Clinical Studies and Thrombin Generation in Patients Homozygous or Heterozygous for the G20210A Mutation in the Prothrombin Gene

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Abstract—A genetic variation in the prothrombin gene, the G→A transition at nucleotide 20210, is a risk factor for venous thrombosis in heterozygotes and is associated with increased prothrombin activity. The homozygous phenotype and the extent of thrombin generation in heterozygous and homozygous subjects are unknown. We investigated a family that included 2 homozygous and 5 heterozygous carriers of the 20210 A allele. The homozygous propositus and his presumably heterozygous father suffered from deep-vein thrombosis. His presumably heterozygous mother and his homozygous sister had recurrent phlebitis at a young age. The remaining 5 affected family members are still asymptomatic. We studied thrombin generation in the family and in 22 unrelated carriers of the 20210 A allele by measuring (1) prothrombin fragment F1+2 (F1+2) as an index of ongoing thrombin generation and (2) the endogenous thrombin potential (ETP) as an index of the possible thrombin-forming capacity. Their F1+2 levels were not different from those of age-matched controls, and thus, ongoing hemostatic system activation was not detectable. A significantly increased ETP was found in the heterozygous carriers of the 20210 A allele compared with the controls (527.8 ± 114.9 versus 387 ± 50.1 nmol/L · min, \( P < 0.0001 \)). In the 2 homozygotes, the ETP was almost twice (639 and 751 nmol/L · min, respectively) as high as in the controls. We conclude that homozygosity for the G20210A mutation in the prothrombin gene is associated with a severe, albeit more benign, thrombotic diathesis compared with homozygosity for deficiencies of antithrombin, protein C, or protein S. In carriers of the 20210 A allele, the pathomechanisms leading to thrombosis should be sought in the higher amounts of thrombin that may be formed once thrombin generation is triggered, rather than in ongoing thrombin generation in vivo. (Arterioscler Thromb Vasc Biol. 1998;18:1287-1291.)

Key Words: G20210A prothrombin mutation ■ deep-vein thrombosis ■ prothrombin fragment F1+2 ■ endogenous thrombin potential

A genetic variation in the 3’-untranslated region of the prothrombin gene, a G→A transition at nucleotide position 20210, has been shown to be associated with an increased risk for venous thrombosis.¹,² The 20210 A allele was found in 18% of selected patients with a personal and family history of venous thrombosis, in 6.2% of unselected consecutive patients with a first episode of deep-vein thrombosis, and in 2.3% of healthy Dutch individuals.¹ Almost identical prevalence data were reported by Swedish investigators.³ Compared with unaffected subjects, heterozygous carriers of the mutation have an almost 3-fold increased risk of venous thrombosis. Thus, together with the factor V (FV) Leiden mutation (R506Q), which causes resistance of FV to activated protein C,¹ the G20210A mutation in the prothrombin gene is one of the most common genetic risk factors for thrombosis.⁴

Whereas the incidence of thrombosis has been established in a fairly large number of heterozygotes, clinical data on homozygous (20210 AA) subjects with regard to the severity of their thrombotic diathesis are lacking. So far, only 1 individual with the 20210 AA genotype has been reported.¹ This female patient suffered from venous thrombosis, but the contribution of the prothrombin mutation to the severity of her thrombotic diathesis is difficult to assess because of the coexistence of another genetic risk factor, the FV Leiden mutation. In this article, we report the clinical characteristics of a family that includes 2 homozygous and 5 heterozygous carriers of the 20210 A allele.

The 20210 A allele is associated with elevated plasma levels of prothrombin, and an increased prothrombin activity, as such, was identified as a risk factor for thrombosis.¹ The mechanisms by which increased prothrombin levels may
promote thrombosis are unclear. It has been speculated that an elevation of prothrombin level may lead to increased rates of thrombin generation and, consequently, to thrombosis. To further investigate this hypothesis, we measured 2 distinct indices of thrombin generation in plasma, prothrombin fragment F1+2 (F1+2) and the endogenous thrombin potential (ETP). F1+2 is released from prothrombin during its activation to thrombin and is therefore an excellent measure of ongoing thrombin generation. The ETP reflects the potential thrombin-forming capacity of the coagulation system when it is in a resting state, i.e., the amount of thrombin that could be formed if triggering in vivo should occur.

Methods

The study was conducted as a prospective, multicenter trial with 4 participating centers. Written, informed consent was obtained from all patients who were enrolled in the study. All clinical studies and informed consent procedures were approved by the Ethics Committee of the University of Vienna.

