Thrombosis Research 125 (2010) 438–443

Contents lists available at ScienceDirect

Thrombosis Research

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

Regular Article

Factor XII Ofunato: Lys346Asn mutation associated with blood coagulation factor XII deficiency causes impaired secretion through a proteasome-mediated degradation

Keijiro Suzuki [a,b,*], Kazunori Murai [c], Akira Suwabe [a], Yoji Ishida [c]

[a] Department of Laboratory Medicine, Iwate Medical University School of Medicine, Morioka, Iwate, Japan
[b] Advanced Medical Research Center, Iwate Medical University School of Medicine, Morioka, Iwate, Japan
[c] Department of Hematology/Oncology, Iwate Medical University School of Medicine, Morioka, Iwate, Japan

ARTICLE INFO

Article history:
Received 3 August 2009
Received in revised form 30 October 2009
Accepted 2 December 2009
Available online 22 December 2009

Keywords:
Factor XII deficiency
Mutation
Proteasome
Expression study
Blood coagulation

ABSTRACT

Introduction: Congenital blood coagulation factor XII (FXII) deficiency is a rare coagulation disease and an autosomal recessive trait. It is found by chance in many cases. We identified a novel mutation (Lys346Asn) in the FXII gene of a patient with FXII deficiency, designated as Factor XII Ofunato.
Methods: The proband was a 75-year-old Japanese woman with a prolonged activated partial thromboplastin time (52.8 s). The FXII activity and antigen were greatly reduced (activity, 5%; antigen, 4.5%). We analyzed FXII gene of this patient using a direct sequencing method and characterized mutant FXII through *in vitro* expression studies.
Results: Sequence analysis of the FXII gene revealed a G→C point mutation at nucleotide 9845, resulting in Lys346 (AAG)→Asn (AAC) replacement in the catalytic domain. Expression studies in Chinese hamster ovary cells demonstrated that mutant FXII (346 N-FXII) showed a lower level of accumulation in the cells than wild-type. Secretion of 346 N-FXII was greatly reduced in culture medium. We also investigated mRNA expression levels of wild-type and 346 N-FXII in transfected cells using quantitative RT-PCR. Both mRNA expressions were equivalent levels. Pulse–chase experiments showed that 346 N-FXII was extensively degraded intracellularly compared to wild-type. Using membrane-permeable inhibitors, we observed that degradation occurred in the pre-Golgi compartment and that proteasome apparently plays a central role in this process.
Conclusions: These results show that most 346 N-FXII is degraded intracellularly through endoplasmic reticulum-associated degradation as the protein quality control system, resulting in an insufficient secretion phenotype.

Introduction

Human blood coagulation factor XII (FXII) is a circulating precursor of a multiple functional serine protease, which is a single chain 80 kDa glycoprotein comprising 596 amino acid residues. It plays a role in blood coagulation, fibrinolysis, and the complement system [1,2]. The protein structure comprises a COOH terminal catalytic domain, Type I and Type II domains, two growth factor domains, and a kringle domain. On a negatively charged surface, cleavage by kallikrein at Arg353–Val354 generates activated FXII (FXIIa), thereby initiating the rapid intrinsic pathway by converting blood coagulation factor XI (FXI) to activated FXI [3,4]. The FXII gene is 12 kb, consisting of 14 exons and 13 introns, and is located at 5q33–qter [4,5].

Congenital FXII deficiency is a rare coagulation disease and an autosomal recessive trait. It is found in many cases by chance through a prolonged activated partial thromboplastin time (APTT). Despite the extremely prolonged APTT, patients of FXII deficiency show no readily apparent clinical symptoms such as a tendency to bleed [6,7]. Several reports in the relevant literature describe that FXII deficiency is associated with thrombotic tendencies such as thromboembolism and myocardial infarction [8–10]. However, the relevance of FXII deficiency to a thromboembolic state remains controversial [11,12]. Factor XII deficiency is fundamentally divisible into three categories: a cross-reacting material (CRM)-positive group, a CRM-negative group, and a CRM-reduced group. In fact, CRM-positive patients have

Abbreviations: APTT, activated partial thromboplastin time; Arg, Arginine; Asn, asparagines; ASRA, Allele-specific restriction enzyme analysis; cDNA, complementary deoxyribonucleic acid; CHO, Chinese hamster ovary; CRM, cross-reacting material; Cys, cysteine; DMEM, Dulbecco's modified Eagle medium; DNA, deoxyribonucleic acid; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FBS, fetal bovine serum; FXI, blood coagulation factor XI; FXII, blood coagulation factor XII; FXIIa, activated blood coagulation factor XII; Gln, glutamine; Gly, glycine; GRP, glucose-regulated protein; HRP, horseradish peroxidase; Leu, leucine; Lys, lysine; Met, methionine; mRNA, messenger ribonucleic acid; PBS, phosphate buffered saline; PCN, Protein C Nagoya; PCR, polymerase chain reaction; Pro, proline; PVDF, polyvinylidene difluoride membranes; RNA, ribonucleic acid; RT, reverse transcription; SDS, sodium dodecyl sulfate; Ser, serine; Trp, tryptophan; Tyr, tyrosine.

* Corresponding author. Department of Laboratory Medicine, Iwate Medical University School of Medicine, 19-1 Uchimaru, Morioka, Iwate 020-8505, Japan. Tel.: +81 19 651 5111x3249; fax: +81 19 624 5030.
E-mail address: ksuzuki-th@umin.ac.jp (K. Suzuki).

0049-3848/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2009.12.004

considerable amounts of FXII protein in their plasma, but the protein is nonfunctional. In contrast, FXII antigen is not detected in CRM-negative patients. Patients with CRM-reduced FXII have reduced plasma antigen levels, irrespective of activity levels. Several genetic abnormalities of FXII deficiency have been described in previous reports. Reportedly, CRM-negative FXII-deficient patients are mainly associated with an additional *Taq*I restriction site in intron B (T→C transition at 224 bp upstream of exon 3) [13], which is well known as the Hageman trait. It has been shown to be associated with a mutation in the 5′-flanking region of the gene, exon 1: -8 (G→C) [14]. Recently, other mutations in the FXII gene of CRM-negative and reduced FXII-deficient patients have been identified. Schloesser et al. reported an acceptor splice site mutation of exon 14 [15] and three mutations (Leu395Met, Arg398Gln and a single base pair deletion in exon 12) [16] in unrelated CRM-negative FXII-deficient patients. Actually, FXII Tenri (Tyr34Cys) [17], Arg123Pro, Gly421Lys [18], Tyr486Cys [19,20], and Ala392Thr [21] characterized the molecular etiology through expression studies. In CRM-positive patients, FXII Washington D.C. (Cys571Ser) [22], FXII Locarno (Arg353Pro) [23], and Asp442Asn and Gly570Arg [16] are reported.

Herein, we report a Japanese patient of CRM-reduced FXII deficiency with a novel amino acid substitution (Lys346Asn) in the catalytic domain, which was associated with marked reduction of plasma FXII activity and antigen level, but these levels were detectable. We designated this mutation as Factor XII Ofunato. Moreover, *in vitro* expression studies and pulse–chase analyses revealed that the synthesis mutant FXII was degraded by proteasome in the pre-Golgi compartment.

Patient and Methods

Patient characteristics

A 75-year-old Japanese woman was incidentally found to have a prolonged APTT (52.8 s) before operation for a cataract. She had no history of abnormal bleeding or thrombosis. Both FXII activity and antigen levels were determined, respectively, as 5% (one-step APTT method) and <10% (Laurell's method) of control plasma (pooled plasma of 50 healthy volunteers). Other hemostatic laboratory data were normal; lupus anticoagulant (diluted Russell's viper venom time) and circulating anticoagulant against FXII (FXII inhibitor) were both negative. Based on results of laboratory tests, we suspected that the proband was FXII deficient. Her parents, who were cousins, were deceased. Her sisters and children also had no history of abnormal bleeding. Unfortunately, we did not examine hemostatic tests of her sisters and children because we were unable to obtain their cooperation for this study. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Committee of our hospital. Informed consent was obtained from this patient.

Plasma FXII assays

The FXII activity was measured using the APTT method with FXII-deficient plasma (Instrumentation Laboratory, Lexington, MA, USA). First, FXII antigen was measured using Laurell's method [24] with goat polyclonal antibodies against human FXII (Cedarlane Laboratories Ltd., Ontario, Canada). Pooled plasma from 50 healthy Japanese volunteers was used as standard plasma (100% in activity and antigen). Plasma FXII antigen was also measured using enzyme-linked immunosorbent assay (ELISA) with unlabeled and horseradish peroxidase (HRP)-conjugated goat polyclonal antibodies to FXII (Cedarlane Laboratories Ltd.) according to the manufacturer's protocol. Purified FXII (Haematologic Technologies Inc., Essex Junction, VT, USA) provided a standard curve for ELISA plate. Samples were run as duplicates on three separate experiments.

