BRIEF REPORT

X-Linked Thrombophilia with a Mutant Factor IX (Factor IX Padua)

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SUMMARY

We report a case of juvenile thrombophilia associated with a substitution of leucine for arginine at position 338 (R338L) in the factor IX gene (factor IX–R338L). The level of the mutant factor IX protein in plasma was normal, but the clotting activity of factor IX from the proband was approximately eight times the normal level. In vitro, recombinant factor IX–R338L had a specific activity that was 5 to 10 times as high as that in the recombinant wild-type factor IX. The R338 substitution causes a gain-of-function mutation, resulting in factor IX that is hyperfunctional.

Venous thrombosis in patients who are younger than 45 years of age, a condition that is often associated with a family history of thrombosis and with recurrent episodes of thrombosis, is characteristic of an inherited tendency toward thrombosis (i.e., thrombophilia). Thrombophilia is most commonly associated with a gain-of-function mutation in the factor V gene (factor V Leiden) or in the prothrombin gene, variant 20210A.[1] Studies have shown that an elevated level of factor IX is also an independent risk factor for venous thrombosis.[2,3] The prevalence of high levels of factor IX is 20% among patients with venous thrombosis and 5% in the general population. The molecular basis for the increased level of factor IX in plasma is unknown, however.[4-6] Here we describe a case of X-linked thrombophilia with a gain-of-function mutation (R338L) in the factor IX gene (factor IX Padua).

CASE REPORT

The proband is a 23-year-old white man whose family origins were in the northeast region of Italy. He was admitted to the Thrombosis Unit at Padua University Hospital with a diagnosis of occlusive femoral–popliteal deep-vein thrombosis in the right leg; the diagnosis was confirmed on compression ultrasonography. The event occurred a few days after mild muscular stretching. There was no clinical or physical evidence of systemic disease or use of drugs. Low-molecular-weight heparin (nadroparin), at a dose of 100 U per kilogram of body weight twice daily, and warfarin, at a dose adjusted to achieve a therapeutic international normalized ratio (INR) of 2.0 to 3.0, were administered, and no recurrent thrombosis has appeared during a 14-month follow-up period. Compression ultrasonography performed 1 year after the initial event showed partial recanalization of femoral–popliteal veins. No family member had a history of venous thrombosis.

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METHODS

COAGULATION ASSAYS

We performed coagulation tests and assessed the patient for thrombophilia as described previously.[7,8] Activity levels of factor IX were measured by means of a one-stage clotting assay with the use of the reagent Actin (Siemens), and factor IX antigen levels were determined with the use of matched-pair polyclonal antibodies (Affinity Biologicals). Plasma was obtained from 80 to 100 healthy subjects of both sexes (age range, 20 to 60 years) for use as a reference pool for all assays. All subjects provided written informed consent.

ISOLATION OF FACTOR IX FROM PLASMA AND SEQUENCING OF THE FACTOR IX GENE

From 40-ml samples of plasma obtained from the proband and from the controls, factor IX was isolated by means of affinity chromatography with the use of antihuman factor IX Sepharose (AHIX-5041-S, Haematologic Technologies) and by means of ion-exchange chromatography with the use of Q Sepharose Fast Flow medium.[9] Genomic DNA was obtained, and the factor IX gene was sequenced with the use of the ABI PRISM 3700 DNA Sequence Detection System (Applied Biosystems). Direct detection of the factor IX–R338L mutation was performed with the use of a polymerase-chain-reaction (PCR) assay (primers: sense, 5′-GCCAATTAGGTCAGTGGTCC-3′; reverse, 5′-GATTAGTTAGTGAGAGGCCCTG-3′), followed by digestion with TaqI endonuclease.

