Traffic of rFVIIa through Endothelial Cells and Redistribution into Subendothelium: Implications for a Prolonged Hemostatic Effect

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ABSTRACT

Background
Clinical evidence suggests that the hemostatic action of recombinant activated factor VII (rFVIIa) exceeds its predicted plasma life. Mechanisms involved in the long-lasting effects of rFVIIa for prophylactic treatment of patients with hemophilia and inhibitors have not been fully elucidated.

Objectives
The traffic of rFVIIa through the endothelial cells (EC) and its redistribution into the subendothelial compartment was investigated. Its possible hemostatic action in experiments with flowing blood was also assessed.

Methods
Cultured EC and umbilical veins were exposed to rFVIIa (6 μg/mL) for up to 2 h. Immunocytochemical techniques were combined with confocal microscopy to localize rFVIIa into EC and to trace its possible redistribution into subendothelial compartments. Vessels exposed to rFVIIa were de-endothelized and exposed to flowing blood to determine possible modifications in their hemostatic capacity.

Results
Immunocytochemical studies revealed a significantly enhanced presence of FVIIa dispersedly distributed in the cytoplasm of EC previously exposed to rFVIIa. rFVIIa relocated into nuclear and peripheral areas when prolonged incubations (24 h) were performed. Immunocytochemical studies revealed that rFVIIa localizes into the endothelium and subendothelium of incubated umbilical vessels. In perfusion studies, this rFVIIa redistributed into the subendothelium improved fibrin generation and enhanced platelet thrombus formation.

Conclusions
These results indicate that rFVIIa can be internalized and redistributed into endothelial and subendothelial compartments. This rFVIIa remains functional and promotes hemostatic activity when vessels are denuded. These findings may explain the prolonged prophylactic action of rFVIIa in some clinical conditions.

Keywords: Pharmacodynamics, endothelial cells, hemostatic action, recombinant activated factor VII traffic, prophylaxis

INTRODUCTION
It is well accepted that around 16–36% of hemophilia A and 6–8% of hemophilia B patients will develop inhibitory antibodies to the infused factor that will neutralize its hemostatic action [1]. Recombinant activated coagulation factor VII (rFVIIa) was developed for the treatment of bleeding episodes in hemophilia patients with inhibitors to coagulation factor VIII (FVIII) or factor IX (FIX) [2], who cannot benefit from prophylaxis with FVIII or FIX.

The introduction of rFVIIa has facilitated the clinical management of these patients. rFVIIa has the same structure and activity as the human factor, restoring hemostasis by favoring thrombin generation [3]. Interestingly, rFVII has proven useful in controlling active bleeding episodes not only in hemophilia,
but also in other hemostatic deficiencies including platelet and coagulation disorders [4, 5]. An enhanced thrombin generation at damaged vessels has been proposed as the main mechanism contributing to the hemostatic action of rFVIIa in the control of active bleeding in congenital and acquired disorders of hemostasis [6, 7]. Tissue factor (TF) exposed at sites of vascular damage would help to localize the hemostatic response, favoring fibrin generation and platelet recruitment in more stable thrombi [8–10].

The pharmacokinetic characterization of rFVIIa by different groups has determined a half-life of 2.7 h in adults and of 1.3 h for children [11–13]. Clinical experience from an exploratory phase II trial (in patients subjected to prophylaxis) suggests that the hemostatic action of recombinant activated factor VII (rFVIIa) exceeds its predicted plasma half-life [14–16]. Recent publications have highlighted the potential role of rFVIIa in the prophylaxis of hemophilic patients with inhibitors [15, 17, 18]. Although the mechanisms of action of rFVIIa in the correction of active bleeding have been widely studied, mechanisms involved in the apparent long-lasting effects of rFVIIa for prophylactic treatment remain to be elucidated. It has been speculated that a portion of the rFVIIa infused into patients could diffuse to the extravascular space and once there become available at the site of injury [19].

Our present studies have attempted to explain the possible mechanism involved in the prolonged hemostatic effectiveness of rFVIIa prophylaxis. Using an in vitro experimental setting, we have investigated the traffic of rFVIIa through the endothelial cells (EC) and its redistribution into the subendothelial compartments. To accomplish these objectives, we used a combination of immunocytochemical techniques and perfusion studies with circulating blood to detect the possible traffic of rFVIIa into endothelial cells and to evaluate the potential implications for its hemostatic activity.

