Severe prekallikrein (Fletcher factor) deficiency due to a compound heterozygosis (383Trp stop codon and Cys529Tyr)

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Summary
We investigated a family with prekallikrein deficiency, using both standard coagulation tests and molecular biology techniques. The propositus was found to be a compound heterozygote for a Trp383 stop codon and a Cys529Tyr point mutation. The former mutation was located in exon 11, the latter in exon 14. The propositus inherited the first defect from his father and the second from his mother. Both parents had slightly low prekallikrein levels, but the combination of the two genetic defects produced a phenotype characterized by an extremely low prekallikrein activity and antigen. The propositus' plasma showed a progressive reduction in APTT when incubated for a long time. Conversely, plasma deficient in factor XII, factor XI or high molecular weight kininogen (HMWK) failed to show shortening of the APTT. No circulating anticoagulant was found because the patient's APTT was fully corrected by pooled normal and factor XII-, factor XI- or HMWK deficient plasma. No associated abnormality was apparent in the propositus or his parents. As expected, no tendency for bleeding was noted even after tonsillectomy.

Keywords
Prekallikrein deficiency, Fletcher factor, contact phase, gene mutations

Introduction
Plasma prekallikrein is a glycoprotein synthesized in the liver as a single polypeptide chain and secreted into the blood as a doublet of 88 and 85 KD. The protein resembles factor XI and circulates as a complex with high molecular weight kininogen (HMWK). The normal plasma concentration of this glycoprotein is about 40 µg/ml. Recently, prolylcarboxypeptidase has been identified as a physiological endothelial cell activator of plasma prekallikrein (1). Plasma prekallikrein activation in vitro is catalyzed by activated factor XII (FXIIa). This results in the formation of a serine protease composed of a heavy chain and a light chain held together by a disulfide bond. The catalytic domain is associated with the light chain of the molecule. The heavy chain contains the binding site for HMWK and is involved in the surface-dependent activation of blood coagulation (2-4).
Prekallikrein deficiency (or Fletcher factor deficiency) is a rare clotting disorder which was first described in 1965 by Hathaway et al. (5). Only about thirty cases had been reported in the literature by 1985 (6-7). A few additional cases were described thereafter (8, 9). The peculiarity of this disorder is that the severe coagulation disturbance is not accompanied by hemorrhagic symptoms (5, 10-13).

Prekallikrein deficiency is one of the abnormal contact phase conditions, which also include factor XII (Hageman’s factor), factor XI and kininogen defects (Williams’s, Fitzgerald’s, and Flaujeac’s). The only one of these four conditions that is clinically relevant is factor XI deficiency because it induces a tendency for bleeding (3).

Fletcher factor deficiency seems to have an autosomal recessive transmission. Genetic studies of prekallikrein are scanty and so far no families have been fully studied using molecular biology techniques.

This report describes a family in which the propositus has severe prekallikrein deficiency and was found to be a compound heterozygote for two different genetic abnormalities, one inherited from his mother and the other from his father. This is the first family with prekallikrein deficiency investigated by means of complete clotting studies and molecular biology methods.

Patients, materials and methods

Case report

The propositus is a 14-year old boy who was sent to us for a complete coagulation evaluation because pre-operative coagulation screening revealed a severely prolonged, activated partial thromboplastin time (APTT). The boy’s APTT was about 110 sec (n.v. 32-42 sec.) while his prothrombin time (PT) and thrombin time (TT) were both normal. He had no history of bleeding diathesis. The planned tonsillectomy was postponed because of the coagulation findings.

The family history was also negative for bleeding disorders. The parents were not consanguineous and came from different geographical areas.

After the defect had been exactly defined, the patient underwent surgery with no bleeding or other complications.

Blood samples and assays

Nine milliliters of whole blood were anticoagulated with 1 ml of sodium citrate (3.8%); after centrifugation at 3000x g for 15 min, aliquots of separated plasma were frozen at -40°C. All clotting tests were performed with fresh plasma. Routine coagulation tests were carried out as reported elsewhere (14). Pooled normal plasma was obtained from at least fifteen normal people of both sexes. The plasma was separated and stored in 1-2 ml aliquots in plastic tubes at -40°C ready for use.

