RAPID COMMUNICATION

Moderation of Hemophilia A Phenotype by the Factor V R506Q Mutation

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Although many examples of unrelated hemophilia A patients carrying identical point mutations in the factor VIII (FVIII) gene have been reported, the clinical phenotype is not always the same among patients sharing the same molecular defect. Possible explanations for this discrepancy include undetected additional mutations in the FVIII gene or coinheritance of mutations at other genetic loci that modulate FVIII function. We report molecular genetic analysis of potential modifying genes in two sets of unrelated patients carrying common FVIII missense mutations but exhibiting different levels of clinical severity. Both mutations (FVIII R1689C and R2209Q) are associated with severe hemophilia A in some patients and mild/moderate disease in others. The common von Willebrand disease type 2N mutation (R91Q) was excluded as a modifying factor in these groups of patients. However, analysis of the recently described factor V (FV) R506Q mutation (leading to activated protein C resistance) identified a correlation of inheritance of this defect with reduced hemophilia A severity. Two moderately affected hemophilia A patients, each with either of two FVIII gene mutations, were heterozygous for FV R506Q, whereas two severely affected patients and two moderately affected patients were homozygous normal at the FV locus. Our results suggest that coinheritance of the FV R506Q mutation may be an important determinant of clinical phenotype in hemophilia A and that modification of the protein C pathway may offer a new strategy for the treatment of FVIII deficiency.

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Hemophilia A, an X-linked recessive bleeding disorder, affects approximately 1 in 5,000 males. The disease results from a deficiency of the coagulation protein factor VIII (FVIII). Molecular genetic studies of hemophilia A patients have identified more than 200 distinct mutations in the FVIII gene. An intrachromosomal inversion of the FVIII gene resulting in the separation of exons 1 through 22 from exons 23 through 26 has recently been shown to account for the disease in ~40% of severe hemophiliacs. Based on the level of measurable FVIII activity, hemophiliacs are classified as severe (<1% normal FVIII activity), moderate (1% to 4% FVIII activity), or mild (5% to 25% FVIII activity). The vast majority of mild/moderate hemophiliacs who have been studied have shown to have a missense mutation in the FVIII gene. Although greater than 80 examples of unrelated hemophilia A patients carrying identical point mutations in the FVIII gene have been reported, the clinical phenotype is variable for some patients sharing the same molecular defect. For example, although 13 different patients have been identified carrying the same arginine for cysteine substitution at codon 1689, 4 are classified as severe hemophilia A with FVIII activity less than 1% and 9 as mild/moderate hemophilia. Fourteen patients have also been identified who share a substitution of glutamine for arginine at codon 2209, with 9 classified as severe hemophilia A and 5 as mild/moderate disease.

Possible explanations for this variable expressivity of hemophilia A phenotype among individuals carrying identical FVIII mutations include undetected additional mutations in the FVIII gene, interlaboratory differences in FVIII assays, or the effects of mutations at other loci. Berg et al recently described a family in which coinheritance of both factor XI and FVIII deficiencies resulted in a more severe bleeding tendency than that associated with either deficiency alone. A new subtype of von Willebrand disease (vWD) was recently identified (type 2N vWD) due to defects within the FVIII binding domain of von Willebrand factor (vWF). The gene frequency for the most common type 2N vWD defect (vWF R91Q) may be as high as 1% in some populations. Although these patients generally carry a normal FVIII gene, homozygosity for type 2N vWD results in very low FVIII activity levels and a phenotype closely resembling hemophilia A. We previously hypothesized that coinheritance of a type 2N vWD allele with hemophilia A might result in a more severe hemophilia phenotype. Another potential modifier gene for hemophilia A is the recently described factor V (FV) R506Q mutation associated with activated protein C (APC) resistance and thrombophilia. This mutation is remarkably common, occurring at a frequency of 2% to 7% in a number of populations. We now report molecular genetic analysis of potential modifying genes in two sets of unrelated patients who share a common FVIII missense mutation but have different clinical phenotypes. Our results suggest that coinheritance of the FV R506Q mutation may be an important determinant of clinical phenotype in hemophilia A and that modification of the protein C pathway may offer a new strategy for the treatment of FVIII deficiency.