**MATERIALS AND METHODS**

**Experimental Design**

The present study was designed to evaluate the possible redistribution of rFVIIa in different intracellular compartments, specifically on the endothelium and subendothelial matrix. Isolated endothelial cells (EC) harvested from umbilical cords and human umbilical vessels were incubated with rFVIIa (6 μg/mL final concentration). The presence of rFVIIa in EC and on endothelial extracellular matrices was detected by immunocytochemical studies using specific antibodies against FVIIa detected by confocal microscopy.

The physiological relevance of rFVIIa redistribution to EC or subendothelial matrices was tested using flow systems. The effects on thrombus formation and fibrin generation were assessed.

**Reagents and Antibodies**

Whole blood was anticoagulated with buffered citrate/phosphate/dextrose solution (CPD) to a final concentration of citrate of 19 mM, or with low molecular weight heparin (LMWH; Fragmin®, Pharmacia, Madrid, Spain) at a final concentration of 20 U/mL. rFVIIa was supplied by Novo Nordisk (NovoSeven®, Novo Nordisk, Bagsvaerd, Denmark).

To detect human rFVIIa, a polyclonal rabbit anti-human antibody was used (Agrisera, Vännäs, Sweden). For immunocytochemical techniques, we used as secondary antibody a goat anti-rabbit immunoglobulin (Ig)G Alexa 488 (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Fluorescent properties were prolonged using the Prolong antifade kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA). AuroProbe One GAR and IntenSE™ M silver enhancement kit were from Amersham (Buckinghamshire, UK). Embedding kit JB-4 was from Polyscience (Warrington, USA).

**Studies on Human Endothelial Cells**

**Incubation of ECs with rFVIIa.**

Endothelial cells were harvested from umbilical cords according to the method described initially by Jaffe et al., which is well established in our group [20, 21]. Coverslips with confluent EC monolayers were incubated in the presence or absence of rFVIIa (6 μg/mL final concentration) in Medium 199 supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20% pooled human serum. Incubation was prolonged for 2 h according to the half-life calculated for rFVIIa, at 37°C. An additional incubation of 24 h was also performed to trace the possible redistribution of rFVIIa into the EC. After incubation, coverslips were rinsed with phosphate-buffered saline (PBS) three times and fixed in 4% paraformaldehyde for 15 min at room temperature until immunolocalization was performed.

**Immunolocalization on Endothelial Cells: Confocal Microscopy.**

After the fixation procedure, EC coverslips were rinsed with PBS three times. Permeabilization and blocking for non-specific binding sites were performed with 0.1% saponin and 1% bovine serum albumin (BSA) in PBS (15 min at room temperature), followed by a washing step with PBS. Afterwards, coverslips were incubated with the primary antibody (14 μg/mL rabbit anti-human FVIIa antibody in 0.1% saponin) for 1 h at 37°C. After removing the excess primary antibody with PBS, a second incubation with a green fluorescent-labeled anti-rabbit antibody Alexa-488 was performed in darkness for 45 min at 37°C. Coverslips were rinsed with PBS and with distilled water to remove the excess antibody, and then mounted in Prolong® Gold antifade mounting media (Molecular Probes) for confocal microscopy analysis. Non-specific
labeling was also assessed by omitting the primary antibody. Confocal images were acquired using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with argon and HeNe lasers attached to a Leica DMIRE2 inverted microscope. The images of fluorescein isothiocyanate (FITC) labeling were acquired as follows: excitation at 488 nm, triple dichroic beam-splitter (TD 488/543/633) and emission detection range of 500–535 nm. All images were obtained using a 63× oil immersion objective lens (NA 1.32) equipped with phase contrast optics and the confocal pinhole set at 1 Airy unit.

**Studies on Human Umbilical Vessels**

**Incubation of Vessels with rFVIIa.**

All the umbilical cords were collected within 24 h after delivery. Once rinsed with PBS, each cord was cut into two pieces of approximately 10 cm. Hank’s buffered salt solution (HBSS), with and without rFVIIa (6 μg/mL final concentration), was infused into a vein with both ends clamped and incubated for 2 h at 37°C. After incubation, veins were rinsed with PBS, accurately separated from the remaining cord, and fixed overnight in glutaraldehyde (2.5% final concentration). Fixed veins were rinsed with PBS and dehydrated in an increasing gradient of ethanol. Finally, veins were embedded in glycol-methacrylate and processed histologically to obtain thin (2 μm) cross-sections [22].

