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Molecular mechanisms of platelet-mediated liver regeneration after partial hepatectomy

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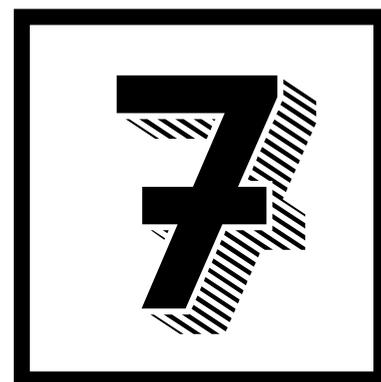
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**INTERMEZZO: IN
VITRO UPTAKE OF
RECOMBINANT
FACTOR VIIA BY
MEGAKARYOCYTES
WITH SUBSEQUENT
PRODUCTION
OF PLATELETS
CONTAINING
HEMOSTATICALLY
ACTIVE DRUG**

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BRITISH JOURNAL OF HAEMATOLOGY

Abstract

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 in hemophilia. Here we show that rFVIIa is taken up by cultured megakaryocytes (MEG-01 cells), and rFVIIa endocytosis was critically dependent on the endothelial protein C receptor. When stimulated, these megakaryocytes produce rFVIIa-containing platelet-like particles (PLPs), whereas rFVIIa is no longer detected in the remnant megakaryocytes. The MEG-01 cell-derived PLPs contain relevant quantities of hemostatically active rFVIIa, as shown by accelerated lag times and increased thrombin peaks in thrombin generation assays using factor VII or VIII depleted plasma when compared to MEG-01 cell-derived PLPs that had not been exposed to rFVIIa. We propose that also *in vivo* redistribution of rFVIIa to the bone marrow compartment results in production of platelets containing 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 activated factor VII (rFVIIa) has been recently shown to prevent spontaneous bleeding in inhibitor-complicated hemophilia when administered once daily (1,2). The prohemostatic effect of rFVIIa prophylaxis is difficult to explain given its plasma half-life of 2 hours.

In the literature, four mechanisms explaining the prophylactic efficacy of once-daily rFVIIa administration have been proposed, including the requirement of much lower doses of rFVIIa to prevent than to treat bleeding, improvement of endothelial cell permeability, uptake of rFVIIa by platelets, and redistribution of rFVIIa into the extravascular space (3). Accumulation of rFVIIa in bone and joints may explain the prophylactic activity of rFVIIa. One study suggested that rFVIIa was taken up by megakaryocytes within the bone marrow (4). The present study tested the hypothesis that megakaryocytes that have taken up rFVIIa will produce rFVIIa-containing platelets, which may explain the prolonged haemostatic effect of rFVIIa in a prophylactic setting.

Materials & Methods

Cell culture

The megakaryoblastic cell line MEG-01 was cultured and differentiated by valproic acid as described earlier (5). After at least 10 days of differentiation, different concentrations of rFVIIa were added to the culture medium and after 2 hours, MEG-01 cells were either harvested or stimulated to produce platelet-like particles (PLPs) by addition of 100 ng/ml recombinant human thrombopoietin (Life Technologies, Carlsbad, CA) 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 (6). 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 Iba using 10 µg/ml R-Phycoerythrin-labeled rat anti-human EPCR antibody or 156 µg/ml R-Phycoerythrin-labeled monoclonal mouse anti-human CD42b (GPIb, clone AN51) 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. Samples were analysed by flow cytometry.

Thrombin generation assay

PLPs were counted using flow cytometry and added to 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 (Thrombinoscope PRP reagent) and protocols (Thrombinoscope BV, 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). All samples were fixed using Vectashield Hardset mounting medium with DAPI (Vector Laboratories Inc., CA, USA).

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 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

We used the megakaryoblastic cell line MEG-01, which was cultured and differentiated by valproic acid as described earlier (5). After at least 10 days of differentiation, different concentrations of rFVIIa were added to the culture medium and after 2 hours, MEG-01 cells were either harvested or stimulated to produce platelet-like particles (PLPs) by addition of 100 ng/ml recombinant human thrombopoietin (rTHPO; Life Technologies, Carlsbad, CA, USA) for 3 days. Cells or PLPs were harvested by centrifugation, washed and lysed by freeze-thawing the samples twice.

We first showed that rFVIIa was dose-dependently taken up by MEG-01 cells (Fig. 1A). After 3 days, 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), which suggests megakaryocytes selectively transfer endocytosed rFVIIa to PLPs. 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 (7).