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MATERIALS AND METHODS

Materials. Normal, FVIII-deficient, and FV-deficient pooled human plasmas were obtained from George King Biomedical, Inc (Overland Park, KS). Monoclonal antibody to the heavy chain of FVIII\(^{c}\) coupled to CL4B-sepharose\(^{c}\) was a gift from Debra Pittman (Genetics Institute, Cambridge, MA). Activated partial thromboplastin (Automated APTT reagent) was purchased from General Diagnostics Organon Teknika Corp (Durham, NC). Wild-type FV (768 \(\mu\)g/mL, 49 UlmL) and FV R5060 (261 \(\mu\)g/mL, 14 UlmL) were purified from human plasma and generously supplied by M.J. Heeb and J.H. Griffin (The Scripps Research Institute, La Jolla, CA).

Patients. Mutation identification in all of the patients studied here has been previously reported. Patients H10\(^{a}\) and JH38\(^{c}\) carry a substitution of cysteine for arginine at codon 1689 (FVIII R1689C). Although patient H10 has severe hemophilia A (undetectable FVlll activity), patient JH38 has only moderate disease (2% to 3% FVlll activity). Patients HP16 and HP17\(^{c}\) have the identical substitution at codon 2209, resulting in a substitution of arginine for glutamine (FVIII R2209Q). Patient HP16 has mild hemophilia A (7% FVlll activity), and patient HP17 has severe disease (<1% FVlll activity). Patients 785 and 818\(^{a}\) also carry the FVlll R2209Q mutation, both with moderate disease (2% to 5% FVlll activity).

DNA analysis for detection of the FV R506Q mutation. Approximately 200 ng of total genomic DNA was amplified by the polymerase chain reaction (PCR) in a 25 \(\mu\)L reaction using 50 ng each of primers 5'-TGCCGCTGCTCTAACAGCCA-3' and 5'-TGT-TATCACACGTGTTGCAAA-3' specific for a 267-bp fragment of the FV gene that includes part of exon 10\(^{11}\). PCR conditions were an initial denaturation for 2 minutes at 94°C followed by 1 minute at 94°C, 1 minute at 54°C, and 1 minute at 72°C for 35 cycles using an MJ Research 96V thermocycler (MJ Research, Inc, Watertown, MA). A final extension for 5 minutes at 72°C was performed to ensure complete extension of the PCR products. After amplification, 7.5 \(\mu\)L of PCR product was digested with Mnl I (New England Biolabs, Beverly, MA) in a 15 \(\mu\)L total reaction volume at 37°C for 1 hour to detect the G to A substitution at bp 111 responsible for the R506Q mutation. Digested PCR products were separated by electrophoresis through 4% composite (3% FMC Bioproducts [Rockland, ME] Nusieve and 1% GIBCO BRL [Gaithersburg, MD]) agarose and visualized by staining with ethidium bromide.

DNA analysis for detection of the vWF R91Q mutation. Detection of the vWF R91Q mutation was performed as previously described\(^{9}\). Briefly, genomic DNA was amplified by PCR using primers specific for exon 20 of the vWF gene. The vWF R91Q mutation was detected by allele-specific oligonucleotide hybridization using specific 15-mers for the normal and mutant alleles.

FVIII assay. FVIII activities were measured in a one-stage clotting assay using FVIII-deficient plasma as a substrate. One unit of FVIII activity is that amount measured in 1 mL of normal human pooled plasma. To determine whether FV R506Q increases FVIII activity in the clotting assay, FV-deficient plasma containing low levels of FVIII were reconstituted with wild-type or R506Q FV. FVIII was immunodepleted from 1 mL of FV-deficient plasma by the addition of 100 \(\mu\)L of anti-FVIII antibody\(^{10}\) coupled to CL4B-sepharose and incubation at 4°C overnight. The plasma supernatant was collected after centrifugation. The amount of FVIII activity remaining in the FV-deficient plasma after immunodepletion was 134 \(\mu\)U/mL. A total of 14 UlmL of purified wild-type or R506Q FV was diluted with the FVIII-depleted FV-deficient plasma to 0.5, and 0.25 UlmL. FVIII activity of these samples was determined by the one-stage clotting assay.