Polymerase chain reaction (PCR) and sequencing of the FXII gene

The primer pairs for amplifying all exons and exon-intron boundaries of the FXII gene used for this study and PCR conditions were described in Kanaji et al. [18]. Then PCR amplification was performed (TaKaRa PCR Thermal Cycler Dice; Takara Bio Inc., Otsu, Japan) using *Taq* DNA polymerase (Takara Bio Inc.). The PCR fragments were sequenced using a Big-Dye terminator cycle sequencing kit and DNA sequencer (ABI Prism 377; Applied Biosystems, Foster City, CA, USA) to detect mutations in the FXII gene.

Allele-specific restriction enzyme analysis (ASRA)

The G to C substitution (see *Results* for mutation in the FXII gene) eliminates a *Hinf*I restriction site. Therefore, this mutation site can be detected using ASRA. Of the amplified products including exons 9–10 (918 bp), 5 μl was digested using *Hinf*I (Takara Bio Inc.) at 37 °C for 60 min. The digests were analyzed using electrophoresis through a 2% agarose gel and stained with ethidium bromide.

Site-directed mutagenesis and construction of the expression vector

The FXII cDNA used for this study was a gift from Dr. Ross T. A. MacGillivray (University of British Columbia, Vancouver, British Columbia, Canada). The *Not*I fragment of FXII cDNA, which contained the entire coding region, was subcloned into a polylinker site of pBluescript II KS (-) (Stratagene, La Jolla, CA, USA). Site-directed mutagenesis using Kukel's method was performed as previously described [25]. Mutagenic oligonucleotides were prepared to mutate codon 346 of the FXII from a Lys (AAG) to an Asn (AAC) (5′-GCGGCTCCGCAACAGTCTGTCTTCG-3′, underlined nucleotide showed the nucleotide position at 9845). Mutant FXII cDNA was sequenced to confirm that it contained the desired sequence without spontaneous errors. Then, *Not*I fragment of wild-type FXII cDNA (FXII-wt) or mutant FXII cDNA (K346N) was prepared and subcloned into a mammalian expression vector pcDNA 3.1+ (Invitrogen Corp., Carlsbad, CA, USA).

Transient expression of FXII-wt and K346N in CHO-K1 cells

The CHO-K1 cells were grown in nutrient mixture F-12 Ham (Sigma-Aldrich Corp., St. Louis, MO, USA) with 10% fetal bovine serum (FBS) under 5% CO_2. They were transiently transfected with FXII-wt and K346N. Transfection was performed using 10 μl of LipofectAMINE 2000 (Invitrogen Corp.) and 4 μg of plasmid DNA (FXII-wt, K346N and pcDNA3.1+ alone) in six-well culture dishes under the manufacturer's recommendation. After 24 h incubation, 1 ml of conditioned media was harvested. The cells were washed twice with ice-cold PBS and lysed in 200 μl of M-PER mammalian protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL, USA) with protease inhibitor cocktail Set I (Calbiochem-Novabiochem AG, Darmstadt, Germany).

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blot were performed as described previously [18]. Briefly, conditioned media (200 μl) were incubated with 2 μl of goat polyclonal anti-FXII antibodies and 50 μl of immobilized Protein G (Pierce Biotechnology Inc.) at 4 °C overnight. The combined beads and equal amount of cell lysates (10 μg protein) were mixed with SDS sample buffer (with 2% 2-mercaptoethanol). After boiling for 5 min, samples were applied onto 5–20% gradient SDS polyacrylamide gels and electrophoresed (SDS-PAGE). The separated proteins were transferred to polyvinylidene difluoride membranes (PVDF; GE Healthcare, Buckinghamshire, UK), blocked in 5% skim-milk/0.1% Tween-20/PBS and incubated with FXII-specific antibodies (mouse anti-FXII antibody, B7C9; Genetex Inc., Irvine, CA, USA) for 1 h. After washing, blots were incubated with HRP-conjugated anti-