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Characterization of mild coagulation factor VII deficiency: activity and clearance of the Arg315Trp and Arg315Lys variants in the Cys310-Cys329 loop (c170s)

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A B S T R A C T

Background and Objectives. Arginine 315 in factor VII (FVII) belongs to a solvent-exposed loop involved in direct interaction with the co-factor (tissue factor, TF), in transmission of TF-induced effects and potentially in FVIIa inactivation. Natural FVII variants at position 315 provide peculiar models for structure-function studies.

Design and Methods. We characterized a mild coagulation FVII deficiency associated with reduced FVII activity (26%) and antigen (67%). Mutations were searched by FVII gene sequencing. FVII variants were created by mutagenesis of FVII cDNA and characterized through expression in HEK293 cells followed by functional studies. FVII antigen in media was estimated by immunoassay while FVII activity was assessed by prothrombin-time based and FXa generation assays. FVII variants were injected into mice to investigate their recovery and half-life. One-way ANOVA was used to test statistical significance.

Results. The patient was double heterozygous for a novel R315W mutation and for the R304Q substitution (FVII Padua) previously demonstrated to impair TF binding. The recombinant 315W-FVII was normally expressed in medium but showed a markedly reduced coagulant function (52%) and activity towards factor X (FX) in plasma (34%). Moreover, the 315W-FVII showed significantly decreased recovery of the protein (20%) and a slightly shorter half-life (8.6 min) as compared to wt-FVII (50% and 10.7 min). We also studied the conservative R315K change that was responsible for low recovery (20%) and a decreased half-life (7 min) of a FVII variant with virtually normal FVII antigen and activity levels.

Interpretations and Conclusions. These findings suggest a dual role of R315 for FVII function and clearance, and indicate that substitutions at this position have appreciable effects on human FVII biology, compatible with residual FVII function and thus with mild FVII deficiency.

Key words: FVII deficiency, missense mutations, 170s loop, recombinant FVII variants, FVII clearance.

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Factor VII (FVII) is a vitamin K-dependent protein belonging to the serine-protease family that plays a key role in the initiation of the extrinsic pathway of blood coagulation.[1] FVII principally circulates in plasma as a zymogen form (99%) at a concentration of approximately 500 ng/mL (10 nM).[2]

Factor VII deficiency is a rare autosomal recessive disorder that is genotypically and phenotypically heterogeneous.[3-5] Cross-reactivity material (CRM$^+$) and CRMred FVII deficiencies,[6] characterized by a complete or partial disparity between the activity and antigen levels, represent valuable models for investigating FVII function and stability. FVII is converted to activated factor VII (FVIIa) by an internal peptide bond cleavage at R152-I153 (human FVII numbering) which gives rise to a light and a heavy chain (Figure 1). This activation triggers extended conformational rearrangement of the molecule, eventually leading to the organization of a catalytic triad (H193, D242, S344 of the heavy chain). Binding to its co-factor, tissue factor (TF), induces reshaping of extended loops in the serine protease domain and increases FVIIa activity.[7] The regions of FVII that are candidates to mediate TF enhancement of FVIIa activity are those directly interacting with TF (i.e. Arg304, Met306, Asp309),[8] and those transmitting TF-mediated effects,[9,10] which include residues 310-329, the 170s loop (chymotrypsin numbering). This solvent-exposed structure presents a high degree of flexibility[11,12] and is also subjected to proteolysis *in vitro* and *in vivo*, potentially leading to FVIIa inactivation.[13,14]

In this report, we describe a mild coagulation FVII deficiency, characterized by disparity between FVII activity and antigen

levels, caused by two different mutations affecting regions in the serine protease (SP) domain (Figure 1) involved in a direct interaction with TF and in transmission of TF-induced effects.

