Characterization of a Thrombin Cleavage Site Mutation (Arg 1689 to Cys) in the Factor VIII Gene of Two Unrelated Patients with Cross-Reacting Material-Positive Hemophilia A


The molecular defect responsible for moderate and severe hemophilia A has been identified for two unrelated patients with the CRM-positive form of this disorder (factor VIII activity of 0.02 and 0.06 U/mL with factor VIII antigen of 0.87 and 2.20 U/mL). In both cases, the immunopurified dysfunctional factor VIII protein is abnormal, in that the 80 Kd light chain is not cleaved by thrombin at arginine-1689. The basis for this failure was identified by polymerase chain reaction amplification of exon 14 of the variant factor VIII genes and direct sequencing of the amplified products. In both cases, a single base substitution (C to T) was identified that produces an arginine to cysteine substitution at amino acid residue 1689. These data identify the molecular defects of the two identical factor VIII variant proteins. The dysfunctional factor VIII has been designated "Factor VIII-East Hartford," the residence of the patient in whom the defect was first identified.

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Submitted August 1, 1989; accepted September 11, 1989.

Supported in part by United States Public Health Service Grants No. HL36099 and RR05737 (LWH) and HL38165 (SEA and HHK).

A portion of these studies was presented at the Annual Meeting of the American Society for Clinical Investigation, Washington, DC, May 1, 1988.

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given in Table 1. No relatives of ARC-5 have had any symptoms suggesting a bleeding disorder. While plasma samples were not obtained, clinical histories were entirely unremarkable for his parents, maternal grandfather, and three children. Studies reported in this paper were carried out on a plasma sample held at -70°C for the 10 years since the patient was last available for evaluation.

The second patient, ARC-10, is from Tennessee and is not related to ARC-5. He also has moderately severe hemophilia A by plasma assays (Table 1), but the clinical presentation is that of a patient with severe disease. The bleeding disorder was detected in childhood, factor VIII replacement therapy has been frequent, and he has chronic arthropathy. No relatives have had any symptoms suggesting a bleeding disorder and DNA studies (vide infra) document that he responded to an infusion of desmopressin (DDAVP) with an increase in factor VIII activity to 0.24 U/mL.

**Table 1. Characterization of CRM-Positive Hemophilic Plasmas in which a Mutation Alters a Factor VIII Light Chain Thrombin Cleavage Site (Arg 1689 to Cys)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>VIII:C (U/mL)</th>
<th>VIII:Ag (U/mL)</th>
<th>vWF:Ag (U/mL)</th>
<th>Clinical Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC-5</td>
<td>0.02, 0.03*</td>
<td>0.87</td>
<td>1.09, 1.35*</td>
<td>Mild; rare factor VIII treatment and no arthropathy</td>
</tr>
<tr>
<td>ARC-10</td>
<td>0.05</td>
<td>2.11</td>
<td>3.12</td>
<td>Severe; frequent factor VIII replacement therapy and chronic arthropathy</td>
</tr>
<tr>
<td>H1016</td>
<td>0</td>
<td>0.96</td>
<td>0.69</td>
<td>Severe; with recurrent hemarthroses</td>
</tr>
</tbody>
</table>

*Values obtained for two different fresh plasma samples.

**Factor VIII measurements.** Factor VIII procoagulant activity was measured by a one-stage method using factor VIII-deficient plasma as substrate. Factor VIII antigen was measured by immunoradiometric assay using 125I-labeled Fab', prepared from the plasma of a patient with an alloantibody to factor VIII, von Willebrand factor antigen was measured by immunoradiometric assay using a rabbit antibody. Citrate-anticoagulated normal plasma, pooled from 10 donors, served as a standard (1 U/mL) for VIII:C, VIII:Ag, and vWF:Ag measurements and was used as the control for immunopurification studies. Factor VIII activity measurements were carried out independently in two different laboratories for both ARC-5 and ARC-10. Similar values were obtained in both cases.

