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A novel congenital dysprothrombinemia leading to defective prothrombin maturation

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ABSTRACT

Introduction: Prothrombin deficiency is a very rare disorder caused by mutations in the F2 gene that generate hypoprothrombinemia or dysprothrombinemia and is characterized by bleeding manifestations that can vary from clinically irrelevant to life-threatening.
Aim: Here we characterize a patient with a novel missense mutation in *F2*, c.1090 T/A (p.Val322Glu), that causes severe dysprothrombinemia.
Methods: Coagulation assays, prothrombin Western Blotting, FII activation by Ecarin, fibrinogen degradation products quantification and thrombin generation assay were carried out to assess prothrombin expression and function. PCR followed by direct sequencing was carried out to characterize the mutation. In silico analysis for missense variant and molecular modeling were applied to predict the mechanism that leads to dysprothrombinemia.
Results and conclusions: The homozygous patient had a markedly prolonged prothrombin time, strongly reduced FII activity (0.82%) but normal antigen levels. In the thrombin generation assay the lag time and the peak height were unmeasurable, suggesting that the Val322Glu mutation results in the inability of the mutant prothrombin to be fully activated to thrombin. In fact, prothrombin activation by ecarin was defective, with a massive accumulation of the meizothrombin intermediate. Molecular modeling and dynamic simulation studies showed that the Val322Glu mutation interferes with protein flexibility at Arg271 and Arg320. This impairs the switch of the protein from zymogen to proteinase, thus preventing the formation of thrombin. Accumulated meizothrombin, however, maintains some fibrinogen-degrading activity, as shown by the formation of FDPs, and this probably explains the patient's mild bleeding phenotype.

Introduction

Prothrombin deficiency (PD, OMIM #613679), i.e. a defect of coagulation factor(F)II, is among the rarest inherited coagulation disorders, with a prevalence of ~1:2,000,000 and an autosomal recessive inheritance. Two main types of the disorder can be identified: hypoprothrombinemia (type I), in which reduced clotting activity and low antigen levels are found, and dysprothrombinemia (type II), in which normal or near-normal antigen levels are observed [1]. Some residual prothrombin coagulant activity is always detectable, because complete PD is probably lethal in humans [2]. Thirty-nine prothrombin deficiency-causing mutations in the factor II gene (*F2*) have been identified so far, most of which missense, but also nonsense, insertions/deletions and splicing mutations [1].

Bleeding manifestations in homozygotes or compound heterozygotes range from not clinically significant to life-threatening. In hypoprothrombinemia, the severity of bleeding is generally related to prothrombin level while in dysprothrombinemia the bleeding phenotype is more heterogeneous, with some patients suffering from severe bleeding and others with mild clinical manifestations [3–5].

The *F2* gene (OMIM #176930) spans ~21 kb within the 11p11-q12 locus and consists of 14 exons [6] encoding a 622-residue prepropeptide with a molecular mass of about 72 kD containing 5 domains: the pre-pro leader sequence, the γ-carboxyglutamic acid (Gla) domain, a kringle-1 domain, a kringle-2 domain and a catalytic serine protease domain. The circulating protein is composed of 579 residues and lacks the pre-pro-leader sequence.

Activation of prothrombin to thrombin is accomplished by the prothrombinase complex, formed by Factor Xa (FXa) and Factor Va (FVa) assembled on phospholipid membranes, following two sequential

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cleavage steps: the first cleavage at Arg320 converts prothrombin, the zymogen, to the serine proteinase meizothrombin (mIIa) and the second at Arg271 generates thrombin and fragment F1.2.

Another pathway is activated by FXa in the absence of FVa and begins with the cleavage of prothrombin at Arg271, giving rise to F1.2 and prethrombin 2 (P2) which is then further cleaved at Arg320 to yield thrombin [7].

Which one of the two pathways is triggered within blood vessels is still unclear. Krishnaswamy and co-workers demonstrated that when prothrombinase assembles on synthetic phospholipid vescicles initial cleavage of prothrombin occurs at Arg320, leading to the formation of an Ile-Val-Glu NH_2 terminal sequence that allows the formation of an internal salt bridge that induces the conformational change transforming the zymogen into proteinase. The proteinase configuration allows then Arg271 to be cleaved by prothrombinase, thus leading to the formation of thrombin [8]. Mann and co-workers recently demonstrated that when prothrombinase is assembled on activated platelets initial cleavage takes place at Arg271, thus bypassing mIIa formation and leading to a rapid procoagulant response [9].

Prothrombin is converted by the snake venom ecarin into mIIa (composed of fragment F1.2 + light-chain and heavy-chain held together by a disulfide bond) via cleavage at Arg320; mIIa is a proteolytically active product that catalyzes further cleavages at Arg284 and Arg155, in this way the F1.2 + light-chain polypeptide is then further degraded into F1, F2 and thrombin light-chain [10].

Here, we describe and characterize a novel missense mutation in *F2* that impairs the conversion of prothrombin into thrombin with accumulation of the mIIa intermediate causing severe dysprothrombinemia but a mild hemorrhagic phenotype.

Materials and Methods

Coagulation assays

Plasma was obtained from citrated venous blood after centrifugation at 4000xg for 10 minutes. Platelet count was performed on a HeCo S automatic cell counter (SEAC, Radim Diagnostic, Calenzano FI, Italy). Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time, FII, V, VII, VIII, IX, X, XI and XII activity and fibrinogen level assays were performed on an ACL Futura Plus coagulometer (Instrumentation Laboratories, Milan, Italy) using HemosIL® reagents (Instrumentation Laboratory, Milan, Italy), as described [11].

Relative quantification of prothrombin by Western Blotting

Protein concentration in plasma was determined by the Bradford assay; 10 μg of proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Membranes were probed with a rabbit anti-human-prothrombin polyclonal antibody, recognizing the F1 portion of prothrombin (aa 130-179) (Abcam, Cambridge, UK). Immunoreactive bands were detected using a peroxidase-conjugated anti-rabbit IgG antibody and chemiluminescence detection. FII expression in plasma from the proband and his children was compared with that of plasmas from healthy controls and of FII-deficient plasma (HemosIL®, Instrumentation Laboratory). Densitometric analysis and relative quantification were performed using the ImageJ software (NIH, USA).

Thrombin generation

Thrombin generation was measured in platelet-poor plasma (PPP) with the calibrated automated thrombogram method (Thrombinoscope BV, Stago, Milan, Italy) in a 96-well plate using 80 μl of PPP with final concentrations of 1 pM tissue factor and 4 μM phospholipids (PPP Reagent, Thrombinoscope BV). A thrombin calibrator (Thrombinoscope BV) with known thrombin-like activity was monitored in parallel. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems, Stago, Milan, Italy) equipped with a 390/460 filter set and thrombin generation curves were calculated using the Thrombinoscope software (Thrombinoscope BV). All samples were analyzed in at least four different repetitive experiments. The height of the thrombin peak was taken as a measure of the amount of thrombin formed (nM), while the lag time of the thrombin generation curves, defined as the time to reach one-sixth of the peak height as a measure of the initiation phase of coagulation [12].

Ecarin activation of prothrombin

For the activation of prothrombin, either plasma (supplemented with 25 mM $CaCl_2$ and 0.1 mM $MgCl_2$) or 8 μg of purified human prothrombin (Sigma Aldrich, Milan, Italy) dissolved in imidazole buffer saline (0.05 M Imidazole, 0.1 M NaCl, pH 7.3) were incubated with ecarin (0.01 U/μl of plasma or 0.01 U/μg of purified protein), at 37 °C for 30 seconds, 1, 4, 7, 15, 30, and 60 minutes. When plasma from healthy controls was used, hirudin (0.1 U/μl) (Sigma Aldrich) was added to avoid clotting, while with patient's plasma the addition of hirudin was not necessary because no clotting occurred, allowing to study prothrombin activation in conditions similar to those used with the purified protein. In selected experiments hirudin was added to patient's plasma too. Reactions were stopped by adding SDS-sample buffer and samples were analyzed by Western blotting using a rabbit anti-human-prothrombin polyclonal antibody recognizing the F1 portion of prothrombin (Abcam, Cambridge, UK) as described above.

Fibrin degradation products

Control-, patient-, or FII deficient-plasma were incubated with ecarin (0.01 U/μl of plasma) for 60 minutes at 37 °C. Clots, when present, were removed and fibrin degradation products (FDP) were measured in the supernatants using the Thrombo-Wellcotest kit (Thermo Scientific, Lenexa, KS, USA).

Molecular diagnosis

DNA was extracted from peripheral blood leukocytes according to standard protocols. We have standardized the polymerase chain reaction (PCR) conditions using primers designed with the Primer 3 software (www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) on the basis of known sequences of *F2* as reported in ENSEMBL database (Wellcome Trust Sanger Institute, Cambridge, United Kingdom): *F2* ENSG00000180210. Briefly, PCR was carried out on 50 μl samples in a Bio-Rad thermal cycler (Bio-Rad Laboratories, Inc., Hercules, California). Each sample contained 0.15 μg genomic DNA, 0.3 μM of each primer, 200 μM of deoxyribonucleotide triphosphates (dNTPs), 1X PCR buffer (with 1.5 mM $MgCl_2$), and 1.5 U of AmpliTaq® Gold Polymerase with appropriate 1X Buffer (Applied Biosystems Inc, Foster City, CA, USA). PCR products were purified and subjected to direct-cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, California), according to the manufacturer's instructions. A control group of 100 healthy and unrelated subjects was used to exclude that novel mutations could be common DNA polymorphisms.

F2 variations were numbered from the A of the ATG initiating codon of the cDNA sequence (Ensembl ID ENSG00000180210). For description of amino acid variants, we used the traditional numbering based on the mature protein of 579 amino acids (without the 43 N-terminal residues of pre-propeptide sequence) according to the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/recs-prot.html).