Six Point Mutations That Cause Factor XI Deficiency

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We have identified six novel types of mutation that cause factor XI deficiency, an inherited bleeding disorder. Two are point mutations that interfere with the normal splicing of exons in the mRNA and four are point mutations that result in amino acid substitutions. One of these amino acid substitutions (Asp 16 → His) is near the amino terminal end of the protein. The other three amino acid substitutions (Leu→Pro, Thr 304 → Ile, and Glu 323 → Lys) are in the fourth apple domain, a region that mediates dimerization of identical subunits of factor XI. All four amino acid substitutions cause a reduction in the amount of factor XI secreted from cells grown in vitro. © 1995 by The American Society of Hematology.

Factor XI is a zymogen of a serine protease that participates in the blood coagulation cascade. It is synthesized in the liver and secreted into the blood as a dimer of identical subunits held together by a single cysteine disulfide bond. In vitro factor XI is converted into its active form, Factor XIa, through proteolytic cleavage, either by factor XIIa in the presence of high molecular weight kininogen and a negatively charged surface or by thrombin in the presence of a negatively charged surface. Factor XIa then activates factor IX.

Both factor XIa and thrombin activate factor XI by cleaving each identical polypeptide into a heavy chain and a light chain. The two heavy chains and the two light chains are held together by three disulfide bonds. Each light chain contains the catalytic domain of the enzyme, which is homologous to the trypsin family of serine proteases. Each heavy chain is composed of four “apple domains” regions of 90 or 91 amino acids with characteristic patterns of cysteine disulfide bonds. The first apple domain contains the site for the binding of factor XI to its cofactor, high molecular weight kininogen. The second apple domain contains the site for the binding of factor XI to its substrate, factor IX. The fourth apple domain mediates dimer formation and also contains a binding site for factor XIIa.

Deficiency in factor XI results in a bleeding disorder that is milder than hemophilia A or B. Spontaneous bleeding is rare. Abnormal bleeding usually occurs only as the result of an accident or a surgical procedure. Factor XI deficiency is inherited as an autosomal, incompletely recessive trait. Homozygotes have severe factor XI deficiency (0% to 20% of the normal plasma factor XI activity level) and heterozygotes have partial factor XI deficiency (30% to 70% of the normal plasma factor XI activity level). Both heterozygotes and homozygotes are at risk of excessive bleeding. Factor XI deficiency is almost always caused by a lack of the protein in the plasma, not by a dysfunctional protein that is present in normal levels. Only 3 cases of cross-reacting material-positive (CRM) factor XI deficiency have been reported, all in German patients.

Factor XI deficiency is rare in the general population but is one of the most common inherited disorders of Ashkenazi Jews. It has been estimated that in this population the frequency of defective factor XI alleles is 13.4%. Asakai et al sequenced the factor XI genes of 6 Ashkenazi Jews homozygous for factor XI deficiency and found 3 types of point mutation (types I, II, and III). The type I mutation interferes with the normal splicing of exons in the mRNA. The type II mutation introduces a premature termination codon. The type III mutation results in the substitution of leucine for phenylalanine 283 in the fourth apple domain.

This substitution causes a reduction in the amount of factor XI secreted from the cell. The type I and type III mutations together account for most of the factor XI deficiency in Ashkenazi Jews, but account for only 12% of the factor XI deficiency in non-Jewish patients. Six other types of mutation to the factor XI gene have since been reported. Two were found in Japanese patients, three were found in English patients, and one was found in an Ashkenazi Jewish patient. Two of these mutations are single-base changes that introduce premature termination codons. One point mutation results in the substitution of glutamic acid for glycine 350 in the fourth apple domain. Two point mutations result in amino acid substitutions in a region of the light chain encoded by exon 12, replacing phenylalanine 442 with valine and threonine 475 with isoleucine. All three of these amino acid substitutions cause a reduction in the amount of factor XI secreted from cells grown in culture. One mutation is a 14-bp deletion involving 3 bp of the 3’ end of exon 14 and 11 bp of the 5’ end of intron N.

We sequenced the factor XI genes of patients with factor XI deficiency not accounted for by the mutations previously described and have identified six novel types of mutation.