**Immunopurification of factor VIII.** The procedure for immunopurifying factor VIII from small volumes of plasma has been published. Patient plasmas or pooled normal plasma were incubated with an immunosorbent prepared by coupling IgG from a high titer factor VIII inhibitor plasma to agarose. The adsorbed protein was analyzed directly or after incubation with highly purified human thrombin (0.2 U thrombin/U VIII:Ag). The protein was eluted with sodium dodecyl sulfate (SDS), analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and identified by immunoblotting using three monoclonal anti-factor VIII. Amplification of genomic DNA and sequencing. Sequence analysis of thrombin cleavage site mutations detected in the two patients was performed using direct sequencing of enzymatically amplified genomic DNA. Because whole blood was not available for patient ARC-5, 10 mL plasma from this patient, stored at -70°C for 10 years, was centrifuged at 27,000g for 20 minutes in order to collect the few remaining leukocytes in the plasma. The pellet was suspended in 100 µL lysis buffer (25 mmol/L EDTA, 0.1 mol/L NaCl) and lysed by addition of 5 µL of 10% SDS. After incubation with 100 µg proteinase K at 37°C overnight and subsequent phenol- and chloroform-extraction, genomic DNA was purified by spin-dialysis on a Centricon 30 (Amicon, Danvers, MA) and subjected to amplification by the polymerase chain reaction (PCR). The 326 bp-region of the FVIII gene, including the thrombin cleavage site at the 3' end of exon 14, was amplified by PCR using *Thermus aquaticus* (Taq) DNA polymerase and directly sequenced as previously described. The amplification primers were 5'-CCT GGG CAA AGC AAG GTA GG-3' (PCR5) and 5'-AGC AGA GCA AAG AAG TAA CCC-3' (PCR6).

Amplification (30 cycles for ARC-10 and 60 cycles for ARC-5) was carried out in volumes of 100 µL containing 500 ng genomic DNA for ARC-10 and all the genomic DNA isolated for ARC-5, 400 mmol/L of each PCR primer, 200 µmol/L of each dNTP, 10 mmol/L Tris-HCl buffer (pH 8.3), 50 mmol/L KCI, 1.5 mmol/L MgCl2, 100 µg/mL gelatin and 2 U of Taq DNA polymerase. Each cycle consisted of 30 seconds denaturation at 94°C, 30 seconds reannealing at 55°C (90 seconds for ARC-5), and 90 seconds extension at 72°C using a DNA Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT). Amplified DNA was desalted and excess dNTPs were removed by spin-dialysis on a Centricon 30. The purified DNA was sequenced directly: 10 ng SP7 sequencing primer (5'-GAT TTT GAC ATT TAT GAT-Y), end-labeled with γ[32P]adenosine triphosphate (ATP) using T4 polynucleotide kinase, was annealed with 80 ng of PCR product on ice after heat denaturation at 95°C for 5 minutes. The reaction mixture was divided into four tubes containing 62 µmol/L unlabelled dNTPs, 6.2 µmol/L dNTPs and 2 U of T7 DNA polymerase (Sequenase USB, Cleveland, OH) in the sequencing reaction buffer (25 mmol/L Tris-HCl, pH 7.5; 10 mmol/L MgCl2, 70 mmol/L NaCl, 7 mmol/L dithiothreitol). After incubation at 37°C for 15 minutes, the reaction was stopped with 95% formamide, 20 mmol/L EDTA, 50 mg/dL bromophenol blue, 50 mg/dL xylene cyanol FF. Samples were boiled for 3 minutes and electrophoresed in a 6% polyacrylamide/8 mol/L urea gel at 58 watts for 2 hours. Gels were dried and exposed to Kodak XAR-5 film (Eastman Kodak Co, Rochester, NY) for 16 hours.

**RESULTS**

**Immunopurification of factor VIII protein.** Factor VIII was directly separated from eight CRM-positive plasmas by immunosorption using a human antibody to factor VIII that had been coupled to agarose beads. Characterization of the protein was then accomplished by SDS-PAGE followed by immunoblotting using monoclonal anti-factor VIII antibodies that react with light chain and heavy chain epitopes (Fig 1).

The factor VIII fragments (before and after thrombin treatment) of six of the eight CRM-positive plasmas could not be distinguished from those of normal plasma. In contrast, while the variant factor VIII immunosolated from

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**Fig 1. Human factor VIII, its domain structure (A1-A3, B, C1, and C2), and three thrombin cleavage sites (arrows). Below this are shown the thrombin cleavage fragments. The fragment mass values in kDa correspond to those of Fulcher et al. The solid boxes represent the regions containing epitopes for the MoAb used to identify factor VIII fragments.**
ARC-5 and ARC-10 plasmas had apparently normal fragments (Fig 2, lanes 6 and 11), the patterns after thrombin treatment were different. The 80 Kd light chain was not cleaved to the 72 Kd fragment in either case (lanes 7-10 and 12-15). Heavy chain cleavage was normal, however, with a 44 Kd fragment detected 0.5 minutes after adding thrombin. The 44 Kd fragment is prominent until further thrombin proteolysis at 60 minutes leads to additional fragmentation, including the accumulation of 17 Kd material. Because the C8 anti-heavy chain monoclonal antibody (MoAb) used in these studies reacts weakly with the intact (92 to 200 Kd) fragments, they are not clearly seen in this figure. The thrombin-resistant minor band at approximately 140 Kd in ARC-5 and ARC-10 eluates was not seen in comparable eluates from normal plasma or other CRM-positive or CRM-negative hemophilic plasmas. It is only detected by MoAb MAB038, suggesting that it consists of the factor VIII light chain and a portion of the B domain. Increasing the ratio of thrombin to 2 U/U of VIII:Ag and increasing the duration of incubation to 16 hours at room temperature did not lead to light chain cleavage by this analysis (data not shown).

