A Frame Shift Mutation in the Fibrinogen Aα Chain Gene in a Kindred With Renal Amyloidosis

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A new American kindred with amyloidosis was found by single-strand conformation polymorphism analysis to have a mutation in the fibrinogen Aα chain gene. Affected members in this kindred have autosomal dominant amyloid nephropathy. DNA sequencing showed a single nucleotide deletion at the third base of codon 524 of the fibrinogen Aα chain gene (4904delG) that resulted in a frame shift and premature termination of the protein at codon 548. Antiserum was produced to a portion of the abnormal peptide predicted by the DNA sequence and amyloid deposits were immunohistologically proven to contain this abnormal peptide. Two of the propositus' 4 children were positive for the mutant fibrinogen Aα chain gene by restriction fragment length polymorphism analysis based on polymerase chain reaction. These two mutant gene carriers now in the second decade of life show no clinical symptoms of amyloidosis as yet but have lower plasma fibrinogen concentrations when compared with their normal siblings. This is the first description of a kindred with renal amyloidosis and low plasma fibrinogen and also the first report of amyloidosis caused by a frame shift mutation.

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CASE REPORTS

A 41-year-old woman (III-2, Fig 1) noticed general fatigue and was found to have grade 3 proteinuria. In the following year, she was produced to a portion of the abnormal peptide predicted by the DNA sequence and amyloid deposits were immunohistologically proven to contain this abnormal peptide. Two of the propositus’ 4 children were positive for the mutant fibrinogen Aα chain gene by restriction fragment length polymorphism analysis based on polymerase chain reaction. These two mutant gene carriers now in the second decade of life show no clinical symptoms of amyloidosis as yet but have lower plasma fibrinogen concentrations when compared with their normal siblings. This is the first description of a kindred with renal amyloidosis and low plasma fibrinogen and also the first report of amyloidosis caused by a frame shift mutation.

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Individuals with proven amyloidosis.

3). and 4 children (IV-I, IV-2, IV-3, and IV-4) using the conventional method. One hundred twenty normal control subjects were also tested by this method. A SSCP analysis was used to screen DNAs of patients with amyloidosis for a mutation in the fibrinogen Aα chain gene. One patient's DNA by PCR was enzymatically amplified using polymerase chain reaction (PCR) directly incorporating radioactive nucleotide. PCR was performed using primers (Fib3 primer, 5'-CTTCGACAC-TGCTCTCAACTG-3'; and Fib2 primer, 5'-TCCTCTGTTGTA-ACTCGTTGCT-3'), GeneAmp PCR reagents (Perkin-Elmer Cetus, Norwalk, CT), and 32P-labeled dCTP (Dupont NEN, Boston, MA). PCR products were then heated at 95°C for 4 minutes, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. PCR products were diluted with 100 vol of buffer containing 50% formamide, 0.05% sodium deoxycyl sulfate (SDS), 0.02% xylene cyanol FF, 0.02% bromophenol blue, and 10 mmol/L EDTA. The PCR products were then heated at 95°C for 5 minutes and loaded onto a nondenaturing polyacrylamide gel (5% T, 2% C, 40 × 20 × 0.04 cm). The gel was electrophoresed at 4°C for 18 hours at 3 W, dried, and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY).

Direct DNA sequence analysis. DNA fragments containing a part of the fibrinogen Aα chain gene were amplified from the patient’s DNA by PCR as described above. They were then separated by the electrophoresis through 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) and the band was excised and melted in 300 μL of TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0). Asymmetric PCR was performed using 1 μL of the gel-purified template and the same sets of primers (primer ratio, 1:30). The sample was extracted with chloroform and subjected to spin dialysis with a Centricon-30 concentrator (Amicon, Beverly, MA). Seven microliters of the sample was extracted with chloroform and subjected to spin dialysis with a Centricon-30 concentrator (Amicon, Beverly, MA).

Restriction fragment length polymorphisms (RFLP) analysis. Because the nucleotide deletion does not create or abolish a restriction endonuclease site to distinguish the mutant allele from normal allele, DNAs from the family members were examined by PCR-induced mutation restriction analysis (PCR-IMRA). A new primer designed to give a recognition site for HindII in the mutant gene (Fib5 primer 5'-ATGTAGGAGATTTGTGCTAGTTA-3') was synthesized. This primer has a T instead of a normal G at the second position from the 3' end and therefore creates a HindII recognition site only when the fibrinogen Aα gene is missing a G at position 4904. PCR was performed with this new primer and Fib2 primer under the same conditions as described above. Ten units of restriction endonuclease HindII (New England Biolabs Inc, Beverly, MA) was directly added to 10 μL of PCR products and incubated at 37°C overnight. These samples were electrophoresed through 4% NuSieve GTG agarose gel, stained with ethidium bromide, and photographed over UV light.