**Immunolocalization on Vessel Cross-sections.**

The presence of rFVIIa in the cross-sections of the umbilical vein was assessed by immunocytochemical techniques at room temperature according to the instructions provided by the manufacturer of the AuroProbe One GAR kit. Non-specific binding sites were blocked with 0.8% BSA and 0.1% fish gelatin provided by the manufacturer, pH 7.4, for 30 min. Sections were incubated with 14 μg/mL rabbit anti-human FVII/VIIa antibody for 1 h. After removing the excess primary antibody by washing it three times with PBS, sections were incubated with a gold-conjugated goat anti-rabbit secondary antibody for 4 h. Excess secondary antibody was removed by washing three times with PBS and then three times with distilled water. Finally, samples were treated with an IntenSE silver enhancement reagent [23].

In all experimental settings, non-specific binding was assessed with non-specific antibody IgG. Samples were visualized using a Reichert-Jung light microscope using epipolarization techniques.

**Haemostatic Activity: Studies in Perfusion Chambers with Flowing Blood**

Hemostatic activity associated with extravascular rFVIIa was assessed using vascular segments from human umbilical vein. Vessels were incubated for 2 h with Hank’s balanced buffer (HBS) or with rFVIIa (6 μg/mL in the same buffer). After incubation, veins were thoroughly washed with PBS, everted, and subjected to a mechanical de-endothelization. Denuded umbilical veins were exposed to flowing blood anticoagulated with LMWH.

Blood (22 mL) anticoagulated with LMWH (20 U/mL) was perfused in an annular chamber using as thrombogenic substrate the damaged vascular segments [24]. Perfusions were performed at a shear rate of 600 s⁻¹ for 10 min. After perfusion, vascular segments were rinsed with PBS (0.15 M), fixed with 2.5% glutaraldehyde (in 0.15 M PBS) at 4°C for 24 h and processed histologically for morphometric evaluation. Fibrin deposition and platelet interaction were evaluated by light microscopy connected to a computer, provided with special software that automatically classifies and quantifies platelet and fibrin coverage [25]. Platelet interaction was expressed globally as a percentage of the surface covered by platelets (%P). The presence of fibrin was also morphometrically quantified and expressed as a percentage of fibrin (%F) [25].

**Statistics**

Results were expressed as mean ± standard error of the mean (SEM). Values provided in immunolocalization experiments on endothelial cells and vascular segments correspond to four to eight experiments. One-way ANOVA test for independent experiments was applied when multiple comparisons were required. Student’s t-test for paired data was used for comparisons in perfusion experiments. The level of statistical significance was established at P<0.01.

**RESULTS**

**Internalization of rFVIIa into Endothelial Cell Cultures**

Experiments performed with human EC cultures confirmed that rFVIIa was inside the EC. Cultures of EC monolayers exposed to rFVIIa (6 μg/mL) for 2 h showed an intense labeling to rFVIIa that appeared homogeneously distributed in the EC cytoplasm (Figure 1A,B). The analysis of control EC cultures, not incubated with rFVIIa, showed very weak signal labeling, probably related to the residual presence of FVII/VIIa in the supplemented culture medium, which contains a 20% serum pool.

Results with prolonged incubations of rFVIIa for 24 h (6 μg/mL) revealed a relocation of this rFVIIa in the EC cytoplasm, being more intense in the periphery and nucleus of the EC (Figure 1C).

Controls with the secondary antibody alone to assess non-specific binding showed no fluorescence, confirming that labeling in the EC cultures resulted from the presence of rFVIIa.
Redistribution of rFVIIa in Umbilical Veins

The immunocytochemical techniques applied to the thin sections of umbilical cord veins revealed positive immunogold labeling for rFVIIa associated with the endothelium and extending to deeper areas in the veins such as the subendothelium and even the internal elastic lamina in those veins that had previously been incubated with rFVIIa for 2 h (Figure 2).

Vessels not incubated with rFVIIa presented a much weaker positive labeling for FVIIa in the EC layer. One possible explanation for this labeling could be the origin of the veins, which have been exposed to blood.

Controls with the secondary antibody alone to assess non-specific binding showed a mild dispersed labeling fluorescence without specific location.

Hemostatic Activity Associated with rFVIIa Associated with the Vessels

Blood was perfused through denuded human umbilical veins previously incubated with HBS or rFVIIa (Figure 3). Perfusion runs over human umbilical veins incubated with HBS resulted in a percentage of platelet coverage equivalent to 11.5 $\pm$ 2.2 %, whereas fibrin formation was minimal (5.2 $\pm$ 4.9 %) (Figure 3A).