To confirm that rFVIIa is taken up by MEG-01 cells (and not just associates with the cell), we performed fluorescence microscopy which showed ubiquitous presence of rFVIIa within the cell in a punctuate pattern 2 hours after addition of rFVIIa (Fig. 1D i), whereas no staining was detected in cells that had not been exposed to rFVIIa (Fig. 1D ii). After 3 days, no rFVIIa staining was detected in the MEG-01 cells (Fig. 1D iii), but the PLPs harvested at day 3 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. Indeed, rFVIIa was predominantly localized within the PLP as $55.6\% \pm 11.3\%$ [mean \pm SD, $n = 3$] of permeabilized cells stained positive for rFVIIa, as compared to $19.9\% \pm 5.1\%$ [mean \pm SD, $n = 3$] of non-permeabilised cells (Fig. 1E).

rFVIIa within PLPs is haemostatically active, as levels were determined in PLP lysates with a functional coagulation assay. To determine whether this functionally active rFVIIa contributes to haemostasis 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-depleted 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 slight but significantly increased peak thrombin generation compared to control PLPs (Fig 1G). Whether rFVIIa in PLPs does not encounter inhibitors (such as tissue factor pathway inhibitor and antithrombin) or whether the rFVIIa measured in our assay only represents a fraction of total rFVIIa in PLPs, with the remainder being in complex with an inhibitor, requires further study.

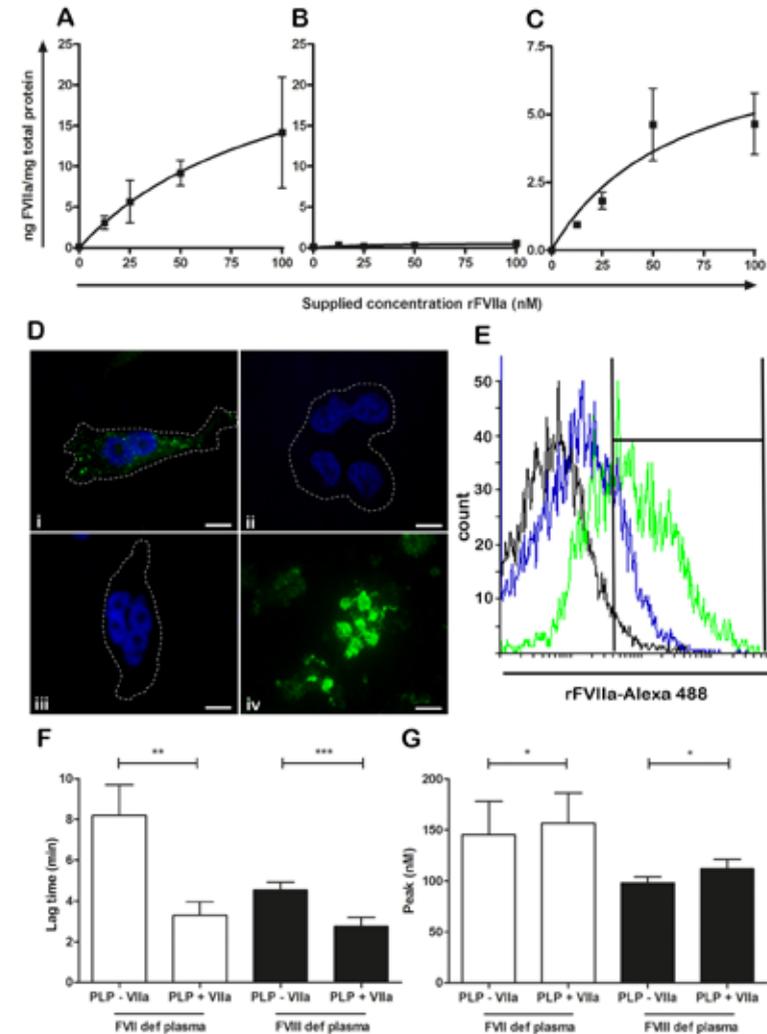


Figure 1: Dose-dependent uptake of rFVIIa by MEG-01 cells with subsequent production of PLPs containing hemostatically active rFVIIa.

(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-depleted plasma. 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-01 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. rFVIIa is stained in green, nuclei in blue, and the cell membrane is represented by the dotted line. Scale bars represent 20 μ m (MEG-01 cells) or 10 μ m (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 factor VII or factor VIII depleted plasma, which was tested by calibrated automated thrombography using the PRP reagent. Shown are lag time (E) and peak (F) values derived from thrombin generation curves. Data represent the mean of three independent experiments. Error bars indicate standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