Design and Methods

Patient

The propositus is a 27-year old African-American female with mild coagulation FVII deficiency. The diagnosis was made when she was 5 years old and was found to have abnormal pre-operative laboratory values prior to a tonsillectomy. In a prothrombin (PT)-based assay using Innovin (Dade Behring, Germany) as a source of recombinant human TF, the FVII coagulant activity (FVII:c) in plasma was 26% whereas the FVII antigen level (FVII:Ag), detected by ELISA (Asserachrom Stago, Asnieres, France), was 67%. The patient does not have spontaneous bleeding episodes and has a normal menstrual cycle. Nevertheless, she has been transfused with fresh frozen plasma for surgery in the past due to abnormally low FVII:c levels. The patient gave informed consent for the study. Family members were not available for this study.

Genomic studies

The coding regions and exon-intron junctions of the FVII gene (*F7*) were sequenced as previously described.[15-18] The patient's genome was further investigated for the presence of polymorphic alleles that could modulate FVII antigen levels. In particular, we investigated the decanucleotide insertion at -323 (alleles A1 and A2) and the R353Q polymorphism, as previously described.[15-18]

Mutagenesis and in vitro expression of FVII recombinant protein

The R315K and R315W mutations were introduced into the FVII cDNA[19] cloned into the pCDNA3.1 Vector (Invitrogen, San Diego, CA, USA) using a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Forward primers for mutagenesis were as follows (underlined residues represent the mutated bases): 5'-ctgcagcagtcatggaaggtgggagac3' (R315W) and 5'-ctgcagcagtcaaagaaggtgggagac3' (R315K). The presence of mutations was confirmed by DNA sequencing of the entire FVII cDNA, thus also excluding the insertion of additional mutations. The HEK293 cell line was transfected with the pwt-FVII, p315W-FVII and p315K-FVII expression vectors using the Lipofectamine 2000 reagent (Gibco, Gaithersburg, MD, USA), following the manufacturer's instructions. Cells were grown in Dulbecco's modified Eagle's medium/F12 in the presence of 10% fetal bovine serum (FBS), 5 μg/mL vitamin-K, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Medium from transiently transfected cells was collected 48-72 hours post-transfection. Stable clones were selected out as previously described.[20] Conditioned medium was collected every 24 hours and stored at -20°C.

Functional assays

FVII clotting assay

Coagulant activity of FVII in conditioned media from stable clones (FVII:c) was determined in a PT-based assay. For each variant, two different concentrations of FVII (0.5-1 nM and 0.3-0.6 nM for 315W-FVII and 315K-FVII, respectively) in conditioned media (75 μL) were added to 75 μL of FVII-depleted plasma (Dade Behring, Germany). Clotting times were measured after the addition of 150 μL of Innovin (Dade Behring, Germany). The assay was standardized using serial dilutions of wt-FVII (100%, 80%, 50% and 40% of the wt-FVII 0.5 nM).

FXa generation assay

FXa generation activity of FVII variants in media from both transiently and stably expressing cells was measured essentially as described elsewhere.[21]

Transient expression

FVII proteins in medium were activated with 0.5 nM FXa at 37°C for 10 min in the presence of 30 μL of Thromborel (Dade Behring, Germany) as the source of human TF, phospholipids and calcium. The activity of the FVII variants was then tested by adding 10 nM FX and 200 μM FXa fluorogenic substrate ($MeSO_2$-D-CHA-Gly-Arg-AMCAcOH, American Diagnostica, CT, USA). Fluorescence (360 nm excitation, 465 nm emission) was monitored over time at 37°C on a SpectraFluorPlus microplate reader (TECAN, Austria).

Stable expression

FVII variants in medium were diluted (1:30) in FVII-depleted plasma. Coagulation was triggered by adding an excess of Thromborel; 200 μM FXa fluorogenic substrate were then added and fluorescence monitored over time. The assays were standardized using serial dilutions of wt-FVII.