Production and purification of specific antibody. A 13 amino acid length peptide (GAQNLAGSQQRN) corresponding to residues 528 through 540 of the abnormal fibrinogen Aα chain sequence was synthesized linked at the C-terminus to an 8-branched MAP resin on a 432A Peptide Synthesizer (Applied Biosystems Inc, Foster City, CA) according to the manufacturer’s protocol. A rabbit was immunized with the cleaved synthetic MAP-peptide and antiserum was collected. Ten milligrams of this peptide was coupled with 2 mL of Affi-Gel 10 gel (Bio-Rad Laboratories, Hercules, CA) in 0.1 mol/L MOPS [3-(N-morpholino) propane sulfonic acid] buffer (pH 7.5) according to the manufacturer’s instruction. Ten milliliters of the rabbit anti-MAP peptide antiserum diaлизed against 0.1 mol/L sodium phosphate buffer (pH 7.2) was loaded onto the column. The column was washed with 1 mol/L NaSCN and 0.5% Triton X-100, and the bound antibody was eluted with glycine-HCl (pH 2.5). Histologic study. Formalin-fixed and paraffin-embedded kidney specimens obtained from the propositus at the autopsy were used for Congo red stain or immunohistochemical staining. Kidney specimens from patients with Ig light chain-related amyloidosis and fibrinogen Aα chain Val 526 amyloidosis were used as control. Congo red staining was performed by the conventional method. For immunohistologic examination, specimens were deparaffinized and incubated with 5 μg/mL affinity-purified rabbit anti-synthetic peptide Ig or with 1:2,000 diluted commercial polyclonal rabbit anti-human fibrinogen (Calbiochem, San Diego, CA). They were then stained with an avidin-peroxidase immunostaining kit (Vectastain; Vector Laboratories Inc, Burlingame, CA) according to the supplier’s protocol.

Plasma fibrinogen studies. Plasma fibrinogen values of the propositus’ father, sister, spouse, and 4 children were examined functionally by fibrin formation and immunologically by radial immunodiffusion. Thrombin and reptilase clotting times were performed as described previously. Western blotting analysis of plasma. Three microliters of plasma from the 4 children were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membrane. The membrane was incubated with polyclonal rabbit antihuman fibrinogen diluted 1:500 for 2 hours and then in alkaline phosphatase-labeled goat antirabbit IgG (Bio-Rad) diluted 1:1,000 for 2 hours. Color was developed using AP Color Development Reagent (Bio-Rad).

Purification of plasma fibrinogen and CNBr cleavage. Fibrinogen was isolated from plasma as previously described. Briefly, 30 mL of plasma anticoagulated with trisodium citrate was obtained from one of the propositus' children identified as a mutant fibrinogen Aα gene carrier by DNA analysis. Plasma was passed through a lysis sepharose 4B column and a gelatin sepharose 4B column (Pharmacia, Uppsala, Sweden) and fibrinogen was precipitated with 25% ammonium sulfate. Purified fibrinogen was treated with cyanogen bromide and fractionated on a Sephacryl G-50 superfine column. Peaks eluting after the large void volume peak were pooled and fractionated on an Ultrasphere-ODS column (Beckman Instruments, Inc, Fullerton, CA). Isolated peptides were sequenced on an Applied
RESULTS

SSCP analysis showed an abnormally migrating band in the propositus’ sample (Fig 2). This abnormal band was not observed in any normal control subject tested.

Direct DNA sequencing of PCR products showed both a normal gene sequence and a mutant gene sequence with a nucleotide deletion at position 4904 of the fibrinogen $\alpha$A gene\(^{13}\) that corresponded to the third base of codon 524\(^{18}\) (Fig 3). This mutant gene codes for a completely abnormal peptide after codon 524 of fibrinogen $\alpha$A chain and creates a new termination codon at 548 (Fig 4).

PCR-RFLP analysis showed that 2 of propositus’ children had the HincII recognition site associated with the deletion and therefore had a digestion band of 148 bp in addition to a normal band of 171 bp, whereas 2 other children showed only a normal band (Fig 5). The propositus’ healthy father and sister showed a normal digestion pattern (data not shown).

Microscopic examination of Congo red-stained sections showed Congoophilic amorphous deposits in glomeruli (Fig 6A). Only a few foci of residual relatively normal glomeruli remained.

Immunohistochemical staining with the antisynthetic MAP peptide antibody showed a moderately strong immunoreactive product in the amyloid deposits in glomeruli (Fig 6B). This staining was absent when the antibody was preabsorbed with the synthetic peptide. Neither Ig nor fibrinogen $\alpha$A chain Val 526 amyloid deposits were stained with this antibody. Polyclonal antihuman fibrinogen failed to stain the amyloid deposits in kidney from the propositus in this study and from a patient with fibrinogen $\alpha$A chain Val 526.