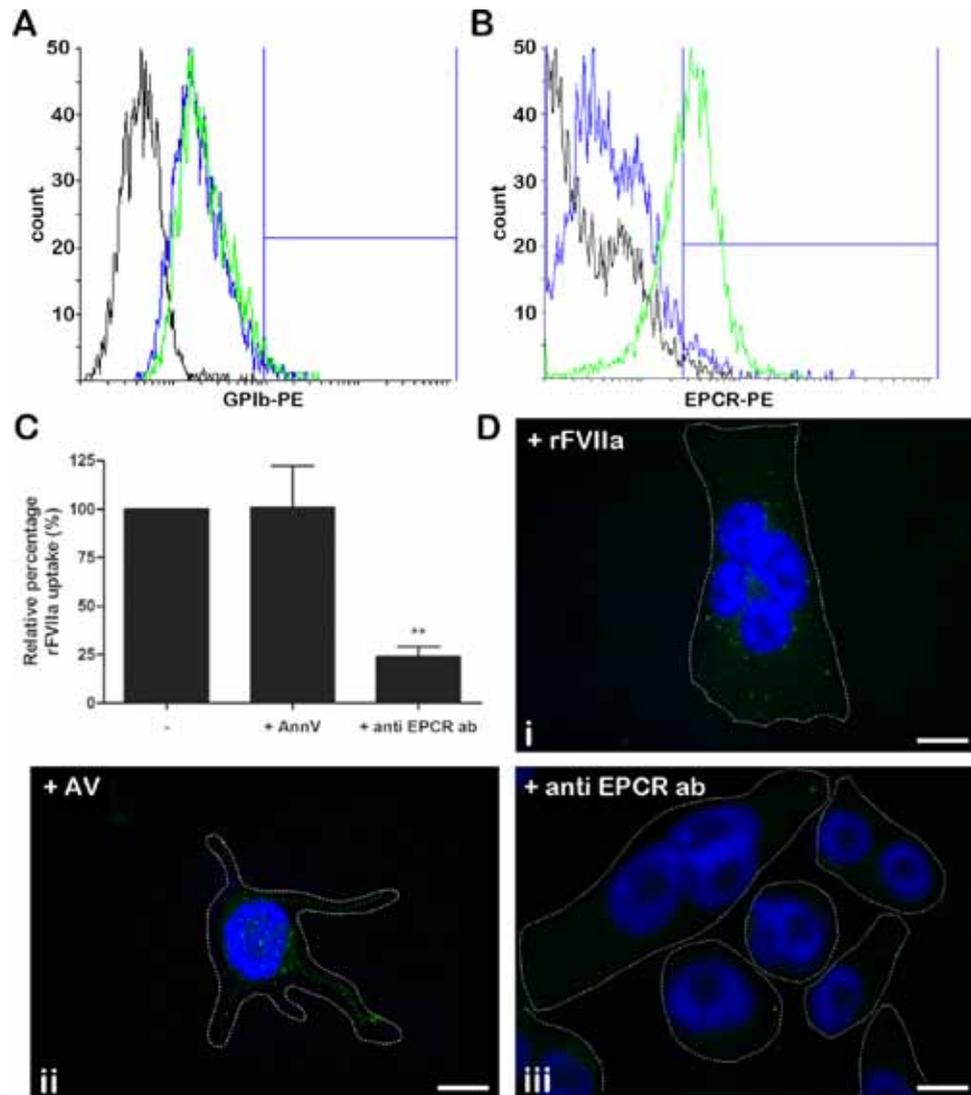


Figure 2: rFVIIa uptake by MEG-01 cells is mediated by EPCR.

(A,B) MEG-01 cells were stained for glycoprotein Iba (A) or EPCR (B) and analyzed by flow cytometry. 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 mg/ml Annexin V (AnnV) or 45 mg/ml rat monoclonal anti-EPCR antibody, and rFVIIa content of cell lysates was determined by a microtitre plate-based clotting assay using factor VII-depleted 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). * $P \leq 0.01$, compared to control (-). (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.

We next assessed the mode of uptake of rFVIIa by MEG-01 cells. We hypothesized a role for negatively charged phospholipids, GPIb α , or EPCR, which are all known binding partners for rFVIIa (8-10). GPIb α was hardly detected on the surface of differentiated MEG-01 cells; $2.5\% \pm 0.6\%$ ([mean \pm SD, n = 3] of cells stained positive for GPIb α (Fig. 2A). In contrast, EPCR was abundantly present on MEG-01 cells with positive staining on $73.2\% \pm 11.8\%$ ([mean \pm 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 $76.0 \pm 8.7\%$ [mean \pm 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). EPCR thus appears to fulfill multiple critical features in the mode of action of rFVIIa, including enhancement of hemostatic activity in the intravascular space (8), improvement of endothelial barrier function (9), transport of rFVIIa to extravascular sites (10), and uptake by megakaryocytes in the bone marrow (this 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.

Author contributions

A.M. Schut and M. Kirschbaum participated in the design of the study, performed experiments, analysed and interpreted data and wrote the manuscript. J. Adelmeijer performed experiments, analysed and interpreted data. P.G. de Groot interpreted data. T. Lisman participated in the design of and supervised the study, interpreted data and wrote the manuscript. All authors revised and approved the manuscript.

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Conflict of interest

This study was supported by an unrestricted educational grant from Novo Nordisk, whose product is studied in the manuscript.

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