Protein concentration

FVII in conditioned medium was concentrated using an ultra-filtration membrane PL-30 (30,000 NMWL, Amicon® Ultra-4, Centrifugal Filter Devices, Millipore Bedford, MA, USA).

In vivo recovery and half-life time of FVII mutants in mice

Experiments were conducted on C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME, USA), 6 weeks old and with an average weight of 25 grams. Concen-

Table 1. PT and FXa generation in plasma.

Recombinant FVII	*FVII:c %*	*FXa generation in plasma%*
wt	100	100 (n=4)
315W	52±8 (n=4)	34±3* (n=4)
315K	79±5 (n=4)	69±6§ (n=5)

*Values are presented as percentage ± standard deviation of activity compared to wt-FVII. *p: 0.009 (wt-FVII vs 315W-FVII); §p: 0.09 (wt-FVII vs 315K-FVII). Statistical comparisons of FXa generation assay were calculated using the raw data expressed as relative fluorescence units/min. FVII clotting assay activity of variants was calculated on a wt-FVII standard curve.*

trated medium from untransfected HEK 293 cells was initially introduced by tail injection into 4 mice to exclude its potential toxic effects. For each FVII variant, four mice were subsequently injected with 200 μL of physiological solution containing 5 μg of the concentrated protein. Blood samples (100 μL) were collected at 5, 15, 30, and 60 min post-injection into capillary tubes containing heparin by retro-orbital bleeding. FVII antigen levels were detected by an ELISA method (Asserachrom Stago) which does not cross-react with murine and bovine FVII. FVII:Ag values at 5 minutes post-injection was used to evaluate the protein recovery. FVII:Ag values at the following time points were considered for the estimate of the half-life of FVII.

Data analysis

One-way ANOVA was used to test the significance of all assays. A $p \leq 0.05$ was considered statistically significant. Statistical comparisons of FVII activity in fluorogenic assays (see FXa generation assay, Table 1) were calculated using the raw data expressed as relative fluorescence units/min. Half-life (Figure 2) was determined using Origin software (Origin laboratory, Northampton, MA, USA).

Results

Coagulation and genomic studies

Sequence analysis of PCR fragments encoding exon 8 of the patient's *F7* gene revealed two point mutations (Figure 1), determining the substitution by glutamine for arginine at position 304 (FVII Padua)[22] and by tryptophan for arginine at position 315. While the first is an extensively investigated and frequent mutation, the second is a novel one in human *F7*.[5] We also found that the patient was homozygous for the most frequent alleles (353R and A1, no insertion) of two polymorphisms, R353Q in exon 8[23] and a decanucleotide insert at position -323 in the 5' flanking region of the *F7* gene.[24]

The much lower FVII:c values (26%) than those expected for FVII-deficient heterozygotes, and the absence of FVII-lowering alleles, indicate the presence of allelic mutations in the patient, and thus a possibly

