Thrombosis Research 125 (2010) 262–266

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Impaired secretion of carboxyl-terminal truncated factor VII due to an *F7* nonsense mutation associated with FVII deficiency

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ARTICLE INFO

Article history:
Received 24 June 2009
Received in revised form 7 September 2009
Accepted 18 September 2009
Available online 12 October 2009

Keywords:
FVII deficiency
F7 mutation
C-terminal truncation
Impaired secretion

ABSTRACT

Introduction: Factor VII (FVII) is a vitamin K-dependent glycoprotein secreted into the blood circulation from hepatic cells. We investigated the molecular basis of the congenital FVII deficiency found in a Japanese patient.
Materials and Methods: We analyzed the *F7* gene of the patient, who was diagnosed with a FVII deficiency at pregnancy. We expressed a carboxyl-terminal truncated FVII (Arg462X FVII) corresponding to the identified mutation in CHO-K1 cells. To study roles of the carboxyl-terminus in the secretion of FVII, we also expressed a series of recombinant FVIIs deleted of limited numbers of carboxyl-terminal amino acids (462Arg-466Pro).
Results: We identified a nonsense mutation (c.1384C>T: p.Arg462X) in *F7*, leading to a lack of five amino acids in the carboxyl-terminus. In expression experiments, Arg462X FVII was undetectable not only by Western blotting, but also by ELISA. A Western blot analysis of the truncated FVIIs revealed that all mutants were expressed in the cells the same as the wild type, but were secreted into the culture medium in lesser amounts than the wild type depending on the length of the deletion, which was confirmed by ELISA. Arg462X FVII did not colocalize with the Golgi on immunofluorescence staining, suggesting that it might be retained in the ER and degraded in the cell.
Conclusion: The carboxyl-terminal amino acids of FVII play an important role in its secretion, and the p.Arg462X mutation was likely to have caused the FVII deficiency in this patient.

Introduction

Factor VII (FVII), a vitamin K-dependent plasma glycoprotein, is synthesized in the liver and secreted into the blood as a single-chain zymogen. Mature plasma FVII is composed of 406 amino acid residues with an apparent molecular weight of 50,000 and is present at a concentration of 500 ng/ml in normal plasma [1,2]. Upon vascular injury and in the presence of calcium, FVII forms a one-to-one stoichiometric complex with its cell surface receptor and cofactor, tissue factor (TF). Once in a complex with TF, FVII is rapidly cleaved to its active form, FVIIa, and converts zymogen factor IX and factor X into active enzymes [3,4]. The formation of an active complex between TF and FVIIa is widely thought to be the primary stimulus for blood coagulation.

The gene for FVII has been cloned and completely sequenced [5]. It is located on chromosome 13 (13q34), just 2.8 kb upstream of the factor X gene, and comprises 9 exons spanning about 12.5 kb. The FVII gene shares a common intron-exon structure with the genes of several other vitamin K-dependent blood coagulation proteins further suggesting they evolved through gene duplication events.

Hereditary FVII deficiency is a rare autosomal recessive bleeding disorder with variable clinical expression [6], and has an estimated incidence of 1 per 500,000 in the general population [7]. The hemorrhagic diathesis in affected patients can be highly variable, and does not necessarily correlate with plasma FVII activity levels [8]. Considering the poor relationship between FVII activity and the bleeding tendency, a molecular diagnosis is helpful in unsolved cases or in cases in which the pattern of inheritance is not clear. To date, extensive genetic analyses of patients with FVII deficiency have identified many causative mutations in the FVII gene (*F7*) (HMGD; http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F7).

Abbreviations: FVII, factor VII; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; TF, tissue factor; PT, Prothrombin time; APTT, activated partial thromboplastin time; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PVDF, polyvinylidene difluoride; PDI, protein disulfide isomerase; FITC, fluorescein isothiocyanate; FIX, factor IX; PC, protein C.

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doi:10.1016/j.thromres.2009.09.014

In the present study, we identified a nonsense mutation in the *F7* gene of a Japanese patient deficient in FVII, and investigated the molecular consequence of the mutation by conducting *in vitro* expression experiments.

Materials and Methods

Preparation of samples and coagulation tests

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine. A blood sample was taken from the patient and collected in a 1/10 volume of 3.2% (w/v) trisodium citrate, after informed consent was obtained. The plasma was then separated by centrifugation at 2000×*g* for 20 min, and aliquots were stored at -70 °C until use. Genomic DNA was isolated from the peripheral blood leukocytes by phenol extraction as described previously [9]. Prothrombin time (PT), activated partial thromboplastin time (APTT), and FVII procoagulant activity (FVII: C) were measured as described [10].

Direct sequencing of DNA

All exons and splice junctions of the *F7* gene were amplified by the polymerase chain reaction (PCR) using the gene-specific primers listed in Table 1. The amplification and Cycle sequencing of all PCR products were performed as described previously [11].

PCR-restriction fragment length polymorphism (RFLP) analysis

To confirm the mutation (c.1384C>T) identified in *F7* of the patient, PCR-restriction fragment length polymorphism (RFLP) was performed. Since the mutation did not create any available restriction enzyme sites, we used a mismatched antisense primer (5'-CTGCTGG GCTAGGGAAATGGtGaTC-3'; substituted bases are underlined small letters) to introduce a *Bcl*I restriction site into the products amplified from the mutant allele. The PCR products were then treated with *Bcl*I and electrophoresed on a 2.0% agarose gel.

