Enhanced thrombin generation in plasma of severe thrombocytopenic patients due to rFVIIa

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Summary
RFFVIIa-enhanced thrombin generation has been shown to be dependent on platelets. In previous work we have shown that addition of monocytes and rFVIIa to microparticle free plasma causes a distinct thrombin generation. The aim of our study has been to examine whether there is enough surface provided by microparticles in thrombocytopenic plasma to allow an effect of rFVIIa.

Patients, methods:
Thrombin generation was measured in platelet rich plasma (PRP) and microparticle free plasma (MFP) of thrombocytopenic haemato-oncological patients with and without addition of rFVIIa by means of calibrated automated thrombography. Microparticles were analyzed in PRP by FACS flow cytometry.

Results:
Microparticle free plasma showed no thrombin generation with or without addition of rFVIIa. Addition of rFVIIa to PRP of thrombocytopenic patients led to a significant shortening of lag phase and time to peak in thrombin generation, while ETP and peak remained unchanged. Conclusion: Our results show that even in plasma of severe thrombocytopenic patients enough surface may be provided by microparticles to allow an enhancement of thrombin generation by rFVIIa.

Résumé
L'activité de l'activateur de facteur VII recombinant (rFVIIa) a été développée pour le traitement des hémorragies chez les patients souffrant de l'hémophylie avec inhibiteurs contre le facteur VIII ou facteur IX. Sa mise en application est également bien établie dans le traitement des facteurs rares insuffisants, la thrombosténie, et l'hémorragie intracrânienne (1–3). Les résultats de cette étude ont été publiés sur le rôle de l’rFVIIa dans les traumatismes sévères et les hémorragies non traumatiques et sur l'effet hémostatique de l’rFVIIa dans l’échec du traitement (4–6).

Les résultats sont moins conséquents dans le traitement des thrombocytopenies (7, 8). Des études anecdotiques suggèrent que l’rFVIIa améliore les hémorragies chez des patients avec une thrombocytopenie sévère, mais le mécanisme d'action n'est pas bien compris (9, 10). Même Hoffman décrit qu’l' rFVIIa est platelet-dependent mais ne se fie pas directement sur les facteurs tissulaires, d'autres ont montré que l' rFVIIa-enhanced thrombin generation is dependent on platelets and tissue factor (11, 12). Une analyse rétrospective de patients traités avec rFVIIa Martinowitz a également signalé un comptage de platelets supérieur à 50 000/µl en précondition pour l'utilisation de rFVIIa dans une hémorragie non contrôlée (13). Cependant, la même équipe de chercheurs a démontré que l’rFVIIa-induced thrombin generation and observations by Tonda et al. indique que les plaquettes peuvent ne pas être essentiellement suffisantes pour un effet de rFVIIa.

Les microparticules (MP) sont des fragments submicroniques qui peuvent être éliminés du plasma membranaire de cellules stimulées ou apoptotiques pour agir comme des effекторs bioactifs vasculaires, disseminant la fonction tissulaire des plaquettes et des phospholipides (14, 15). Des données de notre groupe suggèrent que l’addition de monocytes à microparticle free plasma peut fournir suffisamment de phospholipides pour un effet procoagulant de rFVIIa.

Ce travail a été conçu pour étudier si le pouvoir procoagulant du plasma thrombocytopénique est suffisant pour un effet procoagulant de rFVIIa.

Patients, materials, methods
Patients
Ten severe thrombocytopenic patients were recruited for a donation of 4.5 ml citrated blood during their visit at the department of...
internal medicine and informed consent was obtained. Five patients suffering from lymphoid cancer and five leukemia patients constituted the test collective in which criterion of involvement was a maximum platelet count of 20,000/µl platelet rich plasma. The control group consisted of 10 adult volunteers who were clinically healthy and who had no treatment with antithrombotic drugs.

Plasma, reagents

PRP was obtained by centrifugation at 290 × g for 10 min at room temperature. Thrombocytopenic PRP contained platelets in the range of 0 to 20,000/µl plasma and normal PRP ranged from 146,000 to 332,000/µl plasma. Platelet count measurements were done by means of Sysmex KX-21 (Sysmex Corporation, Kobe, Japan). Microparticle free plasma was separated from platelet free plasma (1550 × g for 20 min) by ultra centrifugation at 14,000 × g for 3 min at 21°C using VWR Centrifugal Filters with 0.2 µm pore size (VWR International, West Chester, Pennsylvania, USA).

Measurements of thrombin generation were performed by use of phospholipid-free PRP-reagent with a content of 0.5 pmol/l tissue factor purchased from Thrombinoscope BV (Maastricht, The Netherlands) and fluorogenic substrate Z-Gly-Gly-Arg-amino-methyl-coumarin purchased from Bachem (Bubendorf, Switzerland). Recombinant factor VIIa (Novo7™) has been purchased from Novo Nordisk A/S ( Bagsværd, Denmark).

Thrombin generation, FACS

The method of calibrated automated thrombography (CAT) developed by Hemker allows simultaneous analysis of several samples. By using a fluorogenic thrombin substrate and continuous comparison to a simultaneously run calibrator, thrombin generation can be monitored automatically, on-line, in cloting platelet poor plasma or platelet rich plasma (17). The assays were done by dint of the Fluoroscan Ascent (Thermo Labsystems, Helsinki, Finland) for 90 minutes and processed by means of Thrombinoscope Software (Thrombinoscope BV, Maastricht, The Netherlands).

Flow cytometric assays (FACS) of microparticles in PRP were performed using BD FACScalibur flow cytometer (BD Biosciences, Erembodegem, Belgium). Via differentiation by 1 µm Fluka Micro Particles size standard (Sigma-Aldrich, Buchs, Switzerland) events 1 µm or smaller in size were defined as microparticles (MP) (18). Microparticles were further analyzed, stained with fluorescent labeled FITC Annexin V and monoclonal antibodies PE CD42b and PE CD61, purchased from BD Pharmingen (BD Biosciences, Erembodegem, Belgium).

Statistics

Statistical analysis was performed with the aid of SPSS 15.0© software (SPSS Inc., Chicago, Illinois, USA). After proof of normal distribution by Shapiro-Wilk-test paired t-tests were calculated to compare intrindividually parameters of thrombin generation (lag time, ETP, time to peak, and peak height) with and without addition of rFVIIa. P-values less than 0.05 were considered as statistically significant.

Results

Thrombin generation was measured in microparticle free plasma and in platelet rich plasma by means of calibrated automated thrombography. Each sample was tested with and without addition of rFVIIa (6 µg/ml plasma).
CAT creates thrombograms in which the concentration of thrombin in clotting plasma is represented by the thrombin generation curves. Derived from the curve progression the following parameters are presented:

- lag time, i.e. the time until thrombin formation starts,
- endogenous thrombin potential (ETP) or the area under curve which is proportional to the amount of converted thrombin substrate,
- peak, i.e. the maximum of thrombin concentration,
- time to peak (TTP), i.e. the time for reaching the peak and the start tail, which marks the endpoint of thrombin generation.

When thrombin generation was measured in PRP of thrombocytopenic patients addition of rFVIIa led to a significant shortening of lag time (p < 0.01). Also TTP decreased significantly in PRP of thrombocytopenic patients (p < 0.01) while ETP (p = 0.13) and peak (p = 0.14) remained unchanged after addition of rFVIIa. TTP in thrombocytopenic PRP (mean 23.86 min, 8.15 – 45.44) showed a broader distribution of metered values than TTP in normal PRP (mean 13.42 min, 11.11 – 15.08) as a sign of coagulopathy in thrombocytopenia while the values of lag time were in the similar range in both groups. Thrombin generation in PRP of severe thrombocytopenic patients showed a reduced mean ETP (1516.30 nmol min, 526.33 – 2359.00) and mean peak height (50.20 nmol/l, 33.34 – 67.78) compared to TG in normal PRP, but a distinct TG was detected over the whole range of investigated thrombocytopenic platelet counts (0–20 000/µl PRP).

In microparticle free plasma no distinct thrombin generation was detectable neither with nor without addition of rFVIIa. In flow cytometric analysis a population of microparticles were found in normal PRP as well as PRP of thrombocytopenic patients. Despite low platelet counts in samples of thrombocytopenic patients microparticle formation positive for annexin V was detected and platelet derived MP were identified by expression of CD61 and CD42b in both groups. FACS also was used indirectly to verify the loss of MP in ultracentrifuged plasma.

**Discussion**

Off label use of rFVIIa in thrombocytopenia has been the subject of several studies and reports. Pihusch et al. described the haemostatic potential of rFVIIa in thrombocytopenic patients following haematopoietic stem cell transplantation, but the platelet counts of the study population has not been mentioned in detail (19). We measured thrombin generation in samples of patients who suffered from severe thrombocytopenia, i.e. platelet counts from 0 to 20 000/µl plasma.

Our results demonstrate that similar to normal plasma a clear and significant effect of rFVIIa exists at very low platelet counts, while no distinct thrombin generation could be detected in microparticle-free plasma. The accelerating effect of rFVIIa on thrombin generation, in the form of shortened lag time and TTP, might be clinically relevant in thrombocytopenic plasma down to lowest platelet counts, while the unchanged reduced ETP seems not to be the pivotal criterion of efficacy in coagulation. Increased ETP levels in thrombin generation would be known as reflecting a possible higher risk of thrombosis (17).

Despite the low platelet counts thrombin generation could be enhanced by rFVIIa because of the effectual presence of phospholipids and tissue factor in thrombocytopenic patients’ plasma, which are provided by microparticles. The results of our flow cytometric investigations showed a certain population of microparticles. Annexin V-positive MP were found as evidence of membrane-bound phosphatidylserine as well as platelet derived MP binding anti CD42b and anti-CD 61. In the context of severe thrombocytopenia the origin of MP which are positive for annexin V but not for CD 42b or CD 61 becomes relevant. Some reports have shown that chemotherapy can cause a higher release of endothelial cell-derived MP (20). Further investigations will have to examine the formation of microparticles in haematological patients because they represent a major number of severe thrombocytopenic patients.

**Conclusion**

Even in plasma of severe thrombocytopenic patients enough procoagulant active surface may be provided by microparticles for an enhancement of thrombin generation by rFVIIa.

**Conflict of interest**

All authors declare, that there is no conflict of interest.

**References**


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