP by high concentrations of rFVIIa requires further investigations. Hoffman and colleagues (2, 3) proposed that the haemostatic

efficiency of rFVIIa is related to the capacity of high concentrations of FVIIa to generate a normal amount of thrombin *in vitro* through the direct activation of FX at the surface of activated platelets without TF, FIX or FVIII. In contrast, Butenas and colleagues (1) showed that thrombin generation due to rFVIIa in absence of FVIII or FIX is TF dependent. They also showed that high doses of rFVIIa *in vitro* could activate platelets in a comparable manner in normal or acquired haemophilia B blood. We found that platelet-derived MP account for most of the increase of circulating MP in patients exhibiting a peak after rFVIIa treatment. Preliminary results of soluble GPV levels, a reliable marker of thrombin-induced platelet activation *in vivo* (13, 14), showed that soluble GPV and MP variations are similar in 3 out of 5 patients assessed concomitantly. These results have to be confirmed on larger series, but suggest that the shedding of platelet-derived MP through thrombin-induced platelet activation contributes, at least in part, to the increase of MP levels after rFVIIa treatment. In a recent study (15), it was shown that TF-bearing MP, generated in blood after stimulation with chimeras of the extracellular portion of P-selectin and the Fc portion of human IgG1, have the potential to improve fibrin for-

mation and correct the bleeding phenotype in haemophilia A mice. Such TF-bearing MP were also observed *ex vivo* in blood from patients with severe haemophilia A after stimulation with the chimeras. In 2 patients, at 15 or 30 minutes after rFVIIa injection, MP-borne TF was increased and returned to basal levels after 2 hours. These observations suggest that MP-borne TF may well play a role in the haemostatic activity of rFVIIa treatment. This, however, requires further *in vivo* investigations as other patients assessed for MP-borne TF did not show the same feature, but they were tested at least 2 hours after rFVIIa injection.

In conclusion, our finding of a transient increase in circulating platelet-derived MP in patients treated by rFVIIa provides further *in vivo* evidence, supported by data from earlier *in vitro* studies with purified FVIIa, that rFVIIa specifically activates platelets either directly, or consecutively to the burst of thrombin generation that could account for its haemostatic efficacy.

Acknowledgements

We thank Dr Lélia Grunebaum and Monique Dehan for valuable help.

References

1. Butenas S, Brummel KE, Branda RF, et al. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. *Blood* 2002; 99: 923-30.
2. Hoffman M, Monroe DM, 3rd, Roberts HR. Activated factor VII activates factors IX and X on the surface of activated platelets: thoughts on the mechanism of action of high-dose activated factor VII. *Blood Coagul Fibrinolysis* 1998; 9 Suppl 1: S61-65.
3. Hoffman M, Monroe DM, 3rd. The action of high-dose factor VIIa (FVIIa) in a cell-based model of hemostasis. *Semin Hematol* 2001; 38: 6-9.
4. Butenas S, Brummel KE, Bouchard BA, et al. How factor VIIa works in hemophilia. *J Thromb Haemost* 2003; 1: 1158-60.
5. Hugel B, Socie G, Vu T, et al. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood* 1999; 93: 3451-6.
6. Lee DH, Warkentin TE, Denomme GA, et al. A diagnostic test for heparin-induced thrombocytopenia: detection of platelet microparticles using flow cytometry. *Br J Haematol* 1996; 95: 724-31.
7. Mallat Z, Benamer H, Hugel B, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 2000; 101: 841-3.
8. Kasper CK, Aledort L, Aronson D, et al. A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975; 34: 612.
9. Aupeix K, Hugel B, Martin T, et al. The significance of shed membrane particles during programmed cell death *in vitro*, and *in vivo*, in HIV-1 infection. *J Clin Invest* 1997; 99: 1546-54.
10. Key NS, Slungaard A, Dandele L, et al. Whole blood tissue factor procoagulant activity is elevated in patients with sickle cell disease. *Blood* 1998; 91: 4216-23.
11. Sumner WT, Monroe DM, Hoffman M. Variability in platelet procoagulant activity in healthy volunteers. *Thromb Res* 1996; 81: 533-43.
12. Butenas S, van't Veer C, Mann KG. "Normal" thrombin generation. *Blood* 1999; 94: 2169-78.
13. Blann AD, Lanza F, Galajda P, et al. Increased platelet glycoprotein V levels in patients with coronary and peripheral atherosclerosis. *Thromb Haemost* 2001; 86: 777-83.
14. Ravanat C, Freund M, Mangin P, et al. GPV is a marker of *in vivo* platelet activation - study in a rat thrombosis model. *Thromb Haemost* 2000; 83: 327-33.
15. Hrachovinova I, Cambien B, Hafezi-Moghadam A, et al. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. *Nat Med* 2003; 9: 1020-5.