Construction of recombinant FVII expression vectors

We prepared a wild-type FVII expression vector (pcDNA-FVIIWT) by introducing a FVII cDNA into the vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) as described previously [10]. To prepare expression vectors producing a series of recombinant FVIIs with limited deletions of the carboxyl-terminal sequence (Arg462X, Ala463X, Pro464X, Phe465X and Pro466X), we performed site-directed mutagenesis by PCR using the primers listed in Table 2. The PCR-amplified DNA fragments were isolated as *Sac*II/*Apa*I fragments, and inserted into pcDNA-FVIIWT. The sequences of all FVII expression vectors were confirmed by direct sequencing.

Table 1
Oligonucleotide primers for amplifying the FVII gene.

Exon	Strand	Oligonucleotide Sequence	Product (bp)	Annealing (°C)
1a	S	5'-CACACCTTAACACCTGCACGCT-3'	392	64
	AS	5'-GCCCACTGCCCTTCCACC-3'		
1b	S	5'-GGGGTGGGCTGTGAGGGA-3'	225	68
	AS	5'-ATGGGAGGGGAAGGAGGTGA-3'		
2	S	5'-CAGCGCCGCTCCCCTCCTC-3'	339	65
	AS	5'-TTCACCGCCGCCGTGCAG-3'		
3,4	S	5'-GGTGTGTCCAGTGCTTACCGTT-3'	344	69-64*
	AS	5'-GGCCACCTCCACCAGCTC-3'		
5	S	5'-ACAGCTCATGCCACCTTCC-3'	350	62
	AS	5'-CCAGTCCCACCCGTCTTT-3'		
6	S	5'-GGCCTCTCAGAGGATGGGT-3'	284	62
	AS	5'-TCCCTTCCAGAGCTGTTGTT-3'		
7	S	5'-GGCGAGTCATCAGAGAAACAA-3'	250	60
	AS	5'-GTCGGACAGGGAAGAGTGG-3'		
8-1	S	5'-GTGGCAGGTGGTGGAAAG-3'	394	64
	AS	5'-TTCGTGATATTTGGGGAGT-3'		
8-2	S	5'-CTGGAGCTCATGGTCCTCAA-3'	393	62
	AS	5'-CCAGGACAGTTCGACGCAG-3'		

*69-64: Annealing temperature of touchdown PCR.

Table 2
Oligonucleotide primers for the construction of recombinant FVII cDNA.

	Mutants	Oligonucleotide Sequences
Sense		5'-CCTGATCAACACCATCTGGGTGGTCT-3'
Antisense	pcDNA-Arg462XFVII	5'-GCTGGGCccGGGAAATGGGGCTCaCA-3'
	pcDNA-Ala463XFVII	5'-GGGCTGCTGGGCccGGGAAATGGtcaTCGCA-3'
	pcDNA-Pro464XFVII	5'-GCTGCTGGGCccGGGAAATcaGGCTCGCA-3'
	pcDNA-Phe465XFVII	5'-TGCTGGGCccGGGtcATGGGGCTCGCA-3'
	pcDNA-Pro466XFVII	5'-GCTGGGCcctcaAAATGGGGCTCGCAGG-3'

Small letters are mismatched nucleotides.
Single- and double-underlines indicate the *Apa*I site and stop codon sequence created by the nucleotide substitution, respectively.

Cell culture and recombinant FVII expressions

Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 10 mg/ml of vitamin K_1 (Isei, Yamagata, Japan) at 37 °C in a humidified atmosphere containing 5% CO_2. The transient expression of recombinant FVIIs was achieved by transfection with 20 μg of each expression vector in 100 mm culture plates using the Ca-phosphate method as described previously [12]. The cells were incubated for 24 hrs in serum-free medium containing 10 mg/ml of vitamin K_1. The cell lysate dissolved in Reporter Lysis Buffer (Promega, Madison, WI) and the culture medium concentrated with a Centrisart I kit (Sartorius, Goettingen, Germany) were subjected to Western blotting.

We also established stable transformants expressing recombinant FVIIs as described previously [13]. The media were collected from confluent plates grown for 24 hrs in serum-free medium containing 5 mg/ml of vitamin K_1 and centrifuged at 12,000×*g* for 20 min at 4 °C, and the supernatants were used for ELISA as described below. The protein concentrations of the samples were determined with a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA), using bovine serum albumin as a standard.

Western Blotting Analysis

The samples (15 μg) of cell lysates and culture medium containing the respective recombinant FVIIs were separated by SDS-PAGE (10%), and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ). FVII proteins were detected by immunostaining with a polyclonal rabbit anti-FVII (Nordic Immunological laboratories, Tilburg, The Netherlands) and a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Cell Signaling Technology Inc., Danvers, MA), using an ECL PLUS Western blotting detection system (Amersham Biosciences).

Enzyme-linked immunosorbent assay (ELISA)

To measure concentrations of the recombinant FVIIs in the culture media, an enzyme-linked immunosorbent assay (ELISA) was performed as described previously [14]. A monoclonal anti-FVII antibody (Innovative Research Inc., Minneapolis, MN) was used for capture and a peroxidase-conjugated polyclonal anti-FVII antibody (Innovative Research Inc.) was used for detection, and the immunosorbent signals were detected by color development, using *o*-phenylenediamine