In contrast, previous incubation of the vessel with rFVIIa resulted in a significant increase in fibrin deposition on the damaged areas (55.0 $\pm$ 10.4% vs 5.2 $\pm$ 4.9%; n=4, P<0.01), but no significant changes in the percentage of the surface covered by platelets were detected (14.5 $\pm$ 3.8% vs 11.5 $\pm$ 2.2%) (Figure 3B).

All these results are summarized in a bar diagram (Figure 4).

DISCUSSION

Our experimental results indicate that rFVIIa traffics through EC and redistributes into endothelial and...
subendothelial compartments. Interestingly, our data reveal that rFVIIa reaches the subendothelium, where it remains functional and can still promote hemostasis as shown in the flow model. Redistribution of rFVIIa into vascular compartments may explain the prolonged action of rFVIIa in the prophylaxis of bleeding disorders in some clinical conditions [14–16].

Previous studies reported that the presence of rFVIIa potentiates coagulation and thrombus formation [9, 10]. This effect is probably related to an increase in thrombin generation through a bypassing activity [6, 7]. Several studies have shown that rFVIIa caused a local increase in procoagulant action on the subendothelium under several hemostatic dysfunctions [8–10, 24, 26, 27]. All this evidence indicates that the mechanism of action of rFVIIa under active bleeding situations may mainly be driven by an increase in fibrin and an improvement in thrombus formation on vascular damaged vessels where tissue factor is exposed. Notwithstanding, the previous mechanism would not explain the beneficial action observed in prophylaxis [15, 16].

Recently, it has been hypothesized that rFVIIa could diffuse to the extravascular compartment, increasing the local concentration of rFVIIa available at the site of injury and favoring the formation of the TF–rFVIIa complex [19]. It has already been reported that rFVIIa is able to bind to endothelial cell protein C receptor (EPCR) with similar affinity to protein C or activated protein C (APC) [28, 29]. These authors suggested that this binding would facilitate the internalization of FVIIa into the EC. Further studies have suggested that rFVIIa bound to EPCR would reduce the procoagulant activity associated with the FVIIa–TF complex [30]. The latter findings indirectly imply that the rFVIIa present in EC would have a limited role in promoting procoagulant activities.

The results from our present investigations prove trafficking of FVIIa to the subendothelium through endothelial cells. Our studies indicate that rFVIIa becomes internalized by the EC and reaches deeper areas of the vasculature such as the subendothelium. Although our immunocytochemical studies show an increased presence of rFVIIa in the EC cytoplasm after a short exposure (2 h), prolonged incubations (for 24 h) resulted in further relocation of rFVIIa into the EC towards the cell periphery and nucleus. It is interesting to note that this observation has not been communicated previously by others. However, there is evidence that EPCR can translocate from the plasma membrane to the nucleus, even bound to APC, where it redirects gene expression [31]. Therefore, if rFVIIa is able to bind to EPCR, it is plausible to find rFVIIa in the nucleus. Moreover, it has been suggested that the FVIIa–TF complex may play an active role in the modulation of gene expression [32]. Further studies are required to understand the physiological and clinical implications of this process during rFVIIa treatment.

Interestingly, the rFVIIa redistributed in the subendothelial compartments remains in an active form, as demonstrated by its ability to promote fibrin generation on damaged vessels (Figure 3). These data suggest that, in contrast to the inactivation of the procoagulant action of rFVIIa into EC anticipated by earlier studies [30], a portion of the rFVIIa that reaches the subendothelium is still capable of promoting and enhancing hemostatic activity. It is not surprising that the presence of the TF–rFVIIa complex in deeper damaged areas could also facilitate the formation of a more stable fibrin structure, resistant to fibrinolytic mechanisms, as has been suggested in previous studies [24, 33]. It is known that the presence of rFVIIa normalizes fibrin clot permeability, improving network structure [34].

In summary, our present studies in a homologous human experimental system demonstrate that rFVIIa could redistribute into endothelial cells and subendothelial compartments, where it would still retain its hemostatic activity. The redistribution of rFVIIa into
vascular compartments may explain the prolonged prophylatic action of rFVIIa under some clinical conditions and provide a mechanism of action to explain the effectiveness of rFVIIa observed in prophylaxis. Notwithstanding, the implications of our findings for improving hemostasis in a clinical setting must be confirmed in patients with hemostatic disorders. The existence of an additional reservoir of rFVIIa may provide new insights into the physiological and pathological implications of FVIIa in hemostasis.

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REFERENCES