RESULTS

DNA analysis for detection of the FV R506Q mutation is shown in Fig 1. The G to A substitution responsible for the

arginine to glutamine change at amino acid 506 of FV results in the loss of an Mnl I restriction endonuclease site.\(^{12}\) When digested with Mnl I, the 267-bp PCR fragment derived from a normal FV allele should show fragments of 163, 67, and 37 bp. PCR product derived from a FV allele carrying the G to A substitution should yield fragments of 200 and 67 bp. As seen in Fig 1, patient H10 carrying the FVIII R1689C mutation (lane 2) and patient HP17 carrying the FVIII R2209Q mutation (lane 4) are homozygous for the normal FV allele (R506). Patient JH38 carrying the FVIII R1689C mutation (lane 3) and patient HP16 carrying the FVIII R2209Q mutation (lane 5) are both heterozygous for FV R506Q. Dideoxy sequence analysis of both strands of the PCR products for each patient confirmed the results of the restriction endonuclease analysis (data not shown). Thus, both moderately affected patients (lanes 3 and 5) are heterozygous for FV R506Q, whereas the severely affected patients (lanes 2 and 4) are homozygous normal at the FV locus. Two other patients with moderate hemophilia A (785 and 818) known to carry the FVIII R2209Q mutation are homozygous normal at amino acid 506 of the FV gene (data not shown). Using allele specific oligonucleotide hybridization analysis, none of the patients was shown to carry the vWF R91Q mutation (data not shown).

Patient HP16 was shown to also have a thymine to cytosine substitution at position -601 of the FVIII gene. The
Effects of this substitution on FVIII gene expression was analyzed using previously described methods.26 Transfection of FVIII promoter sequences containing this substitution linked to a luciferase reporter into liver-derived PLC/PRF/5 cells showed no significant difference from the wild-type promoter. DNase I footprint analysis using rat liver nuclear extracts also showed no significant differences in the J footprint between the wild-type and -601 T→C substituted alleles (data not shown).

The effect of R506Q FV on the measurement of FVIII activity was determined by a one-stage clotting assay after reconstitution of FV-deficient/FVIII-depleted human plasma with increasing amounts of wild-type or R506Q purified plasma-derived FV. As seen in Table 1, reconstitution with either wild-type or R506Q FV slightly increased FVIII activity. However, there was no difference in the amount of FVIII activity detected in the presence of R506Q FV compared with wild-type FV.

**DISCUSSION**

Genetic deficiencies in a number of specific clotting factors have been identified in association with inherited bleeding disorders. Phenotypic variability among individuals carrying identical genetic mutations has been identified for a large number of genetic diseases, including hemophilia A and other coagulation and thrombotic disorders.21,22 Possible explanations for this variable expressivity include the contribution of a second, undetected abnormality in the same gene as well as modifying effects of mutations at other genetic loci. Although an additional substitution was identified in the FVIII promoter of one patient in our study (HP16), this change appears to be a silent variation with no significant effect on FVIII gene expression.

Interaction between co-inherited genetic abnormalities have previously been reported for deficiencies of several clotting factors, including FVIII and factor XI,23 as well as factor X and vWF.24,25 The remarkably high general population frequency (2% to 7%)12,14 of the newly discovered FV R506Q mutation and its association with increased risk for venous thrombosis suggest that it could be a particularly important contributing factor to the variable coagulation responses observed among patients with hemophilia A and other clotting factor deficiencies. This effect was recently shown for the highly variable thrombotic risk associated with protein C deficiency22 as well as protein S deficiency.26

In an effort to identify genetic loci outside of the FVIII gene that may modify the hemophilia A phenotype, we analyzed the DNA of two sets of unrelated hemophilic patients sharing the same FVIII gene mutations but differing in disease phenotype (severe v mild/moderate). Mutations in the FVIII binding domain of vWF presented a particularly attractive candidate for this analysis. Binding of FVIII to vWF in plasma is required for FVIII stability. Patients with mutations in this region of vWF (type 2N vWD) exhibit decreased plasma FVIII levels, and the frequency of the vWF R91Q mutation may be as high as 1% in some populations.9,27 Although the vWF R91Q mutation was not detected in our current analysis, type 2N vWD should remain an important consideration as a potential modifier of hemophilia A severity.

