Coagulation factor VIIa: prohemostatic drug and biomarker for thrombosis
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Uptake of recombinant factor VIIa by megakaryocytes with subsequent production of platelet-like particles containing functionally active drug

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Abstract

Background: A once-daily prophylactic administration of recombinant factor VIIa (rFVIIa) has been shown to reduce the number of bleeding events in patients with inhibitor-complicated hemophilia, which is difficult to explain given its plasma half-life of 2 hours. Redistribution of rFVIIa into the extravascular space, including the accumulation of rFVIIa in megakaryocytes has been previously demonstrated, which may explain the prophylactic effect of once-daily rFVIIa prophylaxis in hemophilia patients. Objective: We studied the uptake of rFVIIa by cultured megakaryocytic cells, and assessed whether platelet-like particles produced from these cells contain functionally active rFVIIa. Methods: Cultured megakaryocytes (MEG-01 cells) were differentiated with valproic acid and subsequently incubated with rFVIIa. Cells were either harvested or stimulated to produce platelet-like particles (PLPs). Results: rFVIIa is taken up by MEG-01 cells, and rFVIIa endocytosis was critically dependent on the endothelial protein C receptor. When stimulated, MEG-01 cells produced rFVIIa-containing PLPs, whereas rFVIIa is no longer detected in the remnant megakaryocytes. The MEG-01 cell-derived PLPs contained relevant quantities of functionally active rFVIIa, as shown by accelerated lag times and increased thrombin peaks in thrombin generation assays using NPP, FVII- or FVIII deficient plasma when compared to MEG-01 cell-derived PLPs that had not been exposed to rFVIIa. Conclusion: Cultured megakaryocytes endocytose rFVIIa in an endothelial protein C-dependent manner. rFVIIa within megakaryocytes is transferred to platelet-like particles, and these PLPs contain functionally active rFVIIa. The (delayed) generation of rFVIIa-containing platelets may (partly) explain the efficacy of once-daily rFVIIa prophylaxis in patients with inhibitor-complicated hemophilia.
Introduction

Recombinant factor VIIa (rFVIIa) has been shown to be safe and effective for the treatment of bleeding episodes or prevention of surgical bleeding in inhibitor-complicated hemophilia A and B [1,2]. In addition to the treatment of bleeding episodes, recent clinical data demonstrated that rFVIIa is also useful to prevent spontaneous bleeding episodes in patients with inhibitors [3]. A once-daily prophylactic administration of rFVIIa has been shown to reduce the number of bleeding events [3,4]. Surprisingly, one study showed that the reduction in bleeding events persisted after the study period of rFVIIa prophylaxis had ended [3]. The prohemostatic effect of rFVIIa during prophylactic treatment, and its apparent effect after cessation of prophylaxis, is difficult to explain given its plasma half-life of 2 hours.

In literature, four mechanisms explaining the prophylactic efficacy of a once-daily rFVIIa administration have been proposed. First, although the half-life of rFVIIa is ~2 hours, detectable hemostatic activity is still present 24 hours after infusion [5], and it may be that prevention of bleeding requires much lower plasma levels of rFVIIa than required for treatment of bleeding. Second, rFVIIa was shown to improve endothelial cell permeability in a mouse model of hemophilia [6], which may be relevant in prevention of bleeding. Third, rFVIIa can be taken up by platelets, which extends its circulating half-life [5,7]. Importantly, rFVIIa which is taken up by platelets remains hemostatically active. Fourth, rFVIIa has been shown to enter the extravascular space, with subsequent accumulation in various tissues in a rat and mouse models [8-10]. Accumulation of rFVIIa in bone and joints may explain the prophylactic activity of rFVIIa. Alternatively, rFVIIa may leak back into circulation from these extravascular storage sites. One study suggested that rFVIIa was taken up by megakaryocytes within the bone marrow [10].

We hypothesize that those megakaryocytes that have taken up rFVIIa will produce rFVIIa-containing platelets, which will be another way in which rFVIIa may become present in circulation at time points distant from initial infusion. Here we examined uptake of rFVIIa by cultured megakaryocytic cells, and assessed whether platelet-like particles produced from these cells contain functionally active rFVIIa.

Methods

Cell culture

The megakaryoblastic cell line MEG-01 was cultured and differentiated by valproic acid as described earlier [11]. After at least 10 days of differentiation, different concentrations
of rFVIIa were added to the culture medium and after 2 h, MEG-01 cells were either harvested or stimulated to produce platelet-like particles (PLPs) by addition of 100 ng/ml recombinant human thrombopoietin (rTPO) (Life Technologies, Carlsbad, CA, USA) for 3 days. Cells or PLPs were harvested by centrifugation, washed, and lysed by freeze-thawing the samples twice.