MATERIALS AND METHODS

Identification of patients with unknown types of mutations to the factor XI gene. Blood was obtained from patients by venepuncture after obtaining informed consent. As previously described, a modified activated partial thromboplastin time assay was used to identify patients with factor XI deficiency, and restriction enzyme analysis of amplified regions of the factor XI gene was used to identify patients whose factor XI deficiency could be fully explained by the presence of type I, II, or type III mutations.

Sequence analysis. Genomic DNA was isolated from peripheral leukocytes by established methods. The polymerase chain reaction (PCR) was performed using these genomic DNA samples as templates.

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amplify 9 regions that together include all 15 exons of the factor XI gene and the intron/exon boundaries. The synthetic oligonucleotide primers used for PCR were 5'-CTGACATACCTGATT TTACGAC-3' and 5'-TTTTTGTTGTATCGTGGAGAATTAGACAC-3' for exons 1 and 2, 5'-CAACATAAGCGTGCCTGAC-3' and 5'-GCTGGTATTTGTTGATGAGA-3' for exons 3 and 4, 5'-GAATTTGGAAGATCTCAGTGCTC-3' and 5'-ATGCACATTGCTCGAC-3' for exon 5, 5'-TTCAGTGGAGATTGAGAATA AGACAC-3' and 5'-TATCTTTACTTTCTCTAGGTGCTGT-3' for exons 6 and 7, 5'-ACGTCATCTGATGGTGTG-3' and 5'-ACATCTTGATGATGTATGAA-3' for exons 9 and 10, 5'-ATTGGCTCTGGTGGACAGTGTA-3' and 5'-CCAGTTCACTTTGAGACAA-3' for exons 11 and 12, 5'-AGGAGATTATATTTGCTTCTA-3' and 5'-TTGCCATATCTTCAGGTA-3' for exons 13 and 14, and 5'-GTCGTAGCTCTGATGGCACC-3' and 5'-TACAACGAATCAGAGGAG-3' for exon 15. PCR was performed at 94°C for 2 minutes and 30 seconds, followed by these steps, repeated 40 times: 94°C for 30 seconds, 55°C for 2 minutes and 30 seconds, and then 72°C for 4 minutes. The amplified fragments were isolated from a 1% agarose gel. The deoxynucleotide sequences of the amplified fragments were determined by direct deoxynucleotide sequencing. In cases in which direct sequencing was not sufficient to clearly identify mutant alleles, the amplified DNA fragments were cloned into a plasmid to sequence each allele separately. In such cases, five fragments of each sequence were sequenced to increase the probability of sequencing both alleles. PCRs and sequencing reactions were repeated to verify results.

Construction of expression vectors for normal and mutant factor XI. Polyadenylated RNA was isolated from human liver using the method of Chromczynski and Sacchi. A synthetic DNA copy (cDNA) of the human liver mRNA was synthesized according to the method of Gubler and Hoffman. The cDNA copy of the mRNA for factor XI was amplified by PCR using the same time and temperature conditions as above. The resulting PCR products were ligated into pEXV-3, an expression vector derived by cloning an SV40 origin of replication into pBR322. The deoxynucleotide sequence of the cloned factor XI cDNA insert was determined to insure that it was free of errors caused by the Taq DNA polymerase. Point mutations were introduced into the cloned factor XI cDNA by site-directed mutagenesis. The mutations in the cDNA were confirmed by deoxynucleotide sequencing.

Cell cultures and expression of factor XI. COS-7 cells (ECACC no. 87021302) were grown in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum, and 5,000 IU/mL streptomycin (GIBCO, Paisley, UK) in a 5% CO2 atmosphere. Cells were transfected by electroporation using the Gene Pulser apparatus (BioRad, Hercules, CA) set at 250 V, 250 μFD. Thirty micrograms of plasmid was used to transfet 2.5 × 106 cells. The electroporated cells were transferred to 5 mL growth medium and grown in a 60-mm culture dish for 48 hours. The cells were then washed three times with DMEM and grown in 5 mL of serum-free growth medium for 12 hours. The serum-free growth medium was then collected and polyadenylated RNA was isolated from the cells.