Mixtures of small amounts of normal plasma with ARC-5 plasma were also characterized by immunopurification and analysis in order to determine the sensitivity of the method for detecting light chain cleavage. The incubation conditions were chosen to be sure that there was complete cleavage of normal plasma factor VIII at Arg 1689 (ie, 2 U of thrombin/U VIII:Ag for 2 hours at room temperature). Factor VIII light chain cleavage was detected in the autoradiograms when the mixtures contained 10% normal plasma, but could not be detected when 2% or 5% normal plasma was added to factor VIII variant (ARC-5) plasma.

Characterization of the molecular defects. These data suggest that the molecular abnormality for ARC-5 and ARC-10 is a change in its structure near Arg 1689, the thrombin cleavage site that generates a 72 Kd light chain fragment from the 80 Kd light chain. Exon 14 of the factor VIII gene contains the codon for Arg 1689, and it was amplified from ARC-5 and ARC-10 genomic DNA by PCR. DNA sequence analyses showed the same single nucleotide change, C to T, at the codon for Arg 1689 in both cases (Fig 3). This missense mutation causes a Cys substitution for Arg and thereby prevents thrombin cleavage of the factor VIII light chain. The mutation also creates a new PstI restriction site in the factor VIII gene. This site was used as the diagnostic criterion to detect the mutation in the propositus and relatives of ARC-10, after PCR-amplification of exon 14 of their factor VIII genes. No other family member, including the patient’s mother, had the Arg 1689 to Cys mutation. Therefore, the mutation occurred de novo in the patient’s mother’s germ cells.

These data identify the molecular basis of two identical factor VIII variant proteins that lack procoagulant function because of impaired thrombin activation. The abnormal factor VIII was designated “Factor VIII-East Hartford” according to the residence of the patient (ARC-5) in which the defect was first detected and characterized. In that
instance, the thrombin cleavage site defect was identified first and the molecular studies established the nature of the mutation. In the other patient, ARC-10, the nucleotide change in the codon for Arg 1689 was detected first and the functional defect was confirmed by immunopurifying the variant factor VIII from plasma.

DISCUSSION

Identical factor VIII gene missense mutations have been identified in two unrelated hemophilic patients, ARC-5 and ARC-10. This thrombin cleavage site mutation prevents activation and procoagulant function because of an Arg to Cys substitution at amino acid 1689. The absence of thrombin-generated factor VIII light chain cleavage and loss of procoagulant function in this protein are consistent with the data of Pittman and Kaufman for a factor VIII variant that was generated by site-directed mutagenesis so that Arg 1689 was converted to isoleucine. As is the case for many factor VIII single nucleotide mutations, the substitution in these two hemophilic patients is CpG to TpG.

The clinical and coagulation laboratory data for these two patients are quite different from that reported by Gitschier et al for a patient with severe hemophilia A, and no detectable factor VIII procoagulant activity, in whom the same mutation, Arg 1689 to Cys, was detected by PCR-amplification of genomic DNA. Subsequent studies have documented that the variant factor VIII light chain in that patient’s plasma is resistant to thrombin cleavage. The reason for the spectrum of laboratory and clinical findings for the three patients (Table 1) is not clear. The “specific activity” (ratio of VIII:C to VIII:Ag) is the same for the two patients that we have studied (0.023), while the patient studied by Tuddenham et al had no detectable factor VIII procoagulant activity.

Although the three plasmas have quite different VIII:Ag levels, the VIII:Ag/vWF:Ag ratios are all comparable to those of normal plasma.

The only other published description of CRM-positive hemophilia caused by a thrombin cleavage site mutation is factor VIII-Kumamoto, in which an Arg 372 to His substitution prevents factor VIII heavy chain cleavage. That patient (ARC-1) has a moderately severe bleeding disorder, a plasma VIII:C level of 0.05 U/mL, and an VIII:C/VIII:Ag ratio of 0.015. Data for the three patients that we have studied raise questions about the nature of factor VIII procoagulant activity in plasmas containing variant factor VIII that resist thrombin cleavage. Two possibilities must be considered. The factor VIII activity that is reproducibly detected in these plasmas may indicate that the variant factor VIII can accelerate factor X cleavage by factor IXa, even though the factor VIII light chain (ARC-5 and ARC-10) or the factor VIII heavy chain (ARC-1) is not cleaved by thrombin. The low levels of VIII:C that were detected for recombinant Arg 1689 to Ile could have the same explanation. If this is the case, the H-10 variant factor VIII must differ from ARC-5 and ARC-10 in some way, even though it has an identical substitution at amino acid residue 1689. Our genetic analyses do not include possible coincidental mutations or independent polymorphisms that distinguish the variants, not detectable by protein gel mobility measurements. In view of the low plasma concentration of factor VIII and the very large gene responsible for its synthesis, identifying the nature of such a difference poses a formidable challenge.