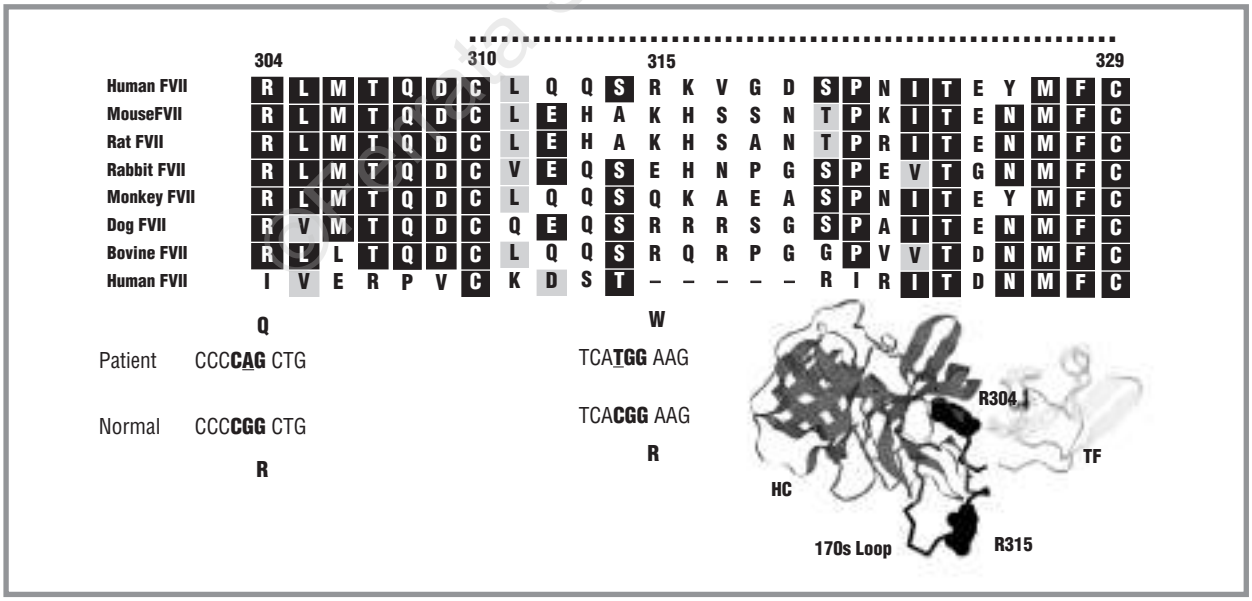

Figure 1. Sequence and structure of the 170s loop. Top: Alignment of the 304-329 region of the mammalian FVII and human FII heavy chain (HC). White on black indicates identical residues; black on gray indicates similar or conserved residues; black on white indicates no similarity. Residues belonging to the 170s loops are indicated with a dotted line. Bottom left: Nucleotide and amino acid changes in the patient at positions 304 and 315. Nucleotide changes are underlined. Bottom right: Crystallographic structure[33] of human FVIIa HC (dark gray) and tissue factor (TF, light gray). 304-329 region, black backbone structure; residues 304 and 315, black spacefill structure.

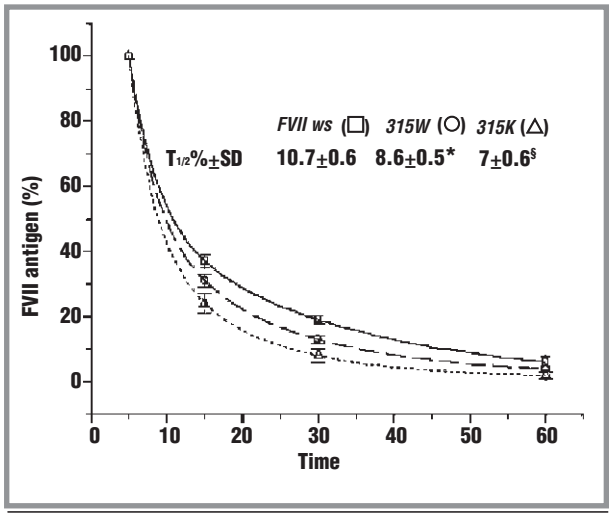

Figure 2. Clearance of recombinant FVII in mouse. Antigen levels measured 5 minutes post-injection were considered as 100%. Wt-FVII (□), 315W-FVII (○) and 315K-FVII (△) variants are indicated with a solid, dashed and dotted line, respectively. Values are given as mean and standard deviation obtained in four animals. The inset reports the mean half-life ± standard deviation of recombinant proteins. *p = 0.01 (wt-FVII vs 315W-FVII); §p < 0.001 (wt-FVII vs 315K-FVII); *§p= 0.014 (315W-FVII vs 315K-FVII).

compound heterozygous condition for R304Q and R315W mutations.