Detection of factor XI secreted from COS-7 cells. The 5 mL volume of serum-free growth medium was reduced to 200 μL by centrifuging the solution through the filter of a Centricon-30 concentrator (Amicon, Beverly, MA). Equal amounts of protein of each sample were electrophoresed on a nonreducing 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins were transferred from the gel to a nitrocellulose membrane by electrophoretic transfer. Factor XI on the nitrocellulose membrane was detected using a biotinylated goat antihuman factor XI polyclonal antibody (GAHXI- PGI; Enzyme Research Laboratories, Swansea, UK). The biotinylated, bound antibody was then detected using a streptavidin-horseradish peroxidase conjugate (Pierce Europe, Luton, UK) and chemiluminescence detection reagents (Amersham International, Amersham, UK) according to the manufacturer’s instructions. The nitrocellulose membrane was exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 30 minutes.

RNA extraction, Northern blotting, slot blotting, and hybridization. Polyadenylated RNA was isolated from transfected COS-7 cells using a commercially available kit (Pharmacia Biotech, St Albans, UK). RNA samples were electrophoresed in a denaturing 1.1% agarose/formaldehyde gel. Polyadenylated RNA was loaded (1.7 μg/lane). The gel was washed in 20× SSC (1× SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate), and the RNA was transferred overnight to a Genescreen membrane (Dupont, New England Nuclear, Boston, MA) using a commercial available kit (Pharmacia Biotech, St Albans, UK). A synthetic DNA copy (cDNA) of the human liver mRNA was synthesized according to the method of Chromczynski and Sacchi. The cDNA copy of the mRNA for factor XI was amplified by PCR using the same time and temperature conditions as above. The resulting PCR products were ligated into pEXV-3, an expression vector derived by cloning an SV40 origin of replication into pBR322. The deoxynucleotide sequence of the cloned factor XI cDNA insert was determined to insure that it was free of errors caused by the Taq DNA polymerase. Point mutations were introduced into the cloned factor XI cDNA by site-directed mutagenesis. The mutations in the cDNA were confirmed by deoxynucleotide sequencing.

Fig. 1. A to G point mutation, which changes the obligatory di-nucleotide AG of the splice acceptor site before exon 10 to GG, preventing normal splicing of exon 9 to exon 10.
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NORMAL

Fig 2. G to C point mutation, which occurs at the fifth nucleotide of intron E after exon 5.

RESULTS

In a previous study, we identified patients with factor XI deficiency not fully accounted for by the mutations previously described. In this study, we sequenced the factor XI genes of these patients to identify unknown types of mutation that cause factor XI deficiency. Genomic DNA isolated from the peripheral leukocytes of these patients was used as the template for the PCR. We amplified by PCR 9 DNA fragments that together included all 15 exons of the factor XI gene and the intron/exon boundaries. The nucleotide sequences of the amplified DNA fragments were determined by direct dideoxynucleotide sequencing. The primers used for sequencing were the same oligonucleotides that were used for PCR. In some cases, the amplified DNA fragments were also cloned into plasmids, and the cloned fragments were sequenced. Each time we identified a mutation, we verified our results by repeating the PCR and then sequencing the newly amplified DNA fragment. This procedure ensured that the mutation was present in the genome of the patient and was not simply an error introduced by the Taq DNA polymerase in the PCR. Using these techniques, we identified 6 novel types of point mutation in the factor XI gene.

Two of the six novel point mutations occur within introns at intron/exon boundaries. One of the point mutations, A to G, occurs at the second to last base of intron N, between exon 9 and exon 10 (Fig 1). Another point mutation, G to C, occurs at the fifth base of intron E, between exon 5 and exon 6 (Fig 2).

Four of the six novel point mutations result in amino acid substitutions. A change of G to C in exon 3 changes GAC coding for aspartic acid 16 to CAC coding for histidine (Fig 3). This amino acid substitution occurs in the first apple domain. The other three novel point mutations that result in amino acid substitutions occur in exon 9, in a region of the gene that encodes for the fourth apple domain. One of these
point mutations, T to C, changes CTG coding for leucine 302 to CCG coding for proline (Fig 4). Another point mutation in exon 9, C to T, changes ACC coding for threonine 304 to ATC coding for isoleucine (Fig 5). The sixth novel point mutation, G to A near the end of exon 9, changes GAA coding for glutamic acid 323 to AAA coding for lysine 323 (Fig 6).

