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Functional characterization of a novel missense mutation, His147Arg, in A1 domain of FV protein causing type II deficiency

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ABSTRACT

Introduction: Congenital factor V (FV) deficiency is a rare inherited disorder. Three compound heterozygous missense mutations, Asp68His, His147Arg, and Arg2074Cys, were observed in a Taiwanese patient with moderately severe FV deficiency.
Method: The novel His147Arg mutation in the A1 domain was investigated by protein modeling, followed by *in vitro* expression studies in COS-1 cells, to elucidate the molecular pathology associated with FV deficiency.
Results: The His147Arg mutation was associated with normal antigen levels, both in cell lysates and conditioned media, whereas FV activity was significantly reduced to 63.5 ± 17.0%. These observations correspond to a type II FV deficiency mutation. Protein modeling by short-duration molecular dynamics (MD) simulation showed that the His147Arg mutation was associated with a conformational change, which could disrupt the stability of FVa by interfering with His1817 coordination of the copper ion. In functional activation assays, the His147Arg mutation did not affect FV protein activation by thrombin; however, reduced cofactor activity of the FVa protein, due to an increased rate of dissociation of heavy and light chains, was observed.
Conclusion: Our results show that the His147Arg mutation in the A1 domain of FV does not impair synthesis or procoagulant activity. Instead, the His147Arg mutation appears to disrupt the stability of FVa, providing a potential explanation for the functional deficiency.

Introduction

The factor V (FV) gene (*F5*) is mapped to human chromosome 1q23, and spans more than 80 kb consisting of 25 exons [1]. The encoded FV protein is composed of six domains, oriented in an A1, A2, B, A3, C1, C2 conformation [2]. FV and FVIII share 35-40% DNA sequence homology for their A and C domains [3]. The three A domains of FV and FVIII are homologous to the copper-binding protein ceruloplasmin (CP) [4], whereas the two C domains of these proteins belong to the lipid-binding discoidin-like protein family [5]. FV is one of the procofactors in the common pathway of the coagulation cascade pathway [3]. Proteolytic removal of the large B domain by thrombin or factor Xa (FXa) converts FV into its active form (FVa), which consists of a heavy chain (A1-A2 domains) and a light chain (A3-C1-C2 domains). The heavy and light chains are linked through a non-covalent association coordinated by two Ca^{2+} ions and one Cu^{+2} ion. This arrangement prevents disruption of domain structure and stabilizes the interactions at the A1/A3 interface [6,7]. FVa is an essential cofactor in the conversion of prothrombin to thrombin by FXa in the presence of calcium and phospholipids [3].

FV deficiency caused by defects in the *F5* gene is an extremely rare disorder with an autosomal recessive mode of inheritance and an incidence of approximately one case per million [8]. Congenital FV deficiency can be classified as type I, with low or undetectable antigen, or as type II, with normal or mildly reduced antigen associated with reduced protein activity. The genetic causes of severe or moderately severe type I FV deficiency have been reported to include mRNA instability, presence of premature stop codons, and/or abnormal protein folding that leads to reduced secretion [9,10]. In contrast, type II deficiency has been

Abbreviations: FV, Congenital factor V; FVIII, coagulation FVIII; FX, coagulation factor X; MD, molecular dynamics; CP, ceruloplasmin; wt, wild-type; Asp, aspartic acid; His, histidine; Arg, arginine; Cys, cystein; PCR, polymerase chain reaction; PDB, protein data bank; EIA, enzyme immunoassay; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HBSS, Hank's Buffered Salt Solution; BSA, bovine serum albumin.

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characterized by secretion of protein variants with impaired activity [10,11]. Mutations associated with type II deficiency usually affect protein function due to altered protein structure [11,12].

In this study, we identified compound heterozygous mutations, Asp68His, His147Arg and Arg2074Cys, in a Taiwanese patient with severe FV deficiency. Two of these mutations, Asp68His and Arg2074Cys, are well characterized and have been shown to decrease FV antigen and activity [13,14]. However, His147Arg, a novel missense mutation located in the A1 domain, has not yet been characterized. Here, we investigated the effect of the His147Arg mutation on protein structural characteristics and protein modeling. We also conducted *in vitro* expression studies to investigate the role of this novel mutation in the pathogenesis of FV deficiency.

Materials and Methods

Subjects and Blood Collection

The subject of this study was a 34-year-old woman with residual FV activity of 2.4%. Prolonged prothrombin time and partial thromboplastin time were incidentally detected prior to the delivery of her second child; prolonged and excessive bleeding had also been reported after a tooth extraction. Venous blood was obtained from the proband, as well as the proband's parents, sister and brother. For each donor, 10 mL of blood was drawn into a plastic tube that contained 0.109 M sodium citrate, for coagulation assays. Platelet-poor plasma was obtained and aliquots were stored at −80 °C. Buffy coat specimens were collected for genomic DNA isolation. The study was conducted according to the Helsinki protocol and informed consent was obtained for all patients prior to their participation.