Recombinant FVII expression

While the phenotype associated with the 304Q-FVII has been previously reported by several groups,[25-27] the contribution of the R315W substitution to the propositus' phenotype was investigated through functional assays of the recombinant molecule. In addition, the R315K-FVII was also studied to characterize the effect of a conservative substitution on human FVII activity and stability. Wt-FVII, 315W-FVII and 315K-FVII variants were transiently expressed in HEK 293 cells and FVII levels were measured as FXa generation activity. In independent experiments (n≥3), FXa generation activity levels of the 315W-FVII (106±13%) and of the 315K-FVII (94±29%) variants in conditioned medium were not statistically different from that of wt-FVII, thus suggesting that the substitutions had a negligible impact on FVII biosynthesis. To provide a more reliable estimate of the FVII specific activity of variants, highly expressing stable clones were selected with G418 and expanded. Levels of secreted FVII in media, determined by ELISA, were 0.9 μg/mL, 1.4 μg/mL and 0.8 μg/mL for the wt-FVII, 315W-FVII and 315K-FVII, respectively. FVII in media from stable clones were used for further functional characterization of variants.

FVII clotting activity

The procoagulant FVII activity was assessed in PT assays based upon normalization of antigen levels. As compared to wt-FVII, the clotting activity of the 315W-FVII and 315K-FVII recombinant variants, tested in duplicate at different concentrations, were 52±8% and 79±5%, respectively (Table 1).

FXa generation in plasma

The activity of recombinant FVII toward its physiological substrate, FX, was investigated in a plasma system. As summarized in Table 1, the 315W-FVII variant showed activity values significantly lower (34±3%) than that of wt-FVII in at least 4 different assays, whereas the activity of 315K-FVII variant (69±6%) was indistinguishable from that of wt-FVII. These data paralleled those obtained in the PT assays.

Recovery and half-life in mice

To provide information on mutant FVII stability *in vivo*, clearance of recombinant proteins was studied in mice. For these experiment, FVII from stable cells was concentrated to a final antigen level of 119 μg/mL for wt-FVII, 94.6 μg/mL for 315W-FVII, and 149.5 μg/mL for 315K-FVII. Normal mice were injected with 5 μg of FVII in medium and antigen levels were monitored over time (Figure 2). At 5 minutes post-injection, the recovery of the wt-FVII in mouse plasma was 905±120 ng/mL which, in a total blood volume of approximately 2.5 mL, corresponds to 50% of the injected FVII. The recovery of the 315W-FVII (434±100 ng/mL, <20%) and 315K-FVII (402±50 ng/mL, <20%) variants was significantly less than that of the wt-FVII (p<0.003). In addition, the half-life of wt-FVII (10.7±0.6 min) appeared to be slightly longer than that of the 315W-FVII (8.6±0.5 min) or the 315K-FVII (7±0.6 min) mutant.

Discussion

In this report, we describe a mild coagulation FVII deficiency caused by a probable compound heterozygous condition for R304Q and R315W mutations, affecting regions (Figure 1) of the SP domain involved in direct interactions with TF, in transmission of TF-induced effects and potentially in FVIIa proteolytic inactivation.[13,14] Functional features of these regions make these mutations, particularly the R315W, interesting models for investigating determinants of FVII activity and antigen levels. The R304Q mutation[25-27] has been reported in more than 15 patients from different countries.[5] Two other variations, R304W[28] and R304E (*IRF7 database, F.B. personal communication*) have been described, indicating that codon 304 (CGG), containing a CpG site, is