**Microtitre plate clotting assay to estimate rFVIIa levels in MEG-01 and PLP lysates**

FVIIa activity in MEG-01 and PLPs lysates was studied by a microtitre plate clotting assay as described earlier [5]. A calibration curve of rFVIIa was used to convert clotting times to rFVIIa concentrations. rFVIIa levels were normalized for total protein content of the samples to correct for enumeration differences between samples using the Pierce BCA protein assay kit (Thermo Scientific, IL, USA).

**Flow cytometry**

Differentiated MEG-01 cells were detached with 5mM (w/v) EDTA in phosphate-buffered saline (PBS) and stained for endothelial protein C receptor (EPCR) or glycoprotein Ibα using 10 µg/ml R-Phycoerythrin-labeled rat anti-human CD201 antibody (EPCR, clone RCR-401, Biolegend, San Diego, CA, USA) or 156 µg/ml R-Phycoerythrin-labeled monoclonal mouse anti-human CD42b (GPIb, clone AN51, Dako Denmark, Glostrup, Denmark) for 20 min at 37°C.

Isolated PLPs were fixed with 2% (v/v) formaldehyde in 0.9% NaCl for 10 min at room temperature. In selected experiments, PLPs were permeabilized with 0.1% (v/v) triton X-100 in PBS for 10 min at room temperature. After washing, samples were stained for rFVIIa using 10 µg/ml monoclonal mouse anti-human FVII (ABIN951602, clone AA-3, antibodies-online, Germany) for 1 h at room temperature followed by 10 µg/ml Alexa Fluor 488-labeled goat anti-mouse antibody (A-11001, Life Technologies, Carlsbad, CA, USA) for 30 min at room temperature. Flow cytometry data acquisition was performed using FlowJo X (TreeStar, Ashland, OR, USA).

**Thrombin generation assay**

PLPs were counted using a MACS Quant flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and added to normal pooled plasma (NPP), FVII- or FVIII deficient plasma (Haematologic Technologies, Inc., Essex Junction, VT, USA) at a final concentration of 10,000 PLPs/µl plasma. Calibrated automated thrombography using the manufacturer's
reagents (Thrombinscope PRP reagent) and protocols (Thrombinscope B.V., Maastricht, the Netherlands) was performed.

**Immunofluorescence staining**

MEG-01 cells or isolated PLPs were permeabilized with 0.1% (v/v) triton X-100 in PBS for 10 min at room temperature, and stained with 5 μg/ml monoclonal mouse anti-human FVII followed by 5 μg/ml Alexa Fluor 488 goat anti-mouse antibody. MEG-01 cell samples were counterstained with 4 units/ml Alexa Fluor 594 phalloidin (A12381, Life Technologies, Carlsbad, CA, USA) which stains F-actin. This staining was used to indicate the cell membrane, but for clarity the staining itself is not shown in the figures. All samples were fixed using Vectashield Hardset mounting medium with 4′, 6-Diamidine-2′-phenylindole dihydrochloride (DAPI) which stains DNA and is used to stain the nuclei (Vector Laboratories Inc., CA, USA). Imaging was performed on a Leica DMI4000B LED. Images were captured using Leica Application Suite Advanced Fluorescence software (LAS AF). Image processing was performed using Imaris 7.1.1 (Bitplane Scientific Software, Zurich, Switzerland).

**Statistical analysis**

To evaluate differences in parameters derived from thrombin generation tests, paired t-tests were used. To evaluate differences in rFVIIa content of MEG-01 cells in absence or presence of blocking agents, one-way analyses of variance with Dunnett’s post-test was used. P values ≤0.05 were considered statistically significant. Statistical analyses were performed using the GraphPad Prism 5.1 Software package (La Jolla, CA, USA).

**Results and discussion**

To investigate uptake of rFVIIa by differentiated MEG-01 cells, cells were incubated with different rFVIIa concentrations (0-100 nM). After 2 hours of incubation, MEG-01 cells were either harvested or stimulated to produce PLPs. rFVIIa was dose-dependently taken up by MEG-01 cells (Fig. 1A). After 3 days of rTPO stimulation, hardly any rFVIIa was detected in the MEG-01 cells (Fig. 1B). In contrast, the PLPs produced from these MEG-01 cells contained appreciable amounts of rFVIIa (Fig. 1C).