To determine whether each of the amino acid substitutions we identified causes factor XI deficiency or is simply a polymorphism, we expressed each of the mutant proteins in vitro and compared their expression with that of normal factor XI. We cloned factor XI cDNA into the expression vector pEXV-3. Point mutations that result in the amino acid substitutions Asp 16→His, Phe 283→Leu (type III), Leu 302→Pro, Thr 304→Ile, and Glu 323→Lys were introduced into factor XI cDNA by site-directed mutagenesis and cloned into pEXV-3.

The expression vectors were introduced into COS-7 cells by electroporation. After the transfected cells had grown for 48 hours, the growth medium was replaced with serum-free growth medium. After 12 hours, the serum-free growth medium was collected, concentrated, and electrophoresed on an SDS-polyacrylamide gel. The separated proteins were transferred from the gel to a nitrocellulose membrane, and factor XI on the nitrocellulose membrane was detected using a polyclonal antibody against factor XI (Fig 7). Factor XI was secreted from COS-7 cells expressing normal factor XI cDNA but was secreted only in reduced amounts from cells expressing each of the mutant factor XI cDNAs.

To determine whether the reduction in secreted factor XI protein was caused by a corresponding reduction in factor XI mRNA transcribed in the transfected COS-7 cells, we assayed for factor XI mRNA in each of the COS-7 cell cultures by both Northern and slot blot analyses (Fig 8). On Northern blot analysis, the factor XI antisense RNA probe hybridized with a doublet of the appropriate molecular weight in RNA isolated from cells transfected with both normal and mutant factor XI cDNA expression constructs. No factor XI mRNA was detected in cells transfected with the expression plasmid. A doublet rather than a single band was observed that was probably caused by alternative splicing of RNA transcribed from the expression vector. Factor XI mRNA was expressed in comparable levels in COS-7 cells transfected with the normal and with each of the mutant factor XI cDNAs. Therefore, the lack of secreted factor XI was caused by posttranslational effects of the amino acid substitutions and not by an absence of factor XI mRNA.

DISCUSSION

Two of the point mutations that we identified interfere with the normal processing of mRNA. The factor XI gene, like almost all eukaryotic nuclear genes, consists of coding (exon) and noncoding (intron) sequences. The intron sequences are spliced out of the initial pre-mRNA transcript before the mRNA is transported into the cytoplasm for translation. A highly conserved sequence of eight nucleotides at the boundary between the 3' end of an exon and the 5' end of an intron, and another highly conserved sequence of four
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The splicing of intron I G to A point mutation in exon 9, which changes GAA coding for glutamic acid 323 to AAA coding for lysine.

One of the point mutations we have found changes the last two bases of intron N from AG to GG, making normal splicing of exon 9 to exon 10 impossible. The sequence AG occurs at the 3' end of introns in virtually all known eukaryotic nuclear genes. In one study, it was shown that any alteration of the 3' AG of an intron abolished splicing in vitro and caused splicing at cryptic, rather than the normal, 3' junctions in vivo. There are at least three other examples of AG to GG mutations at the 3' splice junction site that cause disease in humans. This mutation in the second exon of the β-globin gene causes a form of β thalassemia in the third intron of the gene for apolipoprotein E it causes familial type III hyperlipoproteinemia, and in the sixth intron of the serum albumin gene it causes analbuminemia. The splicing at a cryptic 3' junction that is elicited by this mutation would probably create a premature termination codon in the mRNA, and translation of this abnormal mRNA would produce a truncated protein. Alternatively, the abnormal mRNA might not be transported into the cytoplasm, and so not be translated.

A second splice junction mutation occurs in intron E, five bases after exon 5, replacing G with C. G is the fifth base in 82% of the introns in all known eukaryotic nuclear genes, changing the base at this position reduced the levels of mature mRNA obtained in vitro studies. Three other examples of a mutation in the G at the fifth base of an intron have been found. G to C at position 5 of intron 1 in the β-globin gene was found in a proband with β thalassemia. G to A at the fifth base of intron 14 of the pro-α(1) procollagen gene is one of the causes of lethal osteogenesis imperfecta. A mutation in the pro-α(2) procollagen gene, G to A at the fifth base of intron 33, also causes lethal osteogenesis imperfecta. In all three cases, the mutations cause abnormal processing of RNA transcripts. Because DNA sequence analysis showed that a patient's defective factor XI gene is identical to the normal gene in every region examined, except for this single base substitution, we conclude that this mutation interferes with the normal splicing of exon 5 to exon 6.

