Characterization of Factor XII Tenri, a Rare CRM-Negative Factor XII Deficiency

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Abstract. Factor XII Tenri (Y34C), a rare cross-reacting material (CRM)-negative factor XII deficiency, was identified in a 71-yr-old Japanese woman with angina pectoris. In the patient’s plasma, factor XII activity and antigen levels were only 1.6% and 5.0%, respectively, of those seen in a normal subject. Immunoblot analysis showed that the secreted factor XII Tenri existed not only as a monomer (76 kDa), but also in complexes with apparent molecular weights of approximately 115, 140, 190, 215, and 225 kDa. After reduction with 2-mercaptoethanol, the factor XII Tenri contained in the complexes was completely converted to monomeric form on immunoblot patterns. It appeared that some of the secreted factor XII Tenri formed several types of disulfide-linked complexes, including a factor XII-α1-microglobulin complex, through a newly generated Cys residue. The monomeric form of factor XII Tenri, like normal factor XII, was proteolytically cleaved to generate αFXIIa by plasma kallikrein, plasmin, FXIa, FXIIa, or trypsin [1,2]. Subsequently, cleavage of αFXIIa is induced at Arg 334 and Arg 343 to generate βFXIIa (28 kDa), which is enzymatically much more active than αFXIIa [7]. In vivo, coagulation evidently occurs in an environment completely different from the test tube [8-11], since persons with FXII deficiency do not bleed.

Factor XII deficiency exists as a quantitative defect [designated as a cross-reactive material (CRM)-negative deficiency, with equal levels of FXII activity and antigen concentration] and as a dysfunctional defect [designated as a CRM-positive deficiency]. Hageman trait, which is the best known

Introduction

Blood coagulation factor XII (FXII, Hageman factor), a serine protease with a molecular weight of ~76 kDa [1-3], has been considered to play a role in the initiation of blood coagulation and fibrinolysis, and also in the activation of the kinin system via kallikrein [4,5]. In vitro, FXII becomes bound to a negatively charged surface via the N-terminal region [6], and the bond between Arg353 and Val354 is
FXII deficiency, is induced by the presence of an additional Taq I restriction site (T to C substitution at position 224 bp upstream of exon 3) in intron B [12] and an associated mutation in the 5' flanking region (exon 1: -8 G → C) [13]. Some CRM-negative FXII deficiencies are known to be caused by mutations that induce abnormal splicings [14] or frameshifts [15]. Although point mutations resulting in amino acid substitutions would be expected to generate CRM-positive FXII deficiencies [15-17], some such mutations, such as R398Q and L395M [15], Q421K, and R123P [18], have also been reported to induce CRM-negative FXII deficiencies. However, most of the mechanisms responsible for the CRM-negative FXII deficiencies induced by amino acid substitutions are unknown. It is now widely accepted that no risk of bleeding is associated with FXII deficiency. On the contrary, some reports suggested that thromboembolism is frequent in patients with severe or partial FXII deficiency, as a result of an inactivation of fibrinolysis [19-23], although this has not yet been proven. Recent reviews have questioned the association of FXII deficiency with thrombosis [24,25].

A mutation that induces CRM-negative FXII deficiency, named factor XII Tenri (Tyr 34 → Cys), has been reported [26]. That study showed that in affected individuals, a trace amount of FXII was secreted and formed a disulfide-linked complex with α1-microglobulin. The authors speculated that most of the FXII Tenri was folded incorrectly in the endoplasmic reticulum (ER) due to the introduction of Cys 34, and was finally degraded intracellularly through a quality control mechanism in the ER [27]. Factor XII Tenri was the first deficiency for which the mechanism was analyzed at the cellular level [24], and this was followed by a similar analysis for Q421K and R123P [18].

We report here a second case of FXII Tenri. Our initial finding was a prolonged activated partial thromboplastin time (APTT); the patient (a woman) did not show a bleeding tendency. The small amount of secreted FXII Tenri in her plasma existed both as a monomer and within several types of disulfide-linked complexes. We have characterized this case and have tested whether the FXII Tenri within these complexes can be activated in vitro.

## Materials and Methods

### Subject.

The proband was a 71-yr-old Japanese woman with angina pectoris. She was found to have a prolonged APTT (64.5 sec), although she had no hepatic disease. Her FXII activity and antigen levels, compared with a those of normal subject, were 1.6% and 5.0%, respectively. Other clinical data, including coagulation factors, were as follows: PT, 11.2 sec; fibrinogen, 3,080 mg/L; AT-III, 104%; APL, 88%; FXI, 97%; FX, 103%; FIX, 105%; FVIII, 118%; FV, 200%; and vWF, 174%.

### Amplification and DNA sequence analysis of the FXII gene.

Genomic DNA was extracted from peripheral white blood cells using a Whole Blood DNA Extraction Kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions. Each exon, including the exon-intron boundaries of the FXII gene, was amplified from the proband's DNA by means of the polymerase chain reaction (PCR). Fourteen pairs of primers were designed using the program MacVector. Briefly, approximately 100 ng of the extracted DNA was mixed in 20 µl of 10 mM Tris-HCl, pH8.3, containing 2.0 mM MgCl₂, 0.5 µM of each of the forward and reverse primers, 200 µM of dNTPs, and 0.05 u/µl of Taq DNA polymerase. PCR was done in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA) for 50 cycles: denaturing at 94°C for 0.5 min, annealing at 94°C for 0.5 min, and extending at 72°C for 1.5 min. The amplified products were run on 3% agarose gels in tris-borate-EDTA (TBE) buffer. The separated products were stained with ethidium bromide, extracted using GeneClean II (Bio101 Inc., La Jolla, CA, USA), and directly sequenced using an automated sequencer (ABI Prism 310 genetic analyzer, Applied Biosystems, Foster City, CA, USA) in conjunction with the Big Dye® terminator cycle-sequencing kit (Applied Biosystems).

### Preparation of biotin-conjugated anti-human FXII antibody.

Biotinylation was performed by the method of Ogata et al [28]. Briefly, 20 µl of 1 mg/ml N-hydroxysuccinimide biotin in dimethyl sulfoxide was added to 100 µl of anti-human FXII polyclonal antibody (Cedarlane Co., Ontario, Ontario).
Canada) mixed with 200 µl of 0.2 M NaHCO₃. The mixture was incubated for 4 to 5 hr at room temperature, followed by exhaustive dialysis against phosphate-buffered saline (PBS) solution.

**Measurement of FXII activity and antigen levels.** Blood was collected in tubes containing a one-ninth volume of 3.2% trisodium citrate. The plasma was separated by centrifugation (1,500 x g, 10 min, 4°C). FXII activity was measured by an APTT method using FXII-deficient plasma [29]. Diluted plasma (10- to 200-fold) obtained from a normal subject was used as an arbitrary standard, and 50-fold dilutions of the control plasma and patient’s undiluted plasma were used to measure FXII activity (%). The FXII antigen level (%) was analyzed by enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene microtiter plates (Nunc, Denmark) were coated with anti-human FXII polyclonal antibody in 0.1 M Na₂CO₃, pH 9.6 (10 µg protein/well), and incubated at 4°C overnight. Plates were washed 5 times with PBS-Tween 20 after each of the subsequent incubation steps. Unoccupied sites were blocked with 1% skim milk in PBS-Tween 20 for 2 hr at room temperature. The standards and the samples (diluted or undiluted plasma as described above) were added at 100 µl/well in triplicate, and incubated for 2 hr at room temperature. Biotin-conjugated anti-human FXII polyclonal antibody in PBS (2.5 µg protein/well) was added at 100 µl/well and then incubated for 2 hr at room temperature. Peroxidase-conjugated streptavidine (Dako Cytomation, Kyoto, Japan) diluted 2,000-fold with PBS was added at 100 µl/well, followed by incubation for 20 min at room temperature. After the final washing, the color reaction was developed using 100 µl/well of 5 g/L tetramethylbenzidine dihydrochloride and hydrogen peroxide, followed by 100 µl/well of 0.4 M sulfuric acid to stop the reaction. Absorbance at 450 nm was measured using a Personal LAB spectrophotometer (BioChem ImmunoSystems).

**Immunoblot analysis.** The proband’s plasma, from which IgG was removed using Protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden), was reacted with anti-human FXII antibody bound to Protein A-Sepharose. The bound FXII was eluted by 0.1 M glycine-HCl, pH 2.0, and loaded on an 8-16% gradient polyacrylamide gel containing 0.1% SDS, and then electrophoresed [30]. The separated proteins were electrophoretically transferred onto nitrocellulose membranes, which were incubated with a blocking buffer [50 mM Tris-HCl (pH 8.0)]

![Fig. 1. Schematic representation of the composition of the factor XII gene in our patient. Also indicated are the common genetic polymorphism (46C/T) associated with translation efficiency and the nucleotides at the positions related to Hageman trait (exon 1-8 and exon 3-224). The nucleotide sequences for the PCR-amplified exon 3 fragments and the corresponding amino acid sequences are shown in the lower part, for our patient and for a normal subject.](image-url)
containing 2% (w/v) skim milk] for 30 min at room temperature and then washed 3 times with phosphate buffer containing 0.1% (v/v) Tween 20 (washing buffer). The membrane was incubated with a blocking buffer containing biotinylated anti-FXII or anti-α1-microglobulin (CosmoBio, Tokyo, Japan) antibody for more than 4 hr at room temperature, washed 3 times with washing buffer, and then incubated with horseradish-peroxidase conjugated streptavidin. Finally, the membrane was carefully washed, and the bands containing FXII were visualized using an ECL detection kit (Amersham Life Science, Buckinghamshire, England).

Treatment with APTT reagent. Plasma was mixed with an equal volume of APTT reagent (0.2 mg/ml sephalin, 0.03 mg/ml ellagic acid) followed by incubation for 30 sec at 37°C. Then, FXII and its degraded fragments were analyzed by immunoblotting, as described above.

Results

Fig. 1 shows a schematic representation of polymorphic DNA sequences, together with nucleotide sequences of PCR-amplified exon 3 fragments for both a normal individual and our FXII-deficient patient. We identified homozygosity for an A to G mutation at nucleotide position 7832 in exon 3 in the patient, resulting in a Tyr34 to Cys substitution. Sequence analysis of all other exons revealed no mutations. In the FXII gene of the patient, we observed neither the common genetic polymorphism (46 C to T substitution) in the 5’-untranslated region nor mutation in the 5’ flanking region (exon 1: -8 G to C) associated with the additional Taq I restriction site (T to C substitution at position 224 bp upstream of exon 3) in intron B. The FXII activity and the antigen levels in the patient were <5% of those in the normal subject. This CRM-negative FXII deficiency was thus identified as homogeneous FXII Tenri.

Fig. 2. SDS-PAGE followed by immunoblot analysis using biotinylated anti-FXII antibody was carried out for FXII partially purified using anti-FXII antibody-conjugated Protein A-Sepharose [normal (N) or patient’s (P) plasma]. Panel A: 50-fold diluted normal or undiluted patient’s sample was supplied. Panel B: undiluted normal or the patient’s sample was supplied. Each sample was treated with (+) or without (-) 2-mercaptoethanol (2-ME) before analysis. Whitened bands in the normal plasma (N in panel B) indicate antigen excessive patterns. Arrows indicate expected molecular weights in kDa.
Immunoabsorption followed by immunoblot analysis using anti-FXII antibody was performed to identify the small amount of FXII in the patient's plasma (Fig. 2A). Six bands with approximate molecular weights of 76, 115, 140, 190, 215, and 225 kDa were visualized in the pattern. In contrast, only a 76 kDa band was observed for the normal subject (50-fold dilution). Following reduction with 2-mercaptoethanol, only the 76 kDa band was observed in each. In addition, a normal control was supplied to the immunoblot analysis without dilution (Fig. 2B). An extremely large amount of FXII was observed, as expected at the position for 76 kDa (antigen excessive pattern), and at least 4 other bands with higher molecular weights were visualized at the positions for 115, 128, 144, and 155 kDa. After reduction with 2-mercaptoethanol, the unaffected FXII monomer remained as intense bands at the 76 kDa position together with the 115 kDa band; however, the 128, 144, and 155 kDa bands almost completely disappeared, while the 30 and 45 kDa bands were present at relatively stronger intensity. On the other hand, as shown in Fig. 3 several bands that reacted with anti-α1-microglobulin antibody were observed in immunoabsorbed fractions for both the normal and the patient's plasma. However, nonspecific bands of exactly identical sizes and relatively faint intensities were detected in normal and patient's plasma, respectively, after absorption using anti-FXII antibody unconjugated Protein A-Sepharose. This means that the specific band that reacted with anti-α1-microglobulin antibody was observed only in the patient's plasma (* in Fig. 3).

Immunoblot analysis carried out for a normal subject after treatment with APTT measuring reagent revealed bands with molecular weights of 160, 140, and 45 kDa, and an attenuation of the
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Fig. 4. After reduction, only the 45 kDa band remained in the pattern, not the 76, 140, or 160 kDa bands, and a new band with a molecular weight of 115 kDa developed. In contrast, the pattern for FXII Tenri revealed the disappearance of the 76 kDa band and the appearance of 140 and 45 kDa bands (and also of minor bands with molecular weights of 170, 160, and 30 kDa) with no change in the 115, 190, 215, and 225 kDa bands. After reduction, the pattern was similar to that seen for the normal subject in that 115 and 45 kDa bands were recognized in the pattern; however, the 76 kDa band was still present, in contrast to the finding in the normal subject.

Fig. 5 shows the correlation between FXII activity and antigen levels. The values were determined using the same plasma as standard in each case. The suitably diluted plasmas of 11 normal subjects showed similar correspondence between activity and antigen levels. In contrast, the values for the patient with FXII Tenri were distinctly separated (1.6% and 5.0%).

Discussion

In the present study, we describe a rare case with a homozygous FXII abnormality, Tyr34 to Cys substitution (7382 A→G), of a type previously named as FXII Tenri [26]. No other mutation – such as the common genetic polymorphism (46 C to T substitution) in the 5'-untranslated region [31] or the mutation in the 5' flanking region (exon 1: -8 G to C) associated with the additional Taq I restriction site in intron B [12, 13] – was observed in the FXII gene of our patient, nor were mutations observed in any of the 14 exons. Interestingly, homozygous FXII Tenri introduced a CRM-negative deficiency, with both FXII activity and antigen levels being less than 2-5% of those seen in a normal subject. In the immunoblotting patterns, FXII Tenri monomer has an apparent molecular weight of 76 kDa, which is slightly smaller than that of 80 kDa previously reported [26]. This variance could be induced by difference of experimental conditions, such as gel concentration or molecular weight standard. In addition, the higher molecular weight bands >115 kDa, which were not mentioned in the previous report [26], could be complexes with other proteins or itself. The bands with a molecular weight of 115 kDa observed in both undiluted normal and patient’s plasma presumably represent complexes between FXII and α1-microglobulin. In the normal plasma, the 115 kDa band, which was present in an extremely small amount compared with the FXII monomer (76 kDa), may be formed in the absence of free cysteine residues, like the complexes between vitamin K-dependent proteins and α1-microglobulin described previously [32]. Although the actual mechanism remains obscure, high molecular weight bands >115 kDa in the immunoblotting pattern of the patient could be fundamentally different from those of the normal control.

In the immunoblotting patterns, non-specific binding of α1-microglobulin to Protein A-Sepharose was observed for both the normal subject and the patient. However, non-specific binding of FXII was not detected (data not shown). Actually, α1-micro-
globulin is known to form complexes with itself and with many other plasma proteins, eg, prothrombin, albumin, IgA, fibronectin, and α1-inhibitor-3 \[33,34\]. Although non-specific binding to Protein A-Sepharose could be induced by interactions among these complexes, the 115 kDa band in the patient could include a complex between FXII and α1-microglobulin through an S-S bond induced by a neo-cysteine residue (because a non-specific band, which has slightly larger molecular weight of 117 kDa, was completely distinguished from the 115 kDa band that reacted with anti-α1-microglobulin antibody in the patient).

After treatment with APTT measuring reagent, the 76 kDa band seen in the normal plasma did not completely disappear; however, the remaining faint band faded following reduction with 2-mercaptoethanol. In contrast, in the patient the 76 kDa band almost completely disappeared after treatment with APTT measuring reagent, but reappeared after the reduction. These findings indicate that the remaining faint band for FXII in normal plasma was digested only to the first step (αFXIIa), not to the second step (βFXIIa) \[5-7\]. Then, the αFXIIa was further reduced to components with 30 kDa (βFXIIa) and 47 kDa (45 kDa plus the weight of 19AA) by treatment with 2-mercaptoethanol. On the other hand, in the patient the 76 kDa band was completely digested to the second step because it was present in a relatively small amount; however, a new FXII monomer may be generated from the complexes by reduction. This indicates that the FXII Tenri existing as disulfide-linked complexes with other proteins, or itself, is not be converted to an active form. Attached proteins would delay or inhibit the activation of FXII by interfering with its binding to the negative surface. This would explain the presence of a discrepancy between FXII activity and antigen levels in the patient’s plasma, but not in the normal subjects. The 140 kDa band, which appeared after treatment of the patient’s and the normal plasma with APTT reagent followed by a reduction with 2-mercaptoethanol. In addition, the nature of other high molecular weight bands that react with anti-FXII antibody is not always clear. However, it is easy to conjecture that a homodimer of FXII Tenri could exist as one of these complexes.

Normal FXII has within its molecule 40 cysteine residues, forming 20 pairs of disulfide bonds. It is unclear whether the newly generated Cys34 in FXII Tenri is only a candidate for disulfide bonds with free cysteine or with other proteins. The possibility that some of the newly generated Cys34 forms disulfide bonds with other cysteine residues such as Cys28, Cys42, or Cys54 within a molecule cannot be excluded.

References

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