To confirm that rFVIIa is taken up by MEG-01 cells (and not just associates with the cell), we stained MEG-01 cells for rFVIIa. Analysis by fluorescent microscopy showed ubiquitous presence of rFVIIa within the vast majority of cells (80-90%) in an apparent punctuate pattern at 2 hours after addition of rFVIIa to the culture medium (Fig. 1D i),
whereas no staining was detected in cells that had not been exposed to rFVIIa (Fig 1D ii). After 3 days of rTPO stimulation, no rFVIIa staining was detected in the MEG-01 cells (Fig. 1D iii). The MEG-01 cell-derived PLPs harvested at day 3, however, did stain positive for rFVIIa (Fig 1D iv). We also used flow cytometry for rFVIIa in presence or absence of cell permeabilisation to confirm the presence of rFVIIa within PLPs. rFVIIa was predominantly localized within the PLP as 55.6% ± 11.3% [mean ± SD, n = 3] of permeabilized cells stained positive for rFVIIa, as compared to 19.9% ± 5.1% [mean ± SD, n = 3] of non-permeabilised cells (Fig. 1E).

rFVIIa within PLPs is hemostatically active, as levels were determined in PLP lysates with a functional coagulation assay (Fig 1C). To determine whether this functionally active rFVIIa contributes to hemostasis in a plasma environment, we performed in vitro thrombin generation measurements using plasma to which intact PLPs were added. Addition of rFVIIa-containing PLPs resulted in a profound shortening of the lag time of the thrombin generation curve in normal, FVII- and FVIII- deficient plasma, compared to addition of PLPs generated from MEG-01 cells that had not been exposed to rFVIIa (Fig. 1F). rFVIIa-containing PLPs also had a slightly but significantly increased peak thrombin generation compared to control PLPs (Fig. 1G). Previous studies have shown hemostatically active rFVIIa within platelets after rFVIIa endocytosis by platelets in vitro or in vivo [5,7]. In addition, ectopic expression of FVIIa in platelets resulted in correction of the bleeding phenotype in a mouse model of hemophilia A [12], which again showed that rFVIIa remains hemostatically active within the platelet cytoplasm despite the presence of inhibitors such as TFPI in the platelet [13].

We next assessed the mechanism of uptake of rFVIIa by MEG-01 cells. We hypothesized a role for negatively charged phospholipids, GPIbα and EPCR, which are all known binding partners for rFVIIa [14-16]. GPIbα was hardly detected on the surface of differentiated MEG-01 cells; 2.5% ± 0.6% [mean ± SD, n = 3] of cells stained positive for GPIbα (Fig. 2A). In contrast, EPCR was abundantly present on MEG-01 cells with positive staining on 73.2% ± 11.8% [mean ± SD, n =3] of cells (Fig. 2B), which has to our knowledge not been reported before. Uptake of rFVIIa was not affected by Annexin A5 excluding a role for negatively charged phospholipids in rFVIIa uptake, but a 76.0 ± 8.7% [mean ± SD] reduction of rFVIIa uptake was observed in the presence of an antibody to EPCR (Fig. 2C). Immunofluorescent staining of rFVIIa confirmed these results (Fig. 2D).

The present study shows a dose-dependent uptake of rFVIIa by MEG-01 cells in vitro. Stimulation of these MEG-01 cells with rTPO resulted in the production of platelet-like
particles containing functionally active rFVIIa. Interestingly, 3 days after rFVIIa addition to the culture medium, rFVIIa is almost exclusively present in PLPs, suggesting selective transfer of rFVIIa to platelet-like particles. Active selection of agents to be transferred to platelets in the process of megakaryocyte maturation has been previously demonstrated as megakaryocytes transfer some, but not all, mRNA species examined to platelets [17].

Uptake of rFVIIa by megakaryocytes was critically dependent on EPCR, which we, to our knowledge, for the first time identify on megakaryocytes. EPCR thus appears to fulfill multiple critical features in the mode of action of rFVIIa, including enhancement of hemostatic activity in the intravascular space [18], improvement of endothelial barrier function [6], transport of rFVIIa to extravascular sites [19], and uptake by megakaryocytes in the bone marrow (this study). These interactions may be relevant in the (prophylactic) efficacy of rFVIIa variants. Specifically, glycoPEGylated rFVIIa which has a prolonged half-life, does not bind to EPCR but activation of FX was not impaired compared to unmodified rFVIIa [20]. Initial clinical results do suggest this rFVIIa variant to be well tolerated and safe, however no dose-response in inhibitor-complicated hemophilia patients was established and the clinical development of this product has been terminated [21].

We assessed rFVIIa levels in PLP lysates using a functional assay and found functionally active rFVIIa in PLPs generated from MEG-01 cells which were exposed to rFVIIa 3 days before. Although we have not assessed the exact location of rFVIIa within PLPs, the staining pattern suggests it to be stored in granules. Indeed, when added to normal, FVII- or FVIII deficient plasma, FVIIa-containing PLPs showed enhancement of thrombin generation, suggesting exocytosis of rFVIIa from these PLPs. Whether rFVIIa in platelet-like particles does not encounter inhibitors (such as TFPI and AT) or whether the rFVIIa we measure in our assay only represents a fraction of total rFVIIa in platelet-like particles, with the remainder being in complex with an inhibitor requires further study.