Different reagents were used for the APTT tests, i.e. Actin (Dade Laboratories, Miami, USA), Pathrontin (Berhingswerke, Marburg, Germany) and Thrombosit S (Ortho Diagnostic, Raritan, USA).

Factor XI, factor XII, prekallikrein and HMWK clotting assays were carried out using factor-deficient plasmas as a substrate. These substrate plasmas were either from patients with known deficiencies (factor XII-deficient and factor XI-deficient plasmas) or lyophilized plasmas obtained from Sigma Laboratories (St. Louis, USA) (HMWK-deficient plasma and prekallikrein-deficient plasma). Actin was used as activated cephalin in these assays. A volume of 0.1 ml of deficient plasma (substrate) was incubated with 0.1 ml of actin and 0.1 ml of 1:10 diluted test plasma. After a 2-minute incubation at 37°C, 0.1 ml CaCl₂ was added and the clotting time was measured. Diluted (1:10, 1:20, 1:50 and 1:100) pooled normal plasma was used to obtain a calibration curve.

Cross-correction studies were performed using equal parts of either home-made fresh or deep-frozen plasma (factor XII and factor XI) or using lyophilized preparations supplied by Sigma Laboratories (prekallikrein- and HMWK-deficient plasmas).

Electroimmunoassays were carried out using a rabbit raised polyclonal antisera kindly supplied by Behring Laboratories (Marburg, Germany). The concentration of the antisera in the agarose was 4%. The migration time was 12 hours at 4 V/cm (14). Euglobulin lysis time was measured according to the Cliffton and Canamella method (16).

DNA isolation and amplification using polymerase chain reaction (PCR)

Genomic DNA was extracted from peripheral blood leukocytes with the QUIAamp DNA blood mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

Figure 1: Effect of incubation time on the APTT of the propositus and patients with factor XI, factor XII or high molecular weight kininogen deficiency. The Fletcher defect is the only deficiency to show a progressive reduction in APTT as the incubation proceeds.
The promoter, exons 1-15 and boundary introns including the splice junctions of the prekallikrein gene were amplified by PCR for all members of the family, using the specific primers reported by Yu et al. (17), but with a partially modified method; in particular, the reaction was obtained in a total volume of 50 µl using 200 ng of extracted DNA. The mix contained 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.5 mM MgCl₂, 200 µM dNTPs (Ultrapure dNTP Set, Pharmacia Biotech, Uppsala, Sweden), 300 ng of each primer (MGW spa Biotech.), and 1U Taq DNA polymerase (Promega Madison, WI).

The reaction mixture was then amplified using the cycling steps: 94°C, 5 min for 1 cycle; 94°C, 1 min; annealing temperature, 1 min, 72°C, 1 min for 30 cycles and 72°C, 7 min for 1 cycle.

Sequencing of PCR fragments

The PCR products were electrophoresed on a 1% agarose gel in Tris-borate-EDTA buffer (pH 8.3) stained with ethidium bromide and purified with a Microcon filter (Millipore Corporation, Bedford, MA, USA). The purified PCR product was sequenced directly using a Taq Dye Deoxy Terminator Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABIPRISM 3100 Genetic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Results

APTT was severely prolonged in the proband, while both PT and TT were normal. The APTT varied according to the activating substrate in the reagent, but in all cases it greatly exceeded the norm (Table 1). Other routine clotting tests, bleeding time and platelet count were normal (data not shown).

Cross-correction studies in the APTT system showed full correction by pooled, normal and factor XII-, factor XI- or HMWK-deficient plasma, but not by prekallikrein-deficient plasma (Table 2).

A long period of incubation in the APTT system caused a progressive reduction in the clotting times (Fig.1). This did not occur with factor XI-, factor XII- or HMWK-deficient plasma samples. Prekallikrein activity levels were less than 1% of normal in the propositus, about 50% in the father and about 65% in the mother. Prekallikrein activity was completely normal in the propositus’ brother. All members of the family had a normal APTT. Only trace amounts of prekallikrein antigen were found in the propositus, while its levels were respectively about 60% and 70% of normal in the father and mother, and completely normal in the brother (Table 3). The levels of all other clotting factors, including factor XI, factor XII and HMWK were within the normal range in the proband and in his relatives. The euglobulin lysis time was also normal.