DNA analysis for the FV R506Q mutation identified this defect in two mild/moderate hemophilia A patients, one with the FVIII R1689C mutation and one with the FVIII R2209Q mutation. In contrast, a severe hemophilia A patient with the FVIII R1689C mutation and one severe and two mild/moderate hemophilia A patients with the FVIII R2209Q mutation were all homozygous normal (R506) at the FV locus. Although these data suggest that the FV R506Q mutation may be an important modulator of disease severity in a subset of hemophilia A patients, the discrepancy in disease severity for the two patients with the FVIII R2209Q mutation and normal FV R506 suggests that a number of other potential inherited and acquired modifying factors remain to be identified. In contrast to our results, a preliminary study by Chan et al28 failed to detect a clear association between the FV R506Q mutation and the clinical severity of hemophilia A. Also, in a study of 18 hemophilia A patients with FVIII activity less than 1% but a milder bleeding phenotype (mild severe hemophilia A) by Arbini et al,29 none of the 18 was found to carry the FV R506Q mutation. A variable modifying effect of the FV R506Q mutation on hemophilia A severity, dependent on the precise underlying FVIII defect, could explain this discrepancy. The effect of the FV R506Q mutation might be most pronounced in the context of specific FVIII gene mutations, such as the R1689C mutation that occurs at a thrombin cleavage site. More detailed correlation of the clinical course in hemophilia A for different FV and FVIII genotypes should provide a clearer picture of this potential interaction.

The biochemistry of the protein C pathway suggests a plausible mechanism for the attenuation of FVIII deficiency-related bleeding by the FV R506Q mutation. APC inactivates activated FV (FVa) by proteolysis first at R506, followed by cleavage at R306 and R679 that results in loss of cofactor activity.27 Recent studies have shown that FVa R506Q is not completely APC resistant30,31 FVa from patients lacking the R506 cleavage site is still inactivated after cleavage at R306 and R679. However, the rate of inactivation is much slower than in normal (wild-type) FVa.

Although FV-deficient/FVIII-depleted human plasma reconstituted with FV R506Q had similar FVIII activity as that reconstituted with wild-type FV (Table 1), this would not exactly mimic in vivo the hemophilic patient carrying

| Table 1. The Effect of R506Q FV on the Measurement of FVIII Clotting Activity |
|--------------------------|--------------------------|
| **FV Final Concentration** | **FVIII Clotting Activity (mU/mL)** |
|                          | Wild-Type FV | R506Q FV |
| 1.0                      | 171 (1.28)   | 164 (1.22) |
| 0.5                      | 150 (1.12)   | 139 (1.04) |
| 0.25                     | 182 (1.13)   | 147 (1.10) |

*Data represent the average of duplicate measurements. The number in parenthesis indicates the ratio of FVIII activity in the presence to that in the absence of FV.*
FV R506Q. FVα R506Q could function as a competitive inhibitor of APC in the degradation of activated FVIII (FVIIIa)18 leading to prolonged survival of the limited quantity of FVIII activity produced in some cross reacting material-positive (CRM+) hemophilia A patients. Consistent with this hypothesis, FV has been shown to enhance the proteolytic degradation of FVIII by APC with very little FVIII degradation occurring in the presence of FVα.19 FV R506Q could be defective with respect to this APC cofactor activity during FVIIIa inactivation, a potential effect of this mutation that has not been excluded.20

Alternatively, the prolonged activity of FVα R506Q could provide an amplification of the limited signal produced by a defective FVIIIa. Although not all individuals homozygous for FV R506Q present with a thrombotic episode, in one family all homozygotes had elevated levels of prothrombin fragment. Because these data suggest continuous activation of the coagulation mechanism in patients with FV R506Q, Kalafatis et al21 also recently speculated that the FV R506Q mutation could compensate for a defective FVIII in some hemophiliacs. They predicted that a hemophilic carrying FV has been shown to enhance the proteolytic action factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. N Engl J Med 332:912, 1995


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