The plasma fibrinogen concentrations in individuals with the fibrinogen mutation measured by both functional and immunochromatographic assays were below or at the low end of the normal range and lower than those in their normal siblings (Table 1). Thrombin and reptilase clotting times were also prolonged. Western blotting analysis of plasma after SDS-PAGE showed identical patterns in all 4 of the propositus’ children and no additional band was observed in the 2 children who were predicted to have abnormal fibrinogen.

In an attempt to identify the abnormal $\alpha$A chain in plasma fibrinogen, purified fibrinogen was treated with CNBr and fractionated on a Sephadex G50 superfine column. The residue 518-584 CNBr from normal fibrinogen $\alpha$A chain elutes from the column in a peak after the large void volume peak that contains the large disulfide bridged fragment of fibrinogen. The abnormal $\alpha$A chain should yield a smaller CNBr fragment (residue 518 through 547) that should elute with or later than the normal $\alpha$A chain fragment. All peaks from the Sephadex column after the void volume and up to the column volume were pooled and further fractionated by high performance liquid chromatography (HPLC) before sequence analysis. Although the normal residue 518-584 $\alpha$A chain fragment and other CNBr fragments were identified, no abnormal residue 518-547 $\alpha$A chain peptide was found.

DISCUSSION

Fibrinogen is a 340-kD plasma glycoprotein that plays a major role in the process of blood coagulation through its conversion into fibrin.\(^{19}\) It consists of two identical ensem-
Fig 4. A part of the fibrinogen Aα chain gene sequence showing the nucleotide deletion identified in this kindred. The abnormal amino acid sequence predicted from DNA sequence is shown below the normal sequence.

Fig 5. Ethidium bromide-stained agarose gel of PCR-induced mutation restriction analysis for the detection of the mutant gene in the propositus and her children. Lane 1, III-2; lane 2, IV-1; lane 3, IV-2; lane 4, IV-4; lane 5, IV-3; lane M, DNA size marker (λX174 DNA-HaeIII digest). The figures at the right denote the sizes of the bands in basepairs.
mutation, both thrombin and reptilase clotting times and plasma fibrinogen concentration by immunologic and functional methods were normal for individuals with the Val 526 mutation. Because it is unlikely that the 5' end of the gene that codes the first 19 amino acid portion is more susceptible to mutation than the rest of the gene, the high number of mutations found in this part of the gene is probably due to the fact that this region has been well searched for mutations when dysfibrinogen was suspected, whereas most mutations in the rest of the gene do not show clinical symptoms.

Hypofibrinogenemia is a term usually used when plasma fibrinogen is less than 150 mg/dL measured immunologically, electrophoretically, or by other physicochemical methods. Plasma fibrinogen levels in the mutant gene carriers in this kindred of HRA are lower than those of noncarrier siblings, but do not completely meet the criteria of hypofibrinogenemia.

Table 1. Fibrinogen Concentration and Clotting Time of Plasma From the Kindred With the Fibrinogen Aα Chain Mutation (4904delG)

<table>
<thead>
<tr>
<th>Individual</th>
<th>Fibrinogen Mutation</th>
<th>Fibrinogen (mg/dL) Thrombin Clottable (180-380)</th>
<th>Fibrinogen (mg/dL) Radial Immunodiffusion (170-350)</th>
<th>Thrombin Time (sec) (20-24)</th>
<th>Reptilase Time (sec) (22.5)</th>
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</thead>
<tbody>
<tr>
<td>II-4</td>
<td>-</td>
<td>352</td>
<td>378</td>
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</tr>
<tr>
<td>III-1</td>
<td>-</td>
<td>229</td>
<td>274</td>
<td>24.2</td>
<td>24.6</td>
</tr>
<tr>
<td>III-3</td>
<td>-</td>
<td>323</td>
<td>241</td>
<td>25.2</td>
<td>20.2</td>
</tr>
<tr>
<td>IV-1</td>
<td>-</td>
<td>266</td>
<td>315</td>
<td>22.8</td>
<td>25.5</td>
</tr>
<tr>
<td>IV-2</td>
<td>+</td>
<td>132</td>
<td>189</td>
<td>28.1</td>
<td>28.0</td>
</tr>
<tr>
<td>IV-3</td>
<td>-</td>
<td>229</td>
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<td>24.5</td>
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</tr>
<tr>
<td>IV-4</td>
<td>+</td>
<td>199</td>
<td>219</td>
<td>24.9</td>
<td>27.6</td>
</tr>
</tbody>
</table>
brinogenemia. Considering that hypofibrinogenemia is found to be inherited in an autosomal recessive fashion in some kindreds, this deletion mutation could be responsible for hypofibrinogenemia when patients are homozygous. Therefore, carboxyl terminal region of the fibrinogen Aα chain gene should be examined not only in patients with renal amyloidosis but also in the kindreds showing low plasma fibrinogen concentration.