RECOMBINANT FACTOR IX

Human embryonic kidney 293 cells were transduced by lentivirus vectors that expressed wild-type factor IX or factor IX with the substitution of leucine for arginine at position 338 (R338L) with the use of the ViraPower HiPerform Expression System (Invitrogen). The substitution R338L (CGA→CTA) was introduced with the use of the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Culture media consisted of Dulbecco's modified Eagle's medium containing 10% fetal-calf serum and 6 μg per milliliter of vitamin K. The factor IX concentration was determined with the use of an enzyme-linked immunosorbent assay.[10] Functional factor IX activity was measured by means of a clotting assay with the use of a modified one-stage factor assay.[11] To establish the contribution of factor IX to the activated partial-thromboplastin-time measurements of conditioned medium from lentivirus-transduced cells, the activated partial-thromboplastin-time assay was performed before and after incubation at 37°C for 30 minutes with 2.5% affinity-purified goat antihuman factor IX (Cedarlane Laboratories).

RESULTS

COAGULATION AND THROMBOPHILIA SCREENING TESTS

Samples from the proband and from family members (for family pedigree, see Fig. 1A) contained normal levels of protein C, protein S, and antithrombin. Mutant forms of factor V Leiden and prothrombin variant G20210A were not detected. Tests for lupus anticoagulant, anticardiolipin antibodies, anti–β2-glycoprotein I antibodies, and hyperhomocysteinemia were all negative, and levels of factor VIII and factor XI were normal.

FACTOR IX LEVELS AND DNA ANALYSIS

Table 1 shows the plasma levels of factor IX in family members. The proband (II-1) had a high level of factor IX activity (776% of the normal level of activity), but he had normal levels of factor IX antigen (92% of the normal level); the ratio of activity to antigen was 8.4. Notably, during warfarin therapy, when the INR was 3.4, the level of factor IX antigen decreased to 28% of the normal level; at the same time, however, the factor IX clotting activity was 160% of the normal level. Factor IX antigen levels in the mother (I-2) and a younger brother (II-3) were normal, but factor IX clotting activity in the mother was 337% of the normal activity level and factor IX clotting activity in the brother was 551% of the normal activity level. The other family members we studied had normal activity and antigen levels of factor IX.

DNA analysis of the proband and his younger brother revealed a point mutation in the factor IX gene (G31134T transversion) that caused a substitution of leucine for arginine at position 338 (Fig. 1B and 1C). The proband and his younger brother were hemizygous for the G31134T transversion, whereas the mother was heterozygous for the mutated gene. We screened all exons, splice junctions, the promoter region, and the 3′ untranslated region of the factor IX gene from the mother, the proband, and his younger brother, and only the G31134T transversion was found. The proband's father (I-1) and his other brother (II-2), who had