Taken together, we demonstrate EPCR-dependent uptake of rFVIIa by megakaryocytes with subsequent production of rFVIIa-containing ‘prohemostatic’ platelets. Whether this mechanism acts in vivo requires further study, but delayed generation of rFVIIa-containing platelets appears a plausible mechanism to partly explain the efficacy of once-daily rFVIIa prophylaxis in inhibitor-complicated hemophilia patients.
Chapter 3

A. ng FVIIa/mg total protein vs Supplied concentration rFVIIa (nM)

B. ng FVIIa/mg total protein vs Supplied concentration rFVIIa (nM)

C. ng FVIIa/mg total protein vs Supplied concentration rFVIIa (nM)

D. Images showing cellular structures labeled with rFVIIa-Alexa 488:
   i. Cell with rFVIIa-Alexa 488
   ii. Cells with rFVIIa-Alexa 488
   iii. Cell with rFVIIa-Alexa 488
   iv. Cells with rFVIIa-Alexa 488

E. Histogram showing distribution of rFVIIa-Alexa 488:

F. Bar graph showing Lag time (min) for different conditions:
   - NPP
   - FVII def. plasma
   - FVIII def. plasma

G. Thrombin (nM) vs Time (min) for different conditions:
   - NPP + FVIIa
   - NPP - FVIIa
   - FVII def. + FVIIa
   - FVII def. - FVIIa
   - FVIII def. + FVIIa
   - FVIII def. - FVIIa
Figure 1 Dose-dependent uptake of rFVIIa by MEG-01 cells with subsequent production of PLPs containing functionally active rFVIIa. (A-C) rFVIIa was added in different concentrations (0-100 nM) to valproic acid-stimulated MEG-01 cells, which were harvested after 2 hours (A), or stimulated with 100 ng/ml recombinant human thrombopoietin to produce PLPs. After 3 days, MEG-01 cells (B) or PLPs (C) were isolated by centrifugation. Lysates of MEG-01 cells and PLPs were tested for rFVIIa content using a microtitre plate-based clotting assay in factor VII-deficient plasma and rFVIIa levels in the lysates were expressed relative to the total protein content of these lysates. Shown are means of 3 independent experiments, error bars indicate standard error of mean (SEM). (D) Immunofluorescent stainings of MEG-01 cells (i-iii) or PLPs (iv) using an antibody to factor VII(a). Shown are typical examples of MEG-1 cells that were exposed to 100 nM rFVIIa for 2 hours (i) or 3 days (iii), or cells that were exposed for 2 hours to vehicle (ii). In addition, PLPs derived from MEG-01 cells exposed to 100 nM of rFVIIa harvested at day 3 are shown (iv). rFVIIa is stained in green, nuclei in blue, and the cell membrane is represented by the dotted line. Scale bars represent 20 µm (MEG-01 cells) or 10 µm (PLPs). Original magnification 1000x. (E) Flow cytometry analysis of PLPs derived from MEG-01 cells that have been exposed to 100 nM of rFVIIa using permeabilized (green line) or non-permeabilized (blue line) cells. The black line represents PLPs generated from MEG-01 cells that not have been exposed to rFVIIa. Data shown is representative of three independent experiments. (F,G) PLPs derived from MEG-01 cells exposed to 100 nM rFVIIa or vehicle were added to normal pool plasma (NPP), factor VII- or factor VIII deficient plasma, which was tested by calibrated automated thrombography using the PRP reagent. Shown are lag time values (F) derived from thrombin generation curves and representative thrombin generation curves (G). Data represent the mean of three independent experiments. Error bars indicate standard deviation. *p≤0.05, **p≤0.01, ***p≤0.001 by paired t-test.
Figure 2 rFVIIa uptake by MEG-01 cells is mediated by EPCR. (A, B) MEG-01 cells were stained for glycoprotein Ibα (A) or EPCR (B) and analyzed by flow cytometry (green lines). The black lines represent the background fluorescence of the MEG-01 cells, and the blue lines correspond to appropriate isotype controls. Data shown is representative of three independent experiments. (C) MEG-01 cells were incubated with 100 nM of rFVIIa for 2 hours in presence or absence of 30 µg/ml Annexin V (AnnV) or 45 µg/ml rat monoclonal anti-EPCR antibody (RCR-252, Novus Biologicals, Littleton, CO, USA), and rFVIIa content of cell lysates was determined by a microtitre plate-based clotting assay using factor VII- deficient plasma. Shown is the quantity of rFVIIa in cell lysates expressed as percentage of control. Data represents the mean with error bars indicating SEM (n = 3). *p≤0.01, compared to control (-) by one-way analyses of variance with Dunnett’s post-test. (D) Immunofluorescent stainings of MEG-01 cells under the conditions of the experiment shown in panel C. rFVIIa is stained in green, nuclei in blue, and the cell membrane is represented by the dotted line. Scale bars indicate 20 µm. Original magnification 630x.
References