Although no specimen from the propositus’ mother (II-5, Fig 1), who is the other affected member in this kindred, was available for DNA testing, she is presumed to have been a mutant gene carrier because the propositus’ father (II-4) does not have the mutant gene. Factors supporting the pathogenic role for the mutation include the following: (1) association of the mutation and amyloidosis in two generations, (2) SSCP analysis of controls suggested that this DNA deletion is not a common polymorphism in the population, (3) amyloid deposits in this kindred were immunohistochimically stained with antisera raised to the abnormal fibrinogen peptide, and (4) previous studies have shown the linkage between renal amyloidosis and two other mutations in this portion of the gene.⁹,¹⁰

Many kindreds with familial systemic amyloidosis have been reported. Most of them have transthyretin mutations associated with neuropathy and cardiomyopathy. The kindred described here is the fourth to be described with fibrinogen Aα chain amyloidosis and all show nephropathy without signs of other organ involvement. The disease in these kindreds is similar to the kindred described by Ostertag,⁵ but differs by not having hepatosplenomegaly.

Affected members in the kindred described here developed renal dysfunction in their late 30s and early 40s. This onset age is later than that of fibrinogen Aα chain Leu 554 patients who were affected in their 20s or 30s and earlier than the disease in fibrinogen Aα chain Val 526 individuals who developed the disease in the fifth to seventh decade of life.

As with other renal diseases, renal failure due to fibrinogen amyloidosis has been treated with hemodialysis. One patient with fibrinogen Aα chain Leu 554 received a kidney transplant for renal failure. The kidney functioned for 10 years but then failed due to recurrence of amyloidosis. This suggests that kidney transplantation can be a possible option for patients with hereditary amyloidosis of fibrinogen type, if the age of the patient is considered. In patients with transthyretin-related amyloidosis, liver transplantation has been performed and the results are so far promising as a potentially curable treatment for the disease.⁷ Because fibrinogen is also largely synthesized in the liver, amyloidosis patients with variant fibrinogen may also be candidates for liver transplantation.

In a previous study, we isolated a fragment of fibrinogen Aα chain (amino acid residues 500 through 580) with an amino acid substitution (Pib Aα Leu 554) from renal amyloid deposits in an individual of Peruvian descent. Although insufficient tissue was available in the present case for biochemical studies, immunohistologic studies indicated the presence of the predicted abnormal peptide in amyloid deposits. Polyclonal antihuman fibrinogen antiserum failed to stain amyloid deposits of this patient. Because the antiserum did not stain amyloid deposits of a patient with Val 526 mutation either, it seems likely that the antiserum, although it was polyclonal, did not recognize the region of Aα chain deposited in amyloid. In heterozygous patients with transthyretin-related amyloidosis, amyloid deposits have been shown to include normal as well as variant transthyretin.⁹ We do not know if the amyloid of this kindred contains normal fibrinogen as well as the abnormal peptide. If it is assumed that the amyloid peptide in this kindred starts at approximately amino acid position 500, as determined in a patient with fibrinogen Aα chain Leu 554, the abnormal peptide would share 25 amino acid residues of the normal sequence with fibrinogen Aα chain Leu 554 or Val 526 and have 23 residues of completely different sequence to the early termination. Although computer analysis of this abnormal peptide predicts β-strand structure in this 23 amino acid region, it is reasonable to assume that the normal 25 amino acid sequence (residues 500 through 524) that is shared by all three amyloidogenic fibrinogens plays an important role in amyloidogenesis. Further study based on x-ray crystallography may help to answer this question.

Another factor that must be considered in amyloidogenesis of this abnormal fibrinogen Aα chain is effects on metabolism of the protein. It has been reported that some abnormal fibrinogens exist at very low levels in plasma, probably due to rapid degradation. It is possible that the abnormal fibrinogen Aα chain in this kindred that is missing 14% of the normal carboxyl terminal sequence (residues 525 through 610) and has an abnormal peptide sequence of 23 amino acid residues may be degraded much more rapidly than normal protein. This may explain the findings that individuals with this mutation have low plasma fibrinogen levels and the abnormal fibrinogen is not detected in their plasma; at the same time, rapid degradation of the molecule may also cause the release of the amyloid peptide and lead to amyloid deposition in the kidney.

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REFERENCES

Hereditary Renal Amyloidosis 4203


A frame shift mutation in the fibrinogen A alpha chain gene in a kindred with renal amyloidosis

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