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Factor VII deficiency: Unveiling the cellular and molecular mechanisms underlying three model alterations of the enzyme catalytic domain

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ABSTRACT

Activated factor (F) VII is a vitamin K-dependent glycoprotein that initiates blood coagulation upon interaction with tissue factor. FVII deficiency is the most common of the rare congenital bleeding disorders. While the mutational pattern has been extensively characterized, the pathogenic molecular mechanisms of mutations, particularly at the intracellular level, have been poorly defined. Here, we aimed at elucidating the mechanisms underlying altered FVII biosynthesis in the presence of three mutation types in the catalytic domain: a missense change, a microdeletion and a frameshift/elongation, associated with severe or moderate to severe phenotypes. Using CHO-K1 cells transiently transfected with expression vectors containing the wild-type FVII cDNA (FVIIwt) or harboring the p.I289del, p.G420V or p.A354V-p.P464Hfs mutations, we found that the secretion of the FVII mutants was severely decreased compared to FVIIwt. The synthesis rate of the mutants was slower than the FVIIwt and delayed, and no degradation of the FVII mutants by proteasomes, lysosomes or cysteine proteases was observed. Confocal immunofluorescence microscopy studies showed that FVII variants were localized into the endoplasmic reticulum (ER) but were not detectable within the Golgi apparatus. These findings suggested that a common pathogenic mechanism, possibly a defective folding of the mutant proteins, was triggered by the FVII mutations. The misfolded state led to impaired trafficking of these proteins causing ER retention, which would explain the low to very low FVII plasma levels observed in patients carrying these mutations.

1. Introduction

Inherited factor (F) VII deficiency is the most common among rare congenital bleeding disorders with an estimated prevalence of between 1:300,000 and 1:500,000 [1,2]. It has an autosomal recessive pattern of inheritance and clinically it varies ranging from lethal to mild bleeding or even asymptomatic forms [2–4]. The mutational pattern of the *F7* gene is heterogeneous (see http://www.hgmd.cf.ac.uk/ac/index.php). Between 70 and 80% of the disease-associated mutations are represented by missense mutations associated with reduced FVII plasma levels in patients [3]. The remainder is caused by nonsense mutations, deletions, or splicing site changes [5]. Even though the mutational pattern of FVII deficiency is well characterized and the functional aspects of some of the mutant FVII proteins have been explored, the role of intracellular processing of mutant FVII molecules has only been investigated for a few mutations [6–9]. A nonsense mutation in the carboxy-terminal FVII domain induced an impaired secretion of the mutant protein with retention in the endoplasmic reticulum (ER), which demonstrated that the carboxy-terminal region of FVII is essential for FVII secretion [9]. In another report a compound heterozygous point mutation in the central hydrophobic core of the signal peptide and nonsense mutation in the carboxy-terminal domain, induced defective

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trafficking of the mutant FVII with retention in ER and extensive intracellular degradation [8].

It is known that in eukaryotic cells nearly one third of the newly synthesized proteins are targeted to the ER, which represents the first step in the transport of these proteins to other organelles or to the extracellular space [10]. In the ER the folding of nascent proteins and post-translational modifications, which are important steps for proper folding and function of the proteins, are guided by resident enzymes and molecular chaperones [10–12]. Since only correct folded proteins can leave the ER, proteins that fail to adopt their native state are retained and eventually targeted for ER-associated degradation [11,13,14].

For other blood coagulation disorders, it was previously shown that mutations may cause either impaired transport of the protein from the ER to the Golgi [15] or excessive intracellular degradation [16,17]. Accordingly, the molecular mechanisms underlying the functional effect of the various mutant proteins causing blood coagulation disorders can differ between the genetic defects. Therefore, the aim of the present study was to characterize three types of mutations (p.G420V, p.I289del and A354V-p.P464Hfs) in the FVII catalytic domain in order to unveil the molecular mechanisms responsible for FVII deficiency. The p.G420V missense change is associated with undetectable FVII protein levels in plasma and severe bleeding [7]. The one-residue deletion p.I289del was found in a patient who presented severe intra-cranial bleeding at birth and undetectable levels of FVII protein in plasma (Pinotti M, Chuansumrit A, unpublished). The frame-shifted elongated variant p.A354V-p.P464Hfs, has a phenotype that ranges from asymptomatic to moderate and severe bleedings. Using CHO-K1 cells overexpressing either the FVII wild type (FVIIwt) or one of the mutants, we investigated in vitro the molecular and cellular mechanisms by which these mutations may decrease the FVII levels in the plasma of the patients. We found that the synthesis of the mutant proteins was slow and delayed and their secretion was severely affected. This indicates a possible retention of the mutant proteins in the ER resulting in impaired trafficking.

2. Material and methods

2.1. Cell cultures

Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (ATCC®CCL-61, Rockville, MD, USA). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM, Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, HyClone Thermo Scientific, Northumberland, UK), 100 IU/mL penicillin and 100 μg/mL streptomycin (Lonza).