one of the hot spot mutation sites in the *F7* gene. Differently, natural mutations at residue R315 have not previously been identified, even if its codon (CGG) represents another potential hot spot mutation site. Interpretation of the phenotype-genotype relationships in this patient was assisted by comparison with previous findings in subjects carrying the R304Q substitution, and by the expression of the recombinant 315W-FVII, functional assays in human plasma and clearance studies in mice. All changes at position 304 have been found to cause a CRM^+ phenotype, indicating that they do not significantly alter the biosynthetic and secretion processes. Since 304Q-FVII interacts directly with TF,[8] its substitution causes a conformational change in the FVII molecule resulting in reduced TF affinity and coagulant activity.[22] A review of the literature[5] together with the evaluation of the International Registry of FVII Deficiency (*F.B., unpublished results*) indicate that homozygotes or heterozygotes for the R304Q substitution have normal FVII antigen levels, and median FVII activity levels of 18% and 46%, respectively. Although these values must be considered cautiously, they suggest that the vast majority of FVII antigen in patient's plasma, as well as a proportion of the FVII coagulant activity, derive from the 304Q-FVII.

In accordance with this estimate, the R315W change should be responsible for a pronounced reduction in FVII antigen and activity. As in other doubly heterozygous patients, expression studies are indispensable to corroborate the inferred features of the 315W-FVII molecule. The PT and FXa generation activities in plasma obtained with recombinant 315W-FVII (52% and 34%, respectively) are clearly consistent with the predicted reduction in specific activity. Clearance studies in mice revealed a significantly decreased recovery and a slightly shorter half-life as compared to wt-FVII. On the other hand, the expression levels of 315W-FVII in conditioned medium, similar to or even higher than those of wt-FVII, suggest that this change does not influence biosynthesis of the mutant FVII in eukaryotic HEK 293 cells.

To characterize the relevance of a positive charge at position 315 for FVII activity and stability, the conservative R315K change was also studied. The 315K-FVII variant showed procoagulant and FVII activity towards FX that was comparable to those of wt-FVII, but a low recovery and a decreased half-life, even lower than that of the natural 315W-FVII variant. Half-life and recovery values of FVII variants and wt-FVII were reproducibly obtained using FVII concentrated in conditioned media. Although it is desirable to perform *in vivo* studies with purified protein, the recovery of wt-FVII (50%) was similar to that determined in rats (40%) and humans (~44%) following injection of recombinant FVIIa.[29,30]

The simplified approach used in the present work could be exploited to investigate other FVII mutants needed to study determinants of FVII clearance, an aspect poorly known but of interest for treatment of coagulation disorders.[31,32] However, the study in normal mice does not enable us to estimate the decay of recombinant FVII activity, which could be even more pronounced than that of antigen. As shown in the crystallographic structure of FVIIa,[33] arginine 315 is one of the most exposed residues of the flexible 310-329 loop.

Taken together our findings suggest a dual role of R315 for human FVII function and clearance. R315 is one of the five residues, peculiar to only the mammalian FVII 170s loop (Figure 1),[34] conferring a high degree of flexibility to this region of the catalytic domain.[9-12] The extreme substitution of the positively charged R315 with the hydrophobic tryptophan in this loop only partially decreases FVII activity, in accordance with the wide variability at this position (E, Q, K, R) in mammalian FVII molecules.[34,35] The virtually normal function of the 315K-FVII supports the role of the positive charge for complete activity of human FVII.

Moreover, alterations of the R315 could also influence the stability of the molecule in plasma by removing a proteolytic site potentially involved in FVIIa inactivation.[13,14] However, our experiments suggest that both substitutions are responsible for decreased recovery and increased clearance of the recombinant human FVII in mice (Figure 2). It is worth noting that the 315K-FVII mutant, bearing the topologically equivalent residue present in mouse FVII (Figure 1), showed a half-life similar to or shorter than that caused by the non-conservative substitution R315W. Increased clearance of these variants makes other mutants at this position unlikely candidates for being FVII molecules with increased stability for therapeutic purposes. In conclusion our data indicate that both conservative and non-conservative substitutions of R315 have appreciable effects on human FVII biology and are compatible with residual FVII function and thus with mild FVII deficiency.

CFF: b; RT: b; ESP: patient provider and helpful discussion; VRA: experiment in mice and helpful discussion; MP: a, b; FB: a, b, c. The authors reported no potential conflicts of interest.

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