Coagulation Studies

Venous blood was collected by clean venipuncture with a 19-gauge butterfly infusion set into 1/10 volume of 3.8% sodium citrate. The tubes were centrifuged at 3000g for 20 minutes, and the supernatant was removed and stored at −80°C. F1+2 was measured by ELISA technique (Enzygnost F1+2, Behring). Prothrombin activity was determined by a 1-step clotting assay on a KC-10 coagulometer (Amelung, Lenglo-1) with the use of Thromborel S as thromboplastin and factor II−deficient plasma from Behringwerke. Prothrombin antigen concentrations were measured by Laurell electroimmunoassay by using prothrombin antisera (Asserachrom, Diagnostica Stago). Antithrombin activity was determined on an STA analyzer with the use of the chromogenic test STA antithrombin III (Diagnostica Stago). Protein C antigen was measured by Laurell electroimmunoassay and a protein C antiserum from American Diagnostica. Protein C activity was determined on an STA analyzer and the chromogenic substrate STA protein C (Diagnostica Stago). Total and free protein S levels were determined by ELISA technique (Asserachrom protein S, Diagnostica Stago). Plasminogen was measured by a chromogenic assay system (Baxter Diagnostics AG Dade plasminogen chromogenic assay).

The ETP was performed as described recently. In brief, for defibrination, citrated plasma was mixed with a 1:50 volume of Ancrod (Arvin, Knoll AG) solution, incubated for 10 minutes at 37°C, and then kept on ice for 10 minutes. The fibrin clot was then removed. Measurements of ETP were carried out in a laboratory automaton (Cobas BIO centrifugal analyzers, Hoffmann-La Roche) capable of measuring the course of optical density at 405 nm at 30-second intervals for 15 minutes. In the Cobas machine the volumes were 80 μL of defibrinated plasma, 20 μL of thromboplastin (30 pmol/L recombinant tissue factor), and 20 μL of a prewarmed start solution containing 0.1 mol/L CaCl2 and 3 mmol/L of a chromogenic substrate (SQ68, Serbio Laboratories). All measurements were done in duplicate. The normal range (mean±2 SD) for all assays was obtained from 83 age- and sex-matched healthy subjects who were not taking any medication.

Genetic Analysis

Genomic DNA was extracted from 3 mL citrated blood according to standard procedures. DNA (200 ng) was amplified in 50-μL reaction volumes containing 0.2 mmol/L of each dNTP, 10 mmol/L Tris-HCl (pH 8.3 at 25°C), 50 mmol/L KCl, 0.3 μmol/L of each primer (primer sequences were chosen according to Poort et al’), 1.5 mmol/L MgCl2, and 1 U Ampli Taq Gold polymerase (Perkin-Elmer Cetus). Amplifications were performed in a thermal cycler 480 (Perkin-Elmer Cetus). An initial denaturation step of 10 minutes at 95°C was followed by 40 cycles of 1 minute at 94°C, 1 minute at 45°C, and 1 minute at 72°C. A final extension step of 10 minutes at 72°C assured completion of the reaction. Aliquots of 7 μL of each polymerase chain reaction (PCR) product were subjected to digestion with 10 U HindIII in 1× HindIII buffer (Boehringer Mannheim) in a reaction volume of 30 μL. The digests were incubated for 90 minutes at 37°C. Aliquots were separated by electrophoresis on 6% polyacrylamide gels, followed by ethidium bromide staining. FV Leiden was determined as recently described by Bertina et al. Figure 1 shows the pedigree of the kindred with familial thrombophilia. The propositus (II,1) was hospitalized at the age of 40 years for treatment of venographically confirmed, spontaneous deep venous thrombosis of the right leg. He was given oral anticoagulant treatment for 12 months. Two years after discontinuation of oral anticoagulants, he experienced extensive phlebitis of the left leg. His spouse (II,2) and their 2 sons (III,1 and III,2) are asymptomatic at the ages of 54, 24, and 26 years, respectively. The younger sister (II,4) of the index patient experienced recurrent phlebitis of both legs during and immediately after her 2 pregnancies and at least 3 times per year thereafter. Her 2 daughters (III,5 and III,6) are healthy at the ages of 26 and 27 years. They were never exposed to factors known to trigger venous thrombosis, such as immobilization, surgery, pregnancy, or use of oral contraceptives. The older sister (II,3) and her 2 children (III,3 and III,4) never experienced thromboembolic events. The father of the propositus (I,1) suffered from deep-vein thrombosis at the age of 60 years and died of a cerebral infarction at the age of 71 years. The mother of the index patient (I,2) had several episodes of phlebitis at a young age and died of lung cancer at the age of 80 years.