2.2. Construction of expression vectors and transfections

A plasmid expressing FVIIwt was generated in pcDNA™ $3.1^{(+)}$ (Thermo Fisher Scientific Inc., Waltham, MA, USA) as described previously [7] and named pcDNA3-FVIIwt. To create plasmids expressing FVII variants, mutations were introduced into pcDNA3-FVIIwt using the Quick-Change site directed mutagenesis kit (Stratagen-Agilent, La Jolla, CA, USA) as described elsewhere [1,7]. For transient transfections, CHO-K1 cells were grown in 6-well plates until 80% confluency. Vitamin K1 (Sigma Aldrich, Saint Louis, MO, USA), 10 μg/mL, was then added to the medium and the cells were transfected with 2.5 μg of pcDNA3-FVIIwt, pcDNA3-p.G420V, pcDNA3-p.I289del, or pcDNA3-p.A354V-p.P464Hfs using Lipofectamine LTX Plus (Thermo Fisher Scientific) following the manufacturer's instructions. To generate cells stably expressing the FVII variants, CHO-K1 cells were grown in complete DMEM medium supplemented with 800 μg/mL of Geneticine (G-418 Thermo Fisher Scientific) for three weeks. Clones were tested for expression of FVII using quantitative RT-PCR and the clones with highest mRNA expression levels were isolated and expanded. The cell lines were maintained in complete DMEM containing 400 μg/mL of Geneticine.

2.3. Quantitative RT-PCR (qRT-PCR)

Twenty-four hour post transfection total mRNA was isolated using the MagMAX™-96 Total RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (both from Thermo Fisher Scientific). mRNA was reversely transcribed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). FVII mRNA levels were determined by the 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific) using a TaqMan Gene Expression Assay for FVII (Hs01551992_m1, Thermo Fisher Scientific) according to the manufacturer's instructions. TATA binding protein (TBP, Hs99999910_m1, Thermo Fisher Scientific) was used as endogenous control, and the Ct values of the target gene was normalized against the endogenous control cDNA. Relative mRNA level was calculated using the comparative threshold method ($2^{-\Delta\Delta Ct}$).

2.4. FVII expression levels

Twenty-four hour post transfection the culture medium was collected and the cells were washed three times with pre-chilled phosphate-buffered saline (PBS). The cells were lysed in RIPA buffer (Sigma Aldrich) containing Halt protease and phosphatase inhibitor cocktail 1X (Thermo Fisher Scientific). FVII antigen (FVIIAg) was measured in cell lysates and culture medium using the Visualize FVII antigen ELISA kit (Affinity Biologicals Inc., Ancaster, ON, Canada). Total protein concentrations in the lysates were measured using the BCA Protein Assay kit (Thermo Fisher Scientific) and FVIIAg levels were normalized against the total protein concentration of the corresponding cell lysates. Three independent experiments in duplicates were performed. FVII activity in the medium was determined using the Human FVII Chromogenic Activity Kit (Nordic BioSite AB, Täby, Sweden) according to manufacturer's instructions.

2.5. Synthesis and stability of FVII by pulse chase stable isotope labeling with amino acids in cell culture (pc-SILAC)

CHO-K1 cells were grown in SILAC DMEM medium supplemented with 10% FBS for SILAC, L-lysine 2HCL and L-arginine HCL (all from Thermo Fisher Scientific) (light medium). Proline (Thermo Fisher Scientific) was added to the medium at 200 mg/L. Cells were cultured in this pre-exchange light (L) medium until a minimum of three divisions were reached. Cells were then seeded in 6-well plates and cultured in DMEM light (L) medium until 80% confluency. After 24 h, cells were transiently transfected with pcDNA3-FVIIwt, pcDNA3-p.G420V, pcDNA3-p.I289del, or pcDNA3-p.A354V-p.P464Hfs as described above. Four hours after transfection, the light (L) medium was exchanged for heavy (H) medium containing $^{13}C_6$ $^{15}N_4$ L-arginine, $^{13}C_6$ $^{15}N_2$ L-lysine and proline at 200 mg/L (Thermo Fisher Scientific) for cells transfected with pcDNA3-FVIIwt. For cells transfected with pcDNA3-p.G420V, pcDNA3-p.I289del, or pcDNA3-p.A354V-p.P464Hfs, the light (L) medium was exchanged for medium containing $^{13}C_6$- L-arginine, L-lysine 2HCL (4,4,5,5-D4) and proline 200 mg/L (medium medium M) (Thermo Fisher Scientific). 0, 1, 2, 4, 6, 24, and 48 h after exchange of medium the cells were harvested, washed with pre-chilled PBS (Lonza) and lysed with RIPA buffer containing Halt protease and phosphatase inhibitor cocktail 1X (Thermo Fisher Scientific). Cell lysates, were collected at each time point of FVIIwt (heavy) or the FVII mutants (medium) and were mixed at equal proportions. Proteins were separated by SDS-PAGE using Mini-PROTEAN® TGX™ 10% Precast Gels (Bio-Rad, Hercules, CA, USA) and stained with Coomassie Blue (Bio-Rad). For the liquid chromatography-mass spectrometry (LC-MS), the gel bands were excised and in-gel digestion was carried out with 0.1 μg