Alternatively, low levels of factor VIII procoagulant activity may be detected in ARC-1, -5, and -10 because small amounts of thrombin cleavage are possible in spite of the
amino acid substitution. This possibility cannot be excluded, since the analytic method cannot detect cleavage of 5% of the factor VIII molecules. If 2% to 5% of the factor VIII light chains are cleaved in spite of the Arg to Cys mutation, low levels of factor VIII procoagulant activity would be expected. Further improvement in the analytic methods may make it possible to resolve this question.

The differences between the two patients reported here and H-10 studied by Tuddenham et al.\textsuperscript{10,22,23} also make it necessary to consider possible artifacts in the DNA analysis that might have affected our observations. Because the sequencing was performed on amplified DNA, not by cloning, we are confident that the PCR products accurately reflect the patients’ genetic material. Unless a polymerase error occurs at a very early amplification step in one molecule, it is so diluted among the millions of molecules that it is not visible after the sequencing reaction. Moreover, the mutation identified by the PCR reaction changes a \textit{PstI} cleavage site, and this change was verified by direct analysis of ARC-10 DNA.

Because the isolation of DNA from ARC-5 had to be accomplished by an unusual strategy, PCR-amplification of DNA from the small number of leukocytes present in a 10-year-old frozen plasma sample, the possibility of cross-contamination needs to be considered. It is not possible that the ARC-5 data are due to contamination, however, since DNA from the only other sample in which we have detected the Arg 1689 to Cys mutation (ARC-10) had not yet been sent to the Johns Hopkins laboratory when the ARC-5 mutation was identified. Sequencing the remainder of the factor VIII gene has not been performed for either patient since the gene is so large (168 kb), and because of the convincing biochemical and genetic evidence that the Arg 1689 to Cys mutation causes hemophilia A in these individuals.

Overall, our data suggest that thrombin cleavage site mutations are a relatively common cause of CRM-positive hemophilia. Three of the first 12 such plasmas that we have studied have such a mutation, either at Arg 372\textsuperscript{15} or Arg 1689. The nine other CRM-positive plasma samples studied to date have apparently normal factor VIII chain composition and cleavage patterns following incubation with thrombin. Alternative causes of the dysfunction will be sought for these plasmas; among the possibilities are ineffective interactions with phospholipids, von Willebrand factor, factor IXa, and factor X. Studies to characterize these plasmas will lead to better understanding of the molecular pathology responsible for hemophilia, and should further clarify the structural-function relationships that are critical for factor VIII procoagulant activity.

ACKNOWLEDGMENT

We thank Drs Carol Kasper and James Ballard, and the physicians in the Dept of Clinical Pathology, Tokyo Medical College, for providing some of the patient samples used in this study, Dr Carol Fulcher for providing MoAb J16D-9 used to detect the 72 Kd fragment, Dr John Fenton for providing purified human thrombin, Dr Ashraf Aly for assistance in characterizing the thrombin cleavage assay, and Debbie Wilder for manuscript preparation.

REFERENCES

18. Breckenridge RT, Ratnoff, OD: Studies on the nature of the
circulating anticoagulant directed against antihemophilic factor: 
19. Hoyer LW: Immunologic studies of antihemophilic factor 
(AHF, factor VIII). IV. Radioimmunoassay of AHF antigen. J Lab 
Clin Med 80:822, 1972
20. Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, 
Kazazian HH Jr: Characterization of beta-thalassaemia mutations 
using direct genomic sequencing of amplified single copy DNA. 
21. Fulcher CA, Roberts JR, Holland LZ, Zimmerman TS: 
Human factor VIII procoagulant protein. Monoclonal antibodies 
define precursor-product relationships and functional epitopes. J 
Clin Invest 76:117, 1985
22. Rotblat F, Tuddenham EGD: Immunologic studies of factor 
VIII coagulant activity (VIII:C) 1. Assays based on a haemophilic 
23. O'Brien DP, Tuddenham EGD: Purification and characteriza-
tion of factor VIII 1,689-Cys: A nonfunctional cofactor occurring in 
a patient with severe hemophilia A. Blood 73:2,117, 1989