Four of the point mutations to the factor XI gene that we identified result in amino acid substitutions (Asp 16 → His, Leu 302 → Pro, Thr 304 → Ile, and Glu 323 → Lys). Like the four factor XI amino acid substitutions previously described (Phe 283 → Leu, Gly 350 → Glu, Phe 442 → Val, and Thr 475 → Ile), the four novel amino acid substitutions cause a reduction in the amount of factor XI secreted from cells in vitro. This finding is consistent with the observation that deficiency in plasma factor XI clotting activity is caused, with only 3 reported exceptions, 12,13 by a lack of the protein in the plasma, not by a dysfunctional protein that is present in normal levels.

Factor XI shares 58% amino acid sequence homology
with another plasma protein, prekallikrein. Seven of the eight amino acid substitutions in factor XI now identified replace residues that are conserved between factor XI and plasma prekallikrein. The only exception, Leu 302 → Pro, occurs adjacent to cysteine 303, which forms a disulfide bond with cysteine 309. Robson structural predictions indicate that the substitution of proline for leucine 302 causes a helical conformation at residues Cys 299, Gln 300, and Lys 301 of the normal protein to be changed to turn and coil conformations and a turn at Ala 306 to be changed to an extended conformation. These changes in the conformation of the protein might prevent the formation of the Cys 303-Cys 309 disulfide bond, altering the structure of the fourth apple domain.

Three of the amino acid substitutions identified (Leu 302 → Pro, Thr 304 → Ile, and Glu 323 → Lys) occur in the fourth apple domain, the location of the Phe 283 → Leu (type III) and Gly 350 → Glu substitutions previously described. The fact that 62.5% (5 of 8) of the known amino acid substitutions in factor XI occur in the fourth apple domain, a domain that comprises only 15% of the protein (90 of 607 amino acids of each identical subunit), suggests that amino acid substitutions in the fourth apple domain are more likely than amino acid substitutions in other areas to cause factor XI deficiency.

The fourth apple domain mediates the formation of dimers between identical subunits of factor XI. In a pulse-chase experiment, Meijers et al detected much less of the dimerized form of factor XI in cells transfected with the cDNA for Leu 283 factor XI than in cells transfected with the cDNA for normal factor XI, but detected slightly higher levels of the undimerized form of factor XI in cells transfected with Leu 283 factor XI cDNA. They proposed the theory that the Leu 283 substitution retards dimerization of the identical subunits of factor XI and that the undimerized subunits are unstable and not secreted, but are quickly degraded. The occurrence of so many amino acid substitutions in the fourth apple domain suggests that the integrity of this domain is necessary for the stability of the protein. However, studies such as those performed by Meijers et al will be required for personal use only.
to test whether the 3 novel amino acid substitutions in the fourth apple domain affect the expression of factor XI in the same way as the Leu 283 substitution.

The occurrence of 3 amino acid substitutions outside the fourth apple domain indicates that mechanisms other than interference with dimerization can cause a reduction in secreted factor XI. The replacement of Asp 16 in the first apple domain of factor XI is a radical substitution, because the wild-type aspartate side chain is small and negatively charged at physiologic pH, whereas the histidine group is bulky and hydrophobic with neutral charge. It is difficult to predict the effect of such an amino acid substitution, but the alteration of charge and shape and the resultant conformational change of this region of the molecule might interfere with chain folding.

Most of the factor XI deficiency in Ashkenazi Jews can be accounted for by the type II and type III mutations.18,19 The remainder of cases in Ashkenazi and most of the cases in others are caused by a number of other types of mutation. All 15 types of mutation to the factor XI gene now identified cause factor XI deficiency by causing a lack of the protein in the plasma. Three mutations introduce a premature termination codon, four mutations disrupt normal splicing of exons in the mRNA, and eight mutations result in amino acid substitutions. Five of the eight amino acid substitutions are in the fourth apple domain. This suggests that exons 9 and 10, which encode the fourth apple domain, are more likely than other parts of the gene to include mutations that cause factor XI deficiency.

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