A highly sensitive thrombin generation assay for assessment of recombinant activated factor VII therapy in haemophilia patients with an inhibitor

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Summary
Bypass agents are the common treatment for haemophilia patients who develop inhibitory antibodies. Laboratory assessment of the efficacy of bypassing agent therapy is a challenge. In the present work we modified the conditions triggering thrombin generation (TG) assay in order to find the most sensitive assay for detection of rFVIIa and its analogue NN1731 in haemophilic plasma. TG was measured in samples of normal plasma, plasma of haemophilia patient with inhibitors, as well as haemophilia induced plasma. Recalcification-induced TG was compared to tissue factor (TF) -induced TG in the presence and absence of rFVIIa and NN1731. Recalcification-induced TG (without TF) in haemophilic plasma yielded baseline flat curves, with increased TG as a consequence of spiking the plasma rFVIIa. Using our system, we observed both dose-dependence and time-dependence of rFVIIa effect on TG. Elevated concentrations of TF mask the difference between rFVIIa-treated and non-treated haemophilic plasma. NN1731 yielded normalisation of recalcification-induced TG curves (without TF) which may reflect high potency. In conclusion, we suggest that triggering TG by recalcification-only may be the most sensitive assay for determining the impact of bypassing agents in haemophilic plasma, and may serve as a caution surrogate safety marker in future studies.

Keywords
Haemophilia therapy, factor VIII inhibitors, coagulation factors, haemophilia A / B

Introduction
Haemophilia A is an X-linked genetic haemorrhagic disorder resulting from a deficiency of blood coagulation factor VIII (FVIII) which is treated by replacement concentrates. The impact of therapy is easily measured by plasma FVIII activity testing. About 30% of congenital haemophilia patients develop neutralising anti-FVIII antibodies. Bypassing agents – either APCC (activated prothrombin complex concentrates, i.e. FEIBA®, Baxter, Vienna, Austria) or recombinant activated factor VII (rFVIIa) are the common treatment for bleeding episodes and surgical procedures in these patients (1).

Laboratory assessment of the efficacy of bypassing agents is still a challenge. Albeit FEIBA and rFVIIa shorten clotting time in traditional coagulation tests (prothrombin time [PT], partial thromboplastin time [PTT], activated clotting time [ACT]), the endpoint of clot formation reflects merely the initiation of coagulation. Thus, these assays are relatively insensitive measures of bypass activity and are therefore, unsuitable for monitoring purposes. There is an increasing interest in the development of global laboratory assays that would serve as surrogate markers of the bypassing agents’ activity (2–4). Early data suggest that global tests can provide information regarding the overall haemostasis process and its disorders. Nevertheless, these tests need to be standardised with regard to their reagents, methodology and interpretation (4).

Since the final product of bypass activity is thrombin, thrombin generation (TG) has been used as a global assay to monitor the impact of various coagulation concentrates including rFVIIa and FEIBA (4–6). The calibrated automated TG assay was demonstrated to be in correlation with both haemorrhagic and thrombotic states (7–12) and has been suggested as an assay useful for monitoring treatment with bypass agents (1, 5, 9). However, large inter- and intra-patient variability, precludes interpretation of test results as a predictive tool for clinical tailoring of patients’ therapy (13). Currently, the optimal method for testing bypassing agents’ therapy impact by TG has not yet been standardised.

In the present work, we aimed at finding optimal pre-analytical conditions for assessment of rFVIIa-induced TG in plasma samples of haemophilic patients using a TG assay that is not necessarily based on conditions simulating the site of injury, where...
tissue factor (TF) and phospholipids accumulate and prevail. Throughout the comparison of different assay conditions, we tried to define a sensitive TG method suitable for evaluation of rFVIIa efficacy.

Materials and methods

Subjects and patients

For in vitro studies, blood samples were taken from patients with severe haemophilia A with an inhibitor to FVIII (1–260 BU) during routine clinic visits, at non bleeding state. For ex vivo studies, blood was taken from three patients with severe haemophilia A with inhibitor (1, 2, 6 BU), before and after rFVIIa injection (90 or 180 μg/ml). Additional samples were obtained from healthy volunteers. All subjects gave their consent to blood drawing for study purposes. The study was approved by the institutional ethical committee in concordance with the declaration of Helsinki.

Haemophilia A model

For some of the TG experiments we used experimentally induced haemophilia A model. We produced haemophilia A with undetectable FVIII activity in normal platelet-rich plasma (PRP) by mixing normal PRP with 5% of platelet-poor plasma (PPP) of haemophilia A patient with high titer of inhibitor. Control plasma was prepared similarly, by mixing the same normal PRP with 5% of autologous PPP. Thus, control and the experimentally induced haemophilic PRP had the same count of platelets and similar levels of coagulation factors (apart from FVIII).

Reagents

rFVIIa analogue NN1731 and rFVIIa were obtained from NovoNordisk (Copenhagen, Denmark). Human TF (Innovin) was provided by Dade Behring (Marburg, Germany). The concentration of TF was determined by an immunosorbent assay of American Diagnostica (Greenwich, CT, USA). Synthetic phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The thrombin-specific fluorogenic substrate Z-GGR-AMC was from Bachem (Bubendorf, Switzerland). Heps/bovine serum albumin (BSA) buffer containing 20 mM Hepes, 140 mM NaCl pH 7.3 and 5 mg/ml BSA (Sigma, St Louis, MO, USA) was used as a working buffer. Heps buffer pH 7.3 containing 20 mM Heps with 60 mg/ml BSA was used for diluting the fluorogenic substrate.

Processing of blood samples

Blood was drawn in 0.109 M buffered citrate and centrifuged within 30 minutes (min). PPP was obtained at room temperature by centrifugation at 2,000g for 10 min followed by centrifugation at 14,000g for 5 min. PRP was prepared by centrifugation of blood at 130 g for 10 min and adjustment of the platelet count to 150,000 per μl with autologous PPP.

TG test

TG was measured with a fluorometer (Fluoroskan Ascent, Labsystem, Helsinki, Finland) as previously described (8). Briefly, 20 μl working buffer, containing different concentrations of TF, rFVIIa or NN1731 were placed in microtiter plates (Greiner Bio-1, Frickenhausen, Germany). For TG measurements in PPP, the working buffer was supplemented with a mixture of 4 μM synthetic phospholipids (1PS:1PE:1.25PC). For TG measurements with PRP, no exogenous phospholipids were added. Then 80 μl plasma was added to the working buffer and TG was initiated by adding 20 μl Heps/BSA buffer containing 100 mM CaCl₂ and 5 mM fluorogenic substrate Z-GGR-AMC. Each sample was tested in duplicates and repeated on at least three occasions. Fluorescence was measured with an excitation filter at 390 nm and an emission filter at 460 nm. TG was displayed as plots and derived parameters, i.e. lag time, endogenous thrombin potential (ETP) and peak height were calculated by a computer program attached to the fluorometer.

Results

Initiation of TG by recalcification only, in the absence of exogenous TF, failed to induce TG in PPP (Fig. 1A) or PRP (Fig. 1B). Obtained from haemophilia A patients as indicated by flat curves. In contrast, initiation of TG by increasing concentrations of TF (0.2–10 pM) induced a dose-dependent increase in TG, as shown in haemophilic PPP and PRP (Fig. 1A, B). High concentrations of TF (10 pM) normalised the peak height and ETP in the PPP and PRP and shortened the lag time in comparison to normal plasma. In contrast to the absence of TG in haemophilic PRP, when normal PRP was tested, recalcification by itself was sufficient to induce almost the same extent of TG as 1 pM or 5 pM TF (Fig. 2).

TG assay for detecting rFVIIa in haemophilia A plasma

We next tested the influence of TF concentration on the TG increment between, induced in haemophilic plasma in the presence and absence of rFVIIa. We prepared a model of haemophilia A, as indi-
cated in Methods, and initiated TG applying three different pre-analytical conditions: recalcification, 1 pM and 5 pM TF. Plasma was spiked with 2 μg/ml rFVIIa (a concentration comparable to the predicted concentration of rFVIIa in plasma achieved by the administration of approximately 90 μg/kg rFVIIa).

No TG was detected when TG was triggered by recalcification in the absence of rFVIIa. In contrast, in the presence of rFVIIa, recalcification alone was sufficient to induce TG even though TF was not added (Fig. 3A). It is worth noting that when TF at a concentration of 1 pM was present in the reaction, the increment induced by rFVIIa was minimal (Fig. 3B). Moreover, when TG was triggered with high concentration of TF (5 pM) the baseline TG was similar to the TG induced in the presence of rFVIIa and similar to normal plasma (Fig. 3C), reflecting full activation of the system by TF, thus rendering it insensitive for evaluation of a bypassing agent activity.