Measurement of FV Coagulant Activity and FV Antigen Levels

FV activity was measured in plasma using a functional assay based on the prothrombin time with human recombinant tissue factor (Instrumentation Laboratory Company, Bedford, MA) and FV-deficient plasma obtained from a congenitally deficient patient with undetectable FV plasma levels. Plasma FV antigen was measured using a sandwich enzyme immunoassay (EIA; Hyphen Biomed, Andresy, France) based on a sheep anti-human FV polyclonal antibody [15]. FV antigen and activity were expressed as percentage of normal pooled plasma.

DNA Sequencing

DNA was isolated using a commercial DNA extraction kit (Qiagen, Crawley, UK). All 25 exons and junctions of the *F5* gene were amplified using PCR. Bi-directional sequencing of purified PCR products was performed on both strands by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Nucleotide numbering started with the first nucleotide of *F5* cDNA (as described by Jenny et al.) [1]; amino acid numbering started at the first residue of the mature protein.

Site-Directed Mutagenesis

The pMT2/FV-wild-type (wt) mammalian expression plasmid, containing the human full-length *F5* cDNA, was kindly provided by Dr R. J. Kaufman (Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, MI). The identified mutations were introduced into the pMT2/FV-wt plasmid via site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the following primers: 5'-cctcacacacatcta ttactcccgtgaaaatctgatcgagg-3' and 5'-cctcgatcagattttcacgggagtaataga tgtgtgtgagg-3' for the His147Arg mutation; and 5'- ggaacccttccgtgcctgt ctgaatgcccagg-3' and 5'-cctgggcattcagacaggcacggaagggttcc-3' for the Arg2074Cys mutation. The resulting mutant plasmid, pMT2/FV-His147Arg and pMT2/FV-His147Arg + Arg2074Cys, were confirmed by sequencing.

Cell Cultures and Transfections

COS-1 cells (ATTC No. CRL-1650), SV40-transformed green monkey kidney cells, were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO; Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) and antibiotics (250 ng/mL fungizone, 100 μg/mL streptomycin, and 63.8 μg/mL penicillin) in a 5% CO_2 humidified atmosphere at 37 °C.

Cells were plated at a density of 1×10^5 cells/well into 6-well plates. On the following day, for each well, 2 μL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and 5 μg wt or mutant plasmids, or an equimolar mixture of both plasmids, were diluted in 800 μL OPTI-MEMI (GIBCO), and incubated at room temperature for 20 min. These mixtures were then added to cell preparations containing 1,200 μL OPTI-MEMI. After 6 h, culture medium was replaced with fresh medium and supplemented with fetal calf serum and antibiotics. After 24 h, the medium was removed and cells were washed twice with Hank's Buffered Salt Solution (HBSS) and then cultured for an additional 24 h in serum-free medium supplemented with antibiotics, 2.5 mM $CaCl_2$, and 5 mg/mL bovine serum albumin (BSA; GIBCO). In parallel, all transfections were performed in 10-cm diameter dishes (all volumes were scaled up by a factor of 6), to obtain sufficient FV protein for functional assays on conditioned media.

Conditioned media were collected in pre-chilled tubes and centrifuged to remove cell debris. The media were then concentrated by using Amicon Ultra-15 centrifugal filters (Millipore, Bedford, MA), which contained a cellulose membrane with a molecular weight cut-off threshold of 100 kDa. Concentrated conditioned media were stored at −80 °C. Cell lysates were harvested with lysis buffer (phosphate buffered saline with 10 μL/mL triton X-100) with protease inhibitor cocktail (2 mM AFBSF, 1 μM phosphoramidon, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.2 μM aprotinin and 10 μM pepstatin A) (Sigma, St. Louis, MO).

FV Antigen and Activity Measurements in Conditioned Media and Cell Lysates

FV antigen was measured in10-fold concentrated conditioned media and cell lysates using a sandwich EIA with a horse anti-human FV polyclonal antibody (PAHFV-H; (Haematologic Technologies Inc., Essex Junction, VT) and a biotin-conjugated mouse anti-human FV light chain antibody (AHV-5112B; Haematologic Technologies Inc.), EIA standard curves were constructed with reference plasma dilutions of 1:50 to 1:8000 (sample diluents: 0.1 M HEPES, 10 mM NaCl, 1% BSA, and 0.1% Tween 20). The lower detection limit of the assay was 6 ng/mL. The relative FV antigen of FV mutant proteins was calculated as a percentage of FV-wt.

FV coagulant activity was measured in approximately 40-fold concentrated media using a functional assay based on the degree of normalization of the prothrombin time with diluted FV-immunodepleted plasma (Dade-Behring, Liederbach, Germany). Standard human plasma with a 1:20 dilution was assigned as (100%) and standard curves were constructed with reference plasma dilutions of 1:20 to 1:2000 in imidazole buffer solution (pH = 7.25) according to the manufacturers' instruction. The FV activity was calculated from a standard curve based on human pooled plasma. FV specific activity was calculated as the ratio between FV coagulant activity and corresponding antigen levels, and the relative FV specific activities of FV mutant proteins were compared as a percentage of FV-wt.

Molecular Dynamics and Mutation Modeling

Short-duration (10 ps) molecular dynamics (MD) simulations were performed for the His147Arg mutant protein. Models for mutant's