Figure 2: Prekallikrein gene analysis.
A: Sequence analysis of the family for exon 11 (a stop codon mutation).
Top: propositus; middle: father; bottom: mother.
B: Sequence analysis of the family for exon 14 (a missense mutation).
Top: propositus; middle: father; bottom: mother.
Two genetic abnormalities were detected when comparing the sequence exons with the wild-type sequence (Fig. 2). Prekallikrein gene sequence analysis revealed a G1298A point mutation in exon 11 leading to a Trp383 stop. Both the proband and his father were heterozygous for this nonsense mutation, which was absent in the proband's mother. Furthermore, a missense mutation was also found in exon 14 of the prekallikrein gene: this mutation, a G1736A substitution, leads to a Cys529Tyr exchange in the prekallikrein molecule. The proband and his mother were heterozygous for this mutation, whereas the father was normal. The brother's genotype was completely normal.

Forty healthy subjects of both sexes from the same geographical area as the family underwent genetic analysis for the G1736A substitution in exon 14 and for the G1298A mutation in exon 11 of the prekallikrein gene. No other subject was found to carry these mutations.

### Table 1: Variations in APTT depending on different activating substances in the reagent

<table>
<thead>
<tr>
<th>Substance</th>
<th>Propotus’ plasma APTT in sec</th>
<th>Normal APTT in sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human placenta + kaolin (Behringwerke, Marburg, Germany)</td>
<td>146</td>
<td>40-50</td>
</tr>
<tr>
<td>Rabbit brain + ellagic acid (Dade Laboratories, Miami, USA)</td>
<td>110</td>
<td>32-42</td>
</tr>
<tr>
<td>Rabbit brain silica (Ortho Diagnostics, Raislon, USA)</td>
<td>160</td>
<td>40-50</td>
</tr>
</tbody>
</table>

### Table 2: Cross correction studies on patient’s plasma with different plasma reagents.

<table>
<thead>
<tr>
<th>Mixing</th>
<th>APTT in sec.</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient plasma</td>
<td>110</td>
<td>Rabbit brain phospholipids + ellagic acid</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Patient plasma + pooled normal plasma</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Patient plasma + Factor XI def plasma</td>
<td>40</td>
<td>Fresh plasma</td>
</tr>
<tr>
<td>Patient plasma + HMWK def plasma</td>
<td>46</td>
<td>Lyophilized plasma obtained from Sigma</td>
</tr>
<tr>
<td>Patient plasma + Fac XII def plasma</td>
<td>43</td>
<td>Fresh plasma</td>
</tr>
<tr>
<td>Patient plasma + prekallikrein deficient plasma</td>
<td>112</td>
<td>Lyophilized plasma obtained from Sigma</td>
</tr>
</tbody>
</table>

### Table 3: Main coagulation features in the propotus and members of his family.

<table>
<thead>
<tr>
<th></th>
<th>APTT in sec.</th>
<th>PT in sec</th>
<th>Prekallikrein Activity (%)</th>
<th>Prekallikrein Antigen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>100</td>
<td>15</td>
<td>&lt; 1</td>
<td>traces</td>
</tr>
<tr>
<td>Father</td>
<td>36</td>
<td>15</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Mother</td>
<td>32</td>
<td>14</td>
<td>68</td>
<td>72</td>
</tr>
<tr>
<td>Brother</td>
<td>32</td>
<td>14</td>
<td>90</td>
<td>95</td>
</tr>
<tr>
<td>Normal values</td>
<td>32-42</td>
<td>14</td>
<td>80-120</td>
<td>80-120</td>
</tr>
</tbody>
</table>
Discussion

The main clinical value of finding a prolonged APTT lies in the distinction between a FXI deficiency and other contact phase defects, i.e. factor XII deficiency, prekallikrein deficiency and kininogen deficiencies (Williams’s defect if both high and low molecular weight forms are absent, and Fitzgerald’s or Flaujeac’s defect if only HMWK is deficient). Recently it has been demonstrated that the Fitzgerald’s defect is due to an abnormal HMWK (18). All of which are asymptomatic (3, 4). In contrast, factor XI deficiency is accompanied by a moderate tendency for bleeding. The negative clinical picture and the results of laboratory tests clearly established the diagnosis in our propositus.