Based on the observation that triggering TG by recalcification only was the most sensitive assay to discriminate between baseline and rFVIIa induced TG in haemophilic plasma, we used recalcification-induced TG for testing a wide range of rFVIIa concentrations (Fig. 4A,B). We spiked PPP or PRP taken from a haemophilic patient with rFVIIa (0–5 μg/ml) and initiated the reaction by recalcification. In both PPP and PRP, a dose-dependent increase in TG was observed with increasing rFVIIa concentrations.

We then tested recalcification-induced TG in plasma taken from haemophilia A patients before and after administration of rFVIIa. Blood samples were taken before and 15 min, 1, 2 and 4 hours (h) after rFVIIa infusion. All the PPP samples of each time point presented were tested simultaneously. Figure 5 shows a representative time-dependent TG curve obtained in PPP of haemophilia A patients.
mophilia A patient who received 180 μg/kg rFVIIa. Before rFVIIa administration no TG was induced by recalcification. Maximal peak of TG was achieved 15 min after rFVIIa administration which decreased at 1 and 2 h after administration, and declined to baseline at 4 h after rFVIIa injection. Similar time curves were obtained with samples of two additional haemophilia A patients.

**TG assay and NN1731**

Since the rFVIIa analogue NN1731 was recently reported to be more potent than rFVIIa, we tested whether our recalcification induced TG is sensitive tool to differentiate between this analogue and rFVIIa. We induced TG in PRP or PPP of haemophilic plasma in the presence of increasing concentrations of NN1731 (Fig. 6A, B). We found that the capacity of NN1731 to induce TG was much higher than that of rFVIIa at each concentration examined. Furthermore, NN1731 was even able to induce TG higher than that achieved in normal plasma.

**Discussion**

Haemophilia patients with inhibitors to FVIII or FIX still present a major challenge to treating physicians worldwide. Neither clinical outcome measures nor laboratory assays for evaluation of bypassing agents’ efficacy and safety are currently standardised. The aim of our study was to establish the most sensitive conditions required for a TG assay designed for evaluation of rFVIIa and possibly other bypassing agents in haemophilic plasma.

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**Figure 3:** The effect of TF concentration on the TG increment induced in haemophilic PRP in the presence and absence of rFVIIa. Haemophilia PRP was spiked with 2 μg/ml rFVIIa, and TG was triggered by recalcification (A), 1 μM TF (B) and 5 μM TF (C). Normal PRP was used for comparison (C).

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Since TF physiologically triggers the coagulation cascade, it is applied by most laboratories as a trigger for TG assay (8, 14). TF concentration significantly influences the sensitivity of the assay (15, 16), and has not been standardised so far.

In haemophilic plasma, baseline TG is affected by increasing TF concentration (Fig. 1). Whereas, in normal plasma increasing TF concentrations mostly shortened the lag time and did not significantly affect ETP and peak height (Fig. 2), in haemophilic plasma all TG parameters dramatically increased as higher TF concentrations were applied. Furthermore, recalcification alone (without TF) failed to induce any TG in the haemophilic plasma. In previous studies (17, 18) we showed that recalcification alone also failed to induce TG in FIX- and FXI-deficient plasma with inhibitors. The flat curves do not necessarily correlate with the risk of bleeding since patients with either severe haemophilia A or B certainly differ from those with severe FXI deficiency and inhibitors. We believe that a flat baseline curve is important and beneficial for a sensitive assessment of bypass therapy impact on TG. Another advantage of

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**Figure 4:** Dose-dependent curves of rFVIIa-induced TG triggered by recalcification only (no TF) in PPP (A) and PRP (B) obtained from haemophilia A patients with an inhibitor.

**Figure 5:** TG induced by recalcification only (no TF) in PPP of haemophilia A patient before and after administration of rFVIIa. PPP of each depicted time were tested simultaneously.
the flat baseline curve is the ability to avoid the use of Corn Trypsin Inhibitor (CTI), recommended for TG assays performed with low-dose TF (19–22). Since recalcification alone fails to trigger any TG in haemophilic plasma, no CTI is required for elimination of baseline contact activation.

rFVIIa is a common treatment for bleeding episodes, surgery and prophylaxis in haemophilia A and B patients with inhibitors (23,24) as well as for FXI deficient patients with an inhibitor (18, 25). The drug activity is augmented by TF and platelets localised at the injury site.

In vitro pre-analytical conditions applied for rFVIIa- induced TG, using different concentrations of TF, may mimic the haemostatic effect of rFVIIa achieved at bleeding sites following vascular injury. Indeed, elevated amounts of thrombin may be generated by the same concentration of rFVIIa using increased concentrations of TF (Fig. 3) and increasing phospholipids (17, 18, 26). Previous works recommended the application of low dose TF as a trigger of TG, since elevated TG baseline curves were demonstrated for both deficiencies, addition of similar rFVIIa concentrations completely compensated and "normalised" TG in FXI-deficient plasma with inhibitors, yet induced only slight TG increase in haemophilic plasma. The ability of rFVIIa to normalise TG (in the absence of TF) in FXI deficiency plasma may reflect its thrombogenic potential. These differences between FXI- deficient and haemophilic plasma may escape detection by other, less sensitive, TG assays, applying TF as a trigger. It should be noted that, based on our observations, lower dose rFVIIa therapy was used by our group for inducing haemostasis during major surgical procedures in FXI inhibitor patients (18, 25) in order to minimise potential pro- thrombotic risks.

Furthermore, in contrast to large intra and interpatient variability of TF-induced TG assays (13), our recalcification- only assay demonstrated a good reproducibility of interpatient response to rFVIIa on various occasions. Thus, we were able to consistently define patients as "poor responder" or "high responder" to rFVIIa addition- the potential clinical correlation of this finding should be further studied.

In previous work we used recalcification TG assays to distinguish between rFVIIa impact on FXI-deficient plasma and haemophilic plasma (17, 18). Whereas in the absence of rFVIIa, flat TG baseline curves were demonstrated for both deficiencies, addition of similar rFVIIa concentrations completely compensated and "normalised" TG in FXI-deficient plasma with inhibitors, yet induced only slight TG increase in haemophilic plasma. The ability of rFVIIa to normalise TG (in the absence of TF) in FXI deficiency plasma may reflect its thrombogenic potential. These differences between FXI- deficient and haemophilic plasma may escape detection by other, less sensitive, TG assays, applying TF as a trigger. It should be noted that, based on our observations, lower dose rFVIIa therapy was used by our group for inducing haemostasis during major surgical procedures in FXI inhibitor patients (18, 25) in order to minimise potential pro- thrombotic risks.

The main concern of haemophilia treaters applying bypass therapy is their fear of thrombosis. Consequently, the same sensitive TG method was applied by us for individualised ex vivo tailoring of combined low-dose bypass therapy designed to treat bleeding episodes in haemophilia patients with inhibitors (30, 31).
What is known about this topic?
- Thrombin generation methods have been suggested as a potential tool for monitoring bypass agents’ therapy in haemophilia patients with inhibitors.
- Tissue factor is the common trigger for initiation of thrombin generation (TG) assays.
- Pre-analytical conditions dramatically influence TG results in haemophilia plasma.

What does this paper add?
- Measuring TG in the presence of tissue factor facades the differences between recombinant activated factor VII (rFVIIa)-treated and non-rFVIIa-treated haemophilia plasma.
- A new, sensitive mode of TG assessment in haemophilia plasma is suggested, by recalification only, without tissue factor and without Corn Trypsin Inhibitor.
- The ability of bypass agents to induce higher normal TG in this sensitive system may reflect their thrombogenic potential.
- Future use of this modified TG assay in haemophilia plasma treated with bypass agents, may serve as a surrogate caution marker.

NN1731 analogue, as compared to rFVIIa has a substantially increased intrinsic activity, when not bound to TF, yielding increased TG on the surface of activated platelets (32, 33). NN1731 was found to display a more potent haemostatic effect as compared to rFVIIa in human, animal and cell based model studies (33–38). Not surprisingly, using our system, we found that NN1731, in contrast to rFVIIa, can normalise TG induced by recalification only. This ability of NN1731 to normalise TG in vitro in the absence of TF may indicate its enhanced potency combined with a potentially thrombogenic hazard.

Taken together, we assume that TG induced by recalification only (without exogenous TF) may reflect the activity of bypass agents in plasma (and not at the site of injury). Accordingly, in vitro normalisation of TG in our system may serve as a surrogate marker of caution and/or dose limitation, to be considered while planning individually tailored therapy.

In conclusion, the data presented in this study suggest that recalification-induced TG assay is a sensitive tool for monitoring rFVIIa presence in haemophilic plasma. In future studies, such ex vivo assays may be used to predict the efficacy and safety of new bypass agents.

References

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