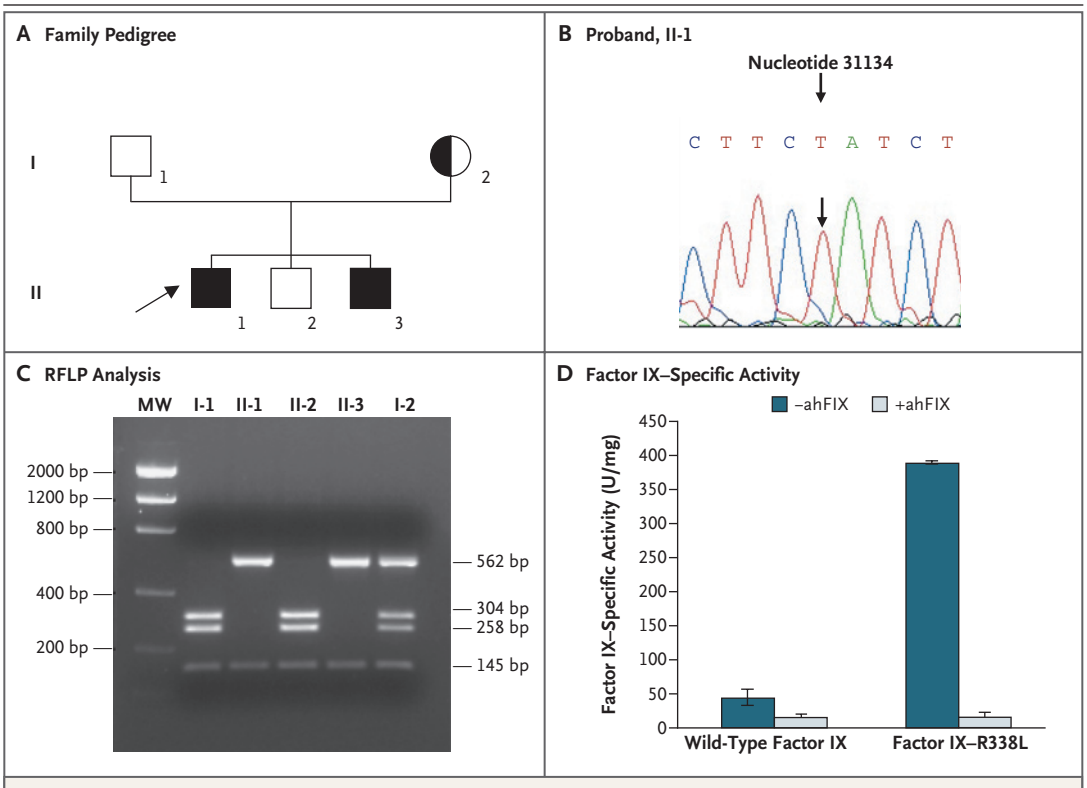

Figure 1. Factor IX Genotype of the Patient and His Family.

Panel A shows the family pedigree. The proband (II-1) is indicated by an arrow. Panel B shows the sequence of the factor IX gene around the site of the mutation in exon 8. A G→T transversion at nucleotide 31134 has occurred in factor IX Padua, resulting in an amino acid substitution of leucine for arginine at position 338. The proband is hemizygous for the mutation (see arrow in the sequence insert). Panel C shows a restriction-fragment–length polymorphism (RFLP) analysis of the polymerase-chain-reaction product of exon 8 of the factor IX gene digested by TaqI endonuclease. Wild-type factor IX exhibits three fragments of 304, 258, and 145 base pairs (bp). The factor IX Padua mutation abolishes one cleavage site, resulting in the formation of only two fragments (562 and 145 bp). Panel D shows the specific activity of recombinant wild-type factor IX and factor IX–R338L. We performed experiments of factor IX–specific activity in human embryonic kidney 293 cells in triplicate and calculated the factor IX–specific activity by dividing the clotting activity by the antigen levels and expressing the results in units per milligram. The dark-blue bars show the averages of specific activity for wild-type factor IX and for three cell lines expressing factor IX–R338L. The light-blue bars show the results of incubation of conditioned medium with antibody to factor IX before measurement of factor IX clotting activity. Factor IX clotting activity was measured in the presence of anti–human factor IX antibodies (+ahFIX) and in their absence (–ahFIX) in the culture medium. MW denotes molecular weight.

normal factor IX activity levels, had neither the G31134T transversion nor other mutations in the factor IX coding region and splice junctions.

We examined the prevalence of the factor IX–R338L mutation in subjects of Caucasian descent from the same geographic area as the proband. They were randomly selected from patients who were being followed in our thrombosis unit and who had mean (±SD) normal levels of factor IX antigen (101.2±15.5%) and activity (100.2±16.1%).[12-14] A total of 200 controls (100 men and 100 women, 20 to 80 years of age) and 200 patients who had had a documented venous thromboembolism (100 men and 100 women, 20 to 80 years of age) were studied. None of these control subjects or patients had the factor IX–R338L allele.

CHARACTERIZATION OF FACTOR IX FROM PLASMA

Factor IX was isolated from plasma that was obtained from the proband and from the pooled control samples. The purity of the preparations was assessed by means of sodium dodecyl sulfate–polyacrylamide-gel electrophoresis with the use of sil-

Table 1. Clinical Characteristics and Laboratory Data from the Family Members.*

Subject	Sex	Age (yr)	Activated Partial-Thromboplastin Time (sec)†	Factor IX Antigen (% of normal level)	Factor IX Activity (% of normal level)	Factor IX Activity-to-Antigen Ratio
II-1, proband	M	23	25.7	92	776	8.4
I-1	M	53	35.2	105	127	1.2
I-2	F	46	28.2	94	337	3.5
II-2	M	21	33.4	116	123	1.0
II-3	M	11	29.1	64	551	8.6

* II-1 refers to the proband, I-1 to his father, I-2 to his mother, II-2 to the older of his younger brothers, and II-3 to the youngest brother.
† The normal range for activated partial-thromboplastin time is 30 to 40 seconds.

ver staining. No difference was found in the apparent molecular weight, as assessed by immunoblotting, between factor IX isolated from plasma obtained from the proband and that isolated from plasma obtained from the controls (data not shown). This result suggests that the protein was properly processed to mature form. After reconstitution of factor IX–deficient plasma with factor IX isolated from plasma obtained from the proband and with factor IX isolated from plasma obtained from the controls, at a final concentration of 5 μg per milliliter (100% of normal), factor IX clotting activity levels were 834% and 100% of normal, respectively.

