A case of glanzmann’s thrombasthenia successfully treated with recombinant factor VIIa during a surgical procedure: observations on the monitoring and the mechanism of action of this drug

Recombinant factor VIIa (rFVIIa) has been shown to be efficient for the treatment of haemorrhages in patients with Glanzmann’s thrombasthenia presenting anti-glycoprotein IIb-IIIa antibodies, but the mechanism of action is not well established and there is no routine laboratory test for the monitoring of rFVIIa. In this study, thrombin generation (TG) test was used to assess the efficacy of rFVIIa ex vivo in a Glanzmann patient with inhibitor, who had a surgery for cholesteatoma. The day before surgery, TG capacity in platelet rich plasma was significantly diminished (Endogenous thrombin potential = 637 nM.min) in comparison with the normal control group (1338±353 nM.min). Thirty minutes after the first infusion of 90 µg/kg of rFVIIa, TG was increased by 59% (1010 nM.min). rFVIIa was administered as intravenous bolus injection of 90 µg/kg q.2h during the first 24h, then 66 µg/kg q.2h during 24h and 53 µg/kg q.2h on post-operative day 3. Residual TG capacity measured before rFVIIa administration mostly remained above 1000 nM.min and the coagulation capacity was not significantly modified after a new injection of rFVIIa. The fibrin network was studied with 3D confocal microscopy using clots obtained with TG test. After rFVIIa infusion, the fibrin network was tighter in comparison with the sample before rFVIIa injection. These results provide further ex vivo evidence on haemostatic efficacy of rFVIIa in Glanzmann’s patients.

Introduction and Case Report

Recombinant factor VIIa (rFVIIa) has been shown to be effective in the treatment of haemorrhages and for the prevention of bleeding during surgery in patients with Glanzmann’s thrombasthenia who present anti-glycoprotein IIb-IIIa (GPIIb-IIIa) antibodies. The mechanism of action is still not completely understood and there is no routine laboratory test for monitoring rFVIIa in terms of haemostatic capacity and potential risk of thrombosis. In this study, a thrombin generation test was used to assess the efficacy of rFVIIa (Novoseven®, Novonordisk, Bagsvaerd, Denmark) in a 55-year-old male with the gypsy mutation modifying the splice donor site of intron 15 of the GP IIb gene responsible for inherited type 1 Glanzmann’s thrombasthenia and anti GP IIb-IIIa isoimmunisation who had a surgery for cholesteatoma. The patient exhibited several post-traumatic bleedings. In 1978, he had an uneventful surgery for a foreign metallic body in the neck using platelet concentrate substitution. In 1999, he underwent surgery for vocal cord nodules, and two polyps were successfully removed under general anaesthesia. In 2004, he was admitted to the gastroenterology department for melena. A coloscopy was performed and two polyps were successfully removed under Novoseven® 90 µg/kg q.2h. Recently, a cholesteatoma was discovered and surgical removal was decided. After a bolus injection of 90 µg/kg thirty minutes before the surgery, rFVIIa was administered as intravenous bolus injections of 90 µg/kg q.2h during the first 24 hours, then 66 µg/kg q.2h during the subsequent 24 hours and 53 µg/kg q.2h on post-operative day 3. No other prohaemostatic agent was given apart from rFVIIa. The blood loss was considered normal by the surgeon and there was no thrombotic complication during the course of rFVIIa treatment.

Materials and Methods

Control Subjects

The control population comprised fifteen healthy volunteers between 21 and 55 years of age, with no bleeding or thrombosis history and not using drugs known to affect the coagulation system.

Blood sampling

Blood was collected into S-Monovette® tubes (Sarstedt, Orsay, France) containing 0.106 mol/l trisodium citrate, with corn trypsin inhibitor 0.8 µM, after obtaining informed consent (CT1 was kindly donated by Prof. Hemker, Synapse bv, Maastricht, The Netherlands). Blood was taken through a 21G needle with a light tourniquet. PRP were prepared within 30 minutes after venipuncture. Platelet rich plasma (PRP) was obtained by centrifugation at 150 g for 10 minutes with collection of the upper PRP fraction. Platelets were counted with an Advia 120® counter (Bayer Diagnostics, USA). Platelet counts were adjusted to 150x10^12/L with autologous plasma. In order to minimize contact activation, polypropylene tubes and pipette tips were used throughout.

Reagents for thrombin generation test

Recombinant relipidated tissue factor (TF) Innovin® was obtained from Dade Behring (Marburg, Germany). Hapes-buffered saline contained 20mM Hapes (Sigma Aldrich, l’Ile d’Abeau Chesnes, France), 140mM NaCl and 5 mg/mL bovine serum albumin (BSA) (Euromedex, Souffelweyersheim, France), pH 7.35. A fresh mixture of fluorogenic substrate and CaCl2 was prepared before each experiment. Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). The mixture of fluorogenic substrate 2.5 mM and CaCl2 0.1M was prepared using buffer containing Hapes 20 mM and 60 mg/mL BSA, pH 7.35. The Calibrator with the activity of 600 nM human thrombin was obtained from Thrombinscope BV (Maastricht, The Netherlands).

Thrombin generation measurement

Thrombin generation was measured in a Fluoroscan Ascent® fluorometer equipped with a dispenser (Thermolabsystems OY, Helsinki, Finland) according to the method described by Hemker et al. Fluorescence intensity was detected at wavelengths of 390nm (excitation filter) and 460 nm (emission filter). Briefly, 80 µL of PPP or PRP were dispensed into the wells of round-bottom 96 well-microwell plates (Greiner, Poitier, France), 20 µL of TF at the final concentration of 0.5 pM was added to the PRP samples. The starting reagent (20 µL per well) contained fluorogenic substrate and CaCl2. A dedicated software program, Thrombinscope® (Thrombinscope BV, The Netherlands) enables the calculation of thrombin activity and displays thrombin activity against time. The measurements lasted 90 minutes. All experiments were carried out in duplicate.

Annexin V labelling

Platelet activation was determined before and thirty
minutes after the first infusion of rFVIIa, by flow cytometry analysis (Coulter EPICS XL MCLTM, USA) after annexin-V labelling using Annexin-V -FITC (BD-Pharmingen, San Diego, CA, USA).

Platelets from platelet-rich plasma (PRP) were diluted at $10^9$ x $10^{-9}$/L in an HEPES buffer (THB), NaCl 137 mM, KCl 2.7 mM, MgCl2.6H2O 0.1 mM, HEPES 20 mM, D-Glc 5.6 mM pH 7.4 with 0.1% BSA. Five microliters of annexin V-FITC were added to 25 µL of platelet suspension with 500 µL of THB containing 2 mM CaCl2, without or with calcium ionophore A23187 (Sigma-Aldrich, Saint Quentin Fallavier, France). Ten minutes later, Annexin-V labelling in FL1 was determined.

3-D confocal microscopy

Study on the fibrin network with 3-D confocal microscopy was realized as previously reported by He et al. using the Leica TCSST2 laser scanning microscope equipped with a laser argon excitation: 488 nm, emission: 512/532 (Leica Microsystems Heidelberg GmbH, Germany).

The kinetic of fibrin polymerization was assessed using Waveform analysis on the MDA system® (Biomerieux, Durham, NC, USA).3

Statistics

Statistical analysis was performed using the Graph Pad Instat 3.0® software package (San Diego, California, USA). The effect of rFVIIa on fibrin network was evaluated using Student t test. A p value of <0.05 was considered statistically significant.

Results and discussion

Ex-vivo effect of rFVIIa on thrombin generating capacity

Laboratory tests showed normal platelet count and coagulation factors were in the normal range. The day before surgery, thrombin generating capacity in platelet rich plasma with CTI was significantly diminished when compared with the normal control group (n=15).

Endogenous thrombin potential (ETP) was 637 nM.min in the Glanzmann patient and 1338±353 nM.min (mean ± 1SD) in the control group. The thrombin generating capacity of 48% of normal as observed in this patient was in agreement with a 40% decreased thrombin generating capacity previously reported in seven Glanzmann patients by Beguin et al.6 This reduction of the thrombin generating capacity could at least partly explain the bleeding phenotype in patients with Glanzmann’s thrombasthenia.

Thirty minutes after the first infusion of 90 µg/kg of rFVIIa, thrombin generation increased by 59% (ETP = 1010 nM.min) and reached the normal ranges (Figure 1). This was accompanied by an increase of 24.7% in the phosphatidyl-serine exposure at the platelet surface after activation by calcium ionophore as indicated by annexin V binding assay (Figure 2). This higher PS exposure obtained after rFVIIa infusion suggests that rFVIIa might have a synergistic activating effect on platelets. Conversely to haemophilia patients with inhibitor, the residual thrombin generating capacity measured before further rFVIIa administrations remained usually above 1000 nM.min. This value lies in the normal ranges (1338 ± 353 nM.min) and the coagulation capacity was not significantly modified after a new injection of rFVIIa (Table 1). This high and persistent thrombin generation capacity induced by rFVIIa could be at least partly explained by an increase in phosphatidyl-serine exposure on the platelet membrane which was probably responsible for the release of circulating micro-particles. This hypothesis is in agreement with the recent data reported by Proulle et al.7 who showed that rFVIIa injection could induce an
increase in platelet-derived circulating microparticles.

In haemophilia patients who present an obvious defect of thrombin generation capacity, rFVIIa activates haemostasis through a tissue factor-dependent and a tissue factor independent enhancement of thrombin generation. As in haemophilia patients, ex-vivo thrombin generation results obtained in this Glanzmann’s patient showed a decreased thrombin generating capacity (ETP 48% of normal) before rFVIIa treatment which normalized after rFVIIa infusions (Table 1). Our results suggest that a thrombin generation test could be used for monitoring and tailoring of the doses of rFVIIa in patients with thrombasthenia.

Ex-vivo effect of rFVIIa on the fibrin network

The fibrin network was studied using 3-D confocal microscopy with clots obtained with the thrombin generation test. Before rFVIIa injection, the clot of the patient with Glanzmann thrombasthenia had a loose aspect. After the Novoseven® infusion, we observed improved clot structure and fibrin fibres were more inter-twined. This result is in agreement with the recently reported in-vitro data showing the formation of a tight fibrin gel structure induced by rFVIIa, with an increased resistance to proteolysis. In addition, we found normal thrombin generation parameters after rFVIIa infusions. We can speculate that this increase of thrombin formation might activate the thrombin activatable fibrinolysis inhibitor (TAFI) which could intensify the resistance of the tighter fibrin fibres against fibrinolysis as demonstrated by Von dem Borne et al. Moreover, a different thrombin activation rate, occurring upon FVIIa infusion, is known to affect the polymerisation rate of fibrin fibres and ultimately the strength of the fibrin gel. In addition, Lisman et al. also reported that activated αIIb,3-deficient platelets in combination with rFVIIa could support thrombin and fibrin generation.

The waveform analysis on the MDA system measuring the kinetics of fibrin polymerization also showed a 3.8-fold accelerated fibrin formation after rFVIIa infusion in comparison with a normal control. This result is in accordance with the recent data published by Galan et al. who showed a marked increase in fibrin deposition after in vitro addition of rFVIIa into blood samples from two patients with Glanzmann’s thrombasthenia.

We report here, to the best of our knowledge, the first ex vivo thrombin generation and fibrin structure results in a patient with Glanzmann’s thrombasthenia. Our results suggest that rFVIIa may be effective through at least two different mechanisms in patients with Glanzmann’s thrombasthenia. Our results on clot structure confirmed previous in vitro results, which have reported a more tightly and more resistant fibrin gel structure after rFVIIa administration. Our data demonstrate that rFVIIa significantly increased the thrombin generating capacity of the patient and emphasised the usefulness of monitoring in patients with thrombasthenia with respect to efficacy and potential thrombogenicity of rFVIIa. They also provide further ex vivo evidence of the haemostatic efficacy of the rFVIIa in Glanzmann’s patients.

### Table 1. Peri-operative evolution of endogenous thrombin potential measured before and after rFVIIa infusions.

<table>
<thead>
<tr>
<th>Day</th>
<th>Residual ETP (nM.min/% of normal)</th>
<th>ETP 30 min after rFVIIa injection (nM.min/% of normal)</th>
<th>rFVIIa treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>637/48</td>
<td>1010/75</td>
<td>1st injection</td>
</tr>
<tr>
<td>Day 0</td>
<td>1148/86</td>
<td>1258/94</td>
<td>90 µg/kg q.2h</td>
</tr>
<tr>
<td>Day 1</td>
<td>1382/103</td>
<td>1353/101</td>
<td>90 µg/kg q.2h</td>
</tr>
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<td>Day 2</td>
<td>1043/80</td>
<td>1081/81</td>
<td>66 µg/kg q.2h</td>
</tr>
<tr>
<td>Day 3</td>
<td>912/68</td>
<td>946/71</td>
<td>53 µg/kg q.2h</td>
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Figure 2. Flow cytometry histograms showing annexin V binding to platelets before and 30 min after the first infusion of rFVIIa 90 µg/kg. After rFVIIa injection, the phosphatidyl-serine exposed at the platelet surface was increased by 24.7% and the mean fluorescent intensity (MFI) was increased by 27%.

Figure 3. Confocal 3-D microscope images at the same magnification of the clots formed during thrombin generation assay showing the fibrin network before (a) and after (b) rFVIIa infusion.
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References