The different APTT prolongation observed in the proband’s plasma when different activating substances were used in the APTT system is hardly surprising. Ellagic acid has proved to be a potent contact phase activator in vivo and in vitro (19-21). Similar differences have been seen by other authors (21, 23).

The behavior of the APTT with longer incubation times confirms previous observations (5, 10, 11) and is important because it indicates that some activation of the contact phase may occur after all (24). This behavior may also be of diagnostic value, since it seems typical of prekallikrein deficiency but does not occur in factor XII, factor XI or kininogen deficiency.

The pattern of inheritance in this family seems to be autosomal recessive. This conclusion was based on the results of the clotting tests and was fully confirmed by the molecular biology analysis, indicating that the parents were carriers of two different defects, which may explain the slightly different prekallikrein levels in the parents, who both had enough to yield a perfectly normal APTT.

The majority of patients described with prekallikrein defects appear to represent cases of true deficiency, though abnormal forms have occasionally been reported. In one study, 5 out of 18 cases showed some cross-reactive material at concentrations of 13 to 30% of normal; the antigen, however, was non-functional (25). These patients should be considered as CRM-reduced, not as CRM+. Patients with prekallikrein Long Beach also had 35% of normal antigen levels and no activity (9). Finally, a variant prekallikrein (prekallikrein Zurich) from a patient whose antigen level was 10% of normal was characterized as non-functional (8). No patients with a normal antigen level, but low activity have been described to date.

Prekallikrein deficiency has been associated in at least two cases with Graves’ disease (26, 27), but the significance of any such association remains to be clarified. There was no history of hyperthyroidism in the family described here. Likewise, an association with thrombotic disorders has also occasionally been reported (28, 29) but, here again, the significance of this association (if confirmed) is unknown. No thrombotic conditions emerged in the family discussed here.

Unfortunately, due to the few molecular biology studies available, no definite conclusions can be drawn on the structure-function relation of prekallikrein. The structure of the human prekallikrein gene has only recently been described: it is composed of 14 introns and 15 exons and is located on chromosome 4q (17).

The amino-terminal portion of the prekallikrein molecule contains a unique structure, called the apple domain. These apple domains, each composed of 90-91 amino acids, contain highly-conserved disulfide bonds linking the first and sixth, the second and fifth and the third and fourth half-cystine residues of each repeat (30).

The mutation in the propositus consisted of a combination of a 383 Trp stop codon in exon 11 with a single amino acid substitution (Cys529Tyr) in exon 14. Residue 383 is close to the area of FXIa activation site (residue 371). It is important to remember that nonsense mutations can be introduced during DNA replication and represent the cause of approximately 30% of genetic diseases (31).

It is surprising that this double heterozygosity gave rise to a complete deficiency of prekallikrein in the plasma of the propositus. Usually this happens in patients who are homozygous for a stop codon. Since one of the molecular abnormalities in the propositus (the Cys to Tyr mutation in exon 14) involves a crucial point responsible for a disulfide bond, it is conceivable that this mutation impedes the proper assembly of the molecule. Since no prekallikrein antigen was found in the propositus’ plasma, we can assume that no fragment of the molecule is secreted. For it to be secreted, but with an epitopic configuration that antiserum cannot detect seems unlikely because we used a polyclonal antiserum.

Another interesting aspect of the family lies in the presence of approximately the same levels of prekallikrein activity and antigen in the parents of the propositus (slightly higher in the mother who has a point mutation in exon 14 causing a Cys529Tyr) in spite of their having two different molecular abnormalities.

A better understanding of the structure-function relationship will only be possible after several families (particularly those with probands showing cross-reactive material in their plasma) have undergone molecular biology studies.

No expression studies could be carried out, but the finding of two defects in the propositus, each inherited from a different parent, strongly supports the conclusion that the combination of the two abnormalities is indeed responsible for the coagulation pattern.

The fact that the propositus’ brother showed no genetic abnormality and had a normal coagulation pattern lends further weight to this interpretation.
References