SPECIFIC ACTIVITY OF RECOMBINANT FACTOR IX–R338L

We developed a recombinant factor IX–expression system to measure the specific activities of the variant factor IX–R338L and of wild-type factor IX. Factor IX–specific activity was determined by the assessment of antigen levels and protein coagulation activity. Figure 1D shows the results from assays of three separate experiments, each performed in triplicate. In conditioned medium, factor IX levels ranged from 906 to 1244 ng per milliliter in wild-type factor IX and from 955 to 1549 ng per milliliter in the mutant factor IX. The protein-specific activity of factor IX–R338L was 390±28 U per milligram, whereas the protein-specific activity for wild-type factor IX was 45±2.4 U per milligram. Incubation with a polyclonal antibody against factor IX reduced the clotting activity of both forms of factor IX, a finding that shows the specificity of the factor IX activity that was measured in the activated partial-thromboplastin-time assay (Fig. 1D).

DISCUSSION

Activated factor IX plays a central role in the intrinsic and extrinsic coagulation pathways; when the level of factor IX is low, there is excessive bleeding, and when the level is elevated, there is thrombophilia. It has been shown in case–control studies that an elevated level of factor IX is an independent risk factor for thrombosis. In contrast to the identification of hundreds of mutations in the factor IX gene that cause deficient clotting activity (hemophilia B),[15] the molecular basis of increased levels of factor IX is unknown.[6,16]

The factor IX mutation reported here was associated with markedly increased activity of factor IX and with thrombosis in the proband. The molecular basis of the increased coagulant activity of factor IX in our patient is a point mutation (factor IX–R338L) that segregates in an X-linked inheritance pattern. The in vitro factor IX activity-to-antigen ratio in the two male members of the family who carry the factor IX–R338L mutation is approximately eight times the normal level. The young brother of the proband, who is currently prepubertal (11 years of age), has a factor IX activity level that is 551% of the normal activity level, and we expect that this level will increase after puberty.[17]

The reconstitution of factor IX–deficient plasma with factor IX isolated from the proband's plasma resulted in clotting activity that was eight times the activity of wild-type factor IX that was isolated from the plasma of normal subjects. Furthermore, recombinant mutant factor IX also had increased specific activity. The factor IX–R338L mutation was not found in 400 subjects (600 X chromosomes) who had normal levels of factor IX antigen and

activity. Thus, it is unlikely that this mutation is a silent polymorphism. These findings implicate the R338L mutation as the cause of the gain-of-function property of the mutant factor IX.

Arginine at position 338 is highly conserved in factor IX from mammals,[18] but not in other vitamin K–dependent proteins in humans. Previously, Chang et al. found that site-specific mutagenesis that substituted alanine for arginine at position 338 (R338A) resulted in a factor IX molecule with clotting activity that was three times the clotting activity in wild-type factor IX.[19] This region of the protein is important for substrate (factor X) binding, and changing from arginine to alanine at position 338 increases the efficiency of the binding of the substrate to the enzyme (factor IX).[19-21] Previously, we found that there was increased hemostatic activity of the factor IX–R338A variant in in vivo models of severe hemophilia B.[10]

Sites at which cytosines precede a guanosine in the DNA sequence (CpG dinucleotides) are considered to be hotspots for mutations,[22] and mutations in CpG dinucleotides comprise 25% of all mutations in hemophilia B.[15] Stop-codon mutations at R338 are common in hemophilia B, but the expected rates of mutations due to transition at R338 are underrepresented.[23] The only missense mutation at this position in hemophilia B is R338P.[15]

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