The propositus, his 2 sisters, and all of their offspring were tested for the 20210 A allele. The propositus and 1 of his sisters were found to be homozygous for the 20210 A genotype while his 2 children, his second sister, and the children of his homozygous sister were heterozygous for the mutation. The levels of antithrombin, protein C, protein S, and plasminogen were normal in all family members investigated. None of the them was a carrier of the FV Leiden mutation.

The clinical and laboratory features of this kindred are shown in the Table. Prothrombin activity in the propositus
Clinical and Laboratory Features in Kindred With a G20210A Mutation in the Prothrombin Gene and Familial Thrombophilia

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Age, y</th>
<th>Genotype</th>
<th>History of Thrombosis</th>
<th>Prothrombin Activity, U/mL</th>
<th>Prothrombin Antigen, U/mL</th>
<th>F1+2, nmol/L</th>
<th>ETP, nmol/L/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>I,1</td>
<td>+</td>
<td>NT</td>
<td>Deep-vein thrombosis</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>I,2</td>
<td>+</td>
<td>NT</td>
<td>Recurrent phlebitis of both legs</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>II,1</td>
<td>56</td>
<td>++</td>
<td>Deep-vein thrombosis, phlebitis of the leg</td>
<td>1.54</td>
<td>1.36</td>
<td>2.1</td>
<td>639</td>
</tr>
<tr>
<td>II,2</td>
<td>54</td>
<td>--/--</td>
<td>None</td>
<td>1.06</td>
<td>1.02</td>
<td>1.0</td>
<td>333.5</td>
</tr>
<tr>
<td>III,1</td>
<td>26</td>
<td>+/+</td>
<td>None</td>
<td>1.53</td>
<td>1.40</td>
<td>0.7</td>
<td>581.9</td>
</tr>
<tr>
<td>III,2</td>
<td>25</td>
<td>+/+</td>
<td>None</td>
<td>1.11</td>
<td>1.32</td>
<td>0.9</td>
<td>430.1</td>
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<tr>
<td>III,3</td>
<td>64</td>
<td>+/-</td>
<td>None</td>
<td>1.72</td>
<td>1.56</td>
<td>1.8</td>
<td>482.7</td>
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<tr>
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<td>40</td>
<td>--/--</td>
<td>None</td>
<td>1.29</td>
<td>1.16</td>
<td>1.1</td>
<td>359.1</td>
</tr>
<tr>
<td>III,5</td>
<td>38</td>
<td>--/--</td>
<td>None</td>
<td>0.95</td>
<td>0.92</td>
<td>0.8</td>
<td>443.2</td>
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<tr>
<td>III,6</td>
<td>52</td>
<td>+/-</td>
<td>None</td>
<td>1.70</td>
<td>1.56</td>
<td>2.4</td>
<td>751</td>
</tr>
<tr>
<td>Controls*</td>
<td>45-17</td>
<td>--/--</td>
<td>None</td>
<td>1.06±0.21</td>
<td>0.99±0.16</td>
<td>1.2±0.45</td>
<td>387±50.1</td>
</tr>
</tbody>
</table>

NT indicates not tested.
*Values are given as mean±SD.

(II,1), his 2 sisters (II,3 and II,4), and 1 of his sons (III,1) was above the mean±2 SD for normal age-matched subjects. Normal levels of F1+2, a measure of prothrombin activation mediated by factor Xa in vivo,5,6 were found in the unaffected and heterozygous family members. In the 2 homozygous subjects, the levels of F1+2 barely exceeded more than 2 SDs above the mean for normal age-matched controls. The ETP was markedly increased in the 2 homozygous individuals but was comparable to that of the controls in the family members without the mutation. In 2 of 5 heterozygotes, the ETP was above the mean±2 SD for normal age-matched subjects.

We also investigated 22 unrelated patients (mean age, 47±15 years; range, 25 to 77) with a history of objectively documented deep-vein thrombosis who were identified as heterozygous carriers of the rare 20210 A allele. The levels of antithrombin, protein C, protein S, and plasminogen were normal in all patients, and none of them was a carrier of the FV Leiden mutation. Carriers of the 20210 A allele had both significantly higher prothrombin antigen and activity levels (mean±2 SD, 1.24±0.50 U/mL and 1.41±0.52 U/mL, respectively) than the control subjects (mean±2 SD, 0.99±0.32 U/mL and 1.06±0.42 U/mL, respectively; P<0.0001 for both comparisons). The levels of F1+2 were determined in multiple samples of these patients at various occasions over a period of 1 year (Figure 2). None of the patients was being treated with antithrombotic drugs. The mean value of each patient over the whole time period was compared with the F1+2 levels obtained from normal sex- and age-matched controls, and no significant difference was found (mean±2 SD, 1.5±1.8 versus 1.2±0.9 nmol/L; P>0.05, Student’s t test). Only 2 subjects with the 20210 A allele had F1+2 values elevated to >2 SDs above the mean for age-matched control subjects on >1 occasion. In 1 of these patients, the consistently increased F1+2 levels are most likely attributable to his advanced age of 77 years.8 The ETP was measured at a single time point after discontinuation of oral anticoagulant therapy. The ETP was significantly higher in the heterozygous carriers of the 20210 A allele than in the control subjects (527.8±114.9 versus 387±50.1 nmol/L·min; P<0.0001). Twelve of the 22 carriers of the mutation had ETP values elevated to >2 SDs above the mean for age-matched control subjects (Figure 3).

**Discussion**

This kindred with a genetic variation in the 3′-untranslated region of the prothrombin gene, a G→A transition at nucleotide position 20210, is an example of familial thrombophilia. Both the propositus, a homozygous carrier of the 20210 A allele, and his presumably heterozygous father experienced deep-vein thrombosis. His presumably heterozygous mother had several episodes of phlebitis at a young age. The younger sister of the propositus, who is also homozygous for the mutation, had phlebitis affecting both legs on >20 occasions. Her 2 pregnancies were complicated by recurrent phlebitic
episodes. The second (heterozygous) sister of the index patient and all 6 family members of the third generation, 4 of them heterozygous for the mutation, are still asymptomatic. Thus, 4 of the 9 affected family members (including the presumably heterozygous parents of the propositus) have already suffered from venous disease, 3 of them early in life. The remaining 5 heterozygous subjects are still relatively young and have not yet been subjected to factors known to trigger venous thrombosis, such as major surgery, prolonged immobilization, or intake of oral contraceptives.

Both carriers of the 20210 AA genotype had several episodes of acute venous disease. From their clinical history, however, it is evident that homozygosity for the mutant prothrombin gene is associated with a far less severe thrombotic diathesis than is homozygous protein C or protein S deficiency, which is often complicated by purpura fulminans of the neonate,10–13 or homozygous type I antithrombin deficiency, which is thought to be incompatible with life.13

In our patients carrying the 20210 A allele, both prothrombin activity and antigen were significantly higher than in control subjects. It was speculated that the elevated prothrombin levels are associated with an increased stability of the transcribed prothrombin mRNA.1 The mechanisms by which increased prothrombin levels may promote thrombosis are, however, unclear. It is tempting to hypothesize that elevated prothrombin levels are associated with an increased rate of thrombin generation and thus with a hypercoagulable state. F1 +2, which is released from prothrombin during its activation to thrombin, is an excellent marker of ongoing thrombin generation.5,6 Elevated F1 +2 levels have been found in many individuals with a defined thrombotic risk factor, such as deficiency of antithrombin, protein C, or protein S; resistance to activated protein C (FV Leiden); and hyperhomocysteinemia, even in the absence of clinical symptoms.14–20

To investigate whether our patients exhibited ongoing hemostatic system activation, we compared their F1 +2 plasma values with those of an age- and sex-matched control group. F1 +2 plasma levels were not increased in the heterozygous patients, and even the 2 homozygous individuals with the highest prothrombin activities had F1 +2 levels that barely exceeded 2 SDs above the mean of normal controls. Thus, a substantially increased thrombin generation was detectable neither in the heterozygous carriers of the mutation nor in the 2 homozygous patients.

In contrast to ongoing thrombin generation as reflected by F1 +2 plasma concentrations, the ETP measures the amount of thrombin that could potentially be formed if triggering of the coagulation system in vivo should occur.7 The ETP decreases on hypocoagulation, such as treatment with heparin, aspirin, or oral anticoagulants, and increases during hypercoagulable states, such as in hereditary thrombophilia and during intake of oral contraceptives.21–24 In our heterozygous patients, the ETP was significantly higher than that in the control subjects, and excessively high levels were found in the 2 homozygous carriers. These data indicate that on stimulation of the coagulation cascade, larger amounts of thrombin can be generated in patients with the G20210A prothrombin mutation than in normal individuals, which may ultimately lead to thrombosis.

In conclusion, our findings do not support the concept that ongoing thrombin generation in vivo is a direct consequence of elevated prothrombin activity in carriers of the 20210 A allele. The thrombotic tendency in these patients may be linked to the larger amounts of thrombin that are formed once thrombin generation is triggered.

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References


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