Mutations in Promoter Region of Thrombomodulin and Venous Thromboembolic Disease

Léna Le Flem, Véronique Picard, Joseph Emmerich, Sophie Gandrille, Jean-Noël Fiessinger, Martine Aiach, Martine Alhenc-Gelas

Abstract—The present study was designed to analyze the thrombomodulin proximal promoter region spanning nucleotides −293 to −12 to search for polymorphisms that could modify thrombomodulin gene expression in patients with venous thromboembolic disease. The study population comprised 205 patients and 394 healthy subjects of similar age and sex distribution. No polymorphisms and only 1 point mutation (G-33A) were found. The G-33A mutation was present at the heterozygous state in 2 patients and in 1 control. Being more frequent in the patients (0.97%) than in the controls (0.25%), the G-33A mutation might be a risk factor for venous thrombosis. To investigate the effect of this mutation on the thrombomodulin promoter activity, the proximal promoter region of the gene (bearing or not bearing the G-33A mutation) was inserted into a promotorless expression vector, upstream of the firefly luciferase gene, and transiently transfected into EA.hy926 endothelial cells. Under the conditions of the assay, the G-33A mutation mildly decreased the promoter activity. This study confirms that abnormalities of the thrombomodulin proximal promoter are not frequent in patients with venous thromboembolism. (Arterioscler Thromb Vasc Biol. 1999;19:1098-1104.)

Key Words: thrombomodulin ■ thrombosis ■ coagulation ■ gene

Thrombomodulin (TM) is an endothelial cell surface glycoprotein receptor that forms a high-affinity complex with thrombin. The thrombin-TM 1:1 complex rapidly activates protein C (PC), which, helped by its cofactor protein S (PS), in turn degrades the clotting cofactors activated (a) factors (F) V and VIII. Moreover, thrombin bound to TM loses all its procoagulant activities such as fibrinogen clotting, activation of FV and FVIII, and activation of platelets. Thus, TM plays an important role in converting thrombin from a procoagulant to a physiological anticoagulant factor.

Thrombophilia is considered to be a multifactorial disorder in that genetic and acquired risk factors often act together in the pathogenic process. Strong evidence of the physiological importance of the PC anticoagulant system has been demonstrated by the thrombotic risk associated with inherited deficiencies of PC, PS, or resistance to activated PC (APCR) linked to the FV Leiden. By analogy with other abnormalities in the PC anticoagulant system, an impaired TM cofactor function could be an additional risk factor for venous or arterial thromboembolic disease (TED). Indeed a point mutation in TM that eliminates the generation of activated PC (a) of a TATA box located between nucleotides (nt) −22 and −22 upstream of the transcription initiation site described by Yu et al., a CAAT box (GCAATC) (nt −110 to −105), 4 possible Sp1 binding sites (nt −12 to −123, −140 to −135, −206 to −201, and −269 to −264), a region responsive to heat shock (nt −77 to −47), and a PyPu box (nt −76 to −56). Actual positive functions for the activity of the TM promoter have been demonstrated for the CAAT box, 2 of the 4 Sp1 binding sites (−206, −140), and the nt −74 to 20 region. The PyPu box contains Ets core motifs able to mediate in endothelial cells both specific positive activation and, together with the TATA box, the TNF-α repression of the TM

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promoter. A silencer element,43 a shear stress–responsive element,44 and 4 retinoic acid–response elements22 have been identified farther upstream.

Analysis of the proximal promoter region of the TM gene to search for gene variations that could influence the TM function in patients with TED has been previously performed by 2 groups (for review, see References 45 and 46). The proximal promoter was normal in more than 300 patients with TED and in 70 controls studied by Ohlin et al.45 Three different mutations (C-133A, G-33A, and GG-9/10AT) were found by Ireland et al46 in, respectively, 1, 1, and 3 of 104 patients with myocardial infarction. These patients were matched by age, sex, and race to controls. One control carried the G-33A mutation. Controls did not carry the 2 other mutations. Taken together, these data do not show a strong association of TM gene defects to an increased thromboembolic risk.

The present study was designed to analyze the TM proximal promoter region to search for polymorphisms that could modify the expression of the gene in patients with venous TED and to study their effects on the TM promoter activity in a transient transfection system.

Methods

Subjects, Blood Collection, and DNA Preparation

Two hundred five patients with venous TED were recruited in a university hospital vascular medicine department between November 1995 and November 1997 and were included in the study if they were younger than 61 years and had had at least one objectively diagnosed episode of deep venous thrombosis (DVT) (compression ultrasonography or venography) or pulmonary embolism (perfusion and ventilation lung scan, conventional pulmonary angiography, or computed tomographic angiography). Three hundred ninety-four healthy subjects aged from 20 to 60 years were recruited between May and September 1996 from a health center specializing in the prevention of cardiovascular disease, to which they had been referred for a routine check-up. Subjects with a history of arterial disease (stroke, myocardial infarction, angina, or peripheral vascular disease), venous TED, or known malignancies were excluded on the basis of a medical questionnaire. The place of birth of the cases and controls was recorded, but there was no geographic or ethnic criteria for eligibility. The study was approved by the local ethics committee and all the subjects gave their informed consent.

Venous blood was collected onto 0.129 mol/L trisodium citrate (1:10). DNA was isolated by the method of Miller et al47 and stored at 4°C.

Genomic DNA Studies

DNA was screened for the FV Q506 allele after polymerase chain reaction (PCR) amplification of exon 10 and restriction enzyme digestion.48 The prothrombin gene G20210A transition was identified after amplification with primers A (5′-TTCAAGCTCTATG-GAAGGGA-3′) and B (5′-CCATGAATAGCAGTTCGGAACAT-TGAAGC-3′). The PCR mixtures contained 25 pmol of each primer, 200 μmol/L dNTPs (Pharmacia Biotech), 300 ng of genomic DNA, and 1× PCR buffer (1 mol/L Tris-HCl, 20 mmol/L KCl, pH 8.3) with 1.5 mmol/L MgCl2 and 0.25 U of Taq polymerase (Super Taq, ATGC Biotechnologie) in a final volume of 50 μL. The thermal profile consisted of a 5-minute denaturation at 94°C, followed by 35 cycles consisting of a 1-minute denaturation at 94°C, a 1-minute annealing at 56°C, and a 1-minute extension at 72°C. The amplified fragments were digested by HindIII (New England Biolabs), giving 2 fragments of 413 and 73 bp for the G20210 allele or 3 fragments of 384, 73, and 29 bp for the A20210 allele.

The TM promoter region spanning nt −293 to −12 was analyzed by using denaturing gradient gel electrophoresis (DGGE) as described by Attree et al,49 after amplification of 2 overlapping fragments of 387 and 303 bp. The design of the primers and the choice of the electrophoretic conditions (6 and 4 hours, respectively, at 160 V in 6.5% polyacrylamide gel containing 40% to 100% denaturant gradient [100% denaturant, 7 mol/L urea and 40% formamide in TEA buffer [2 mol/L Tris, 50 mmol/L EDTA, 1 mol/L sodium acetate, pH 7.6]]) were done to allow detection of abnormalities in the promoter regions spanning from nt −148 to nt −12 and nt −293 to −96, respectively.

Identification of the G-33A mutation was performed by sequencing. The nt −313 to +74 fragment of the TM promoter region was cloned into the pT7Blue Vector using the pT7Blue T-Vector kit (Novagen). Sequencing reactions were performed on plasmid DNAs using the Sequenase 2.0 DNA sequencing kit (USB) with R-20-mer (Novagen). Sequencing reactions were performed on plasmid DNAs cloned into the pT7Blue Vector using the pT7Blue T-Vector kit (Novagen). Sequencing reactions were performed on plasmid DNAs using the Sequenase 2.0 DNA sequencing kit (USB) with R-20-mer and U-19-mer primers (Novagen) as sequencing primers.

Verification of the G-33A mutation identified by sequencing in 1 patient and screening for this mutation in other subjects were performed by using restriction site analysis with Stul (New England Biolabs). The positions of the primers used for amplifications of genomic DNA to screen for TM promoter variations by DGGE or to identify mutations by sequencing or restriction site analysis are shown in Figure 1. The sequences of the primers and the PCR conditions are given in Tables 1 and 2. The whole PCRs were performed on mixtures containing 1 μg genomic DNA, 200 μmol/L dNTP (Pharmacia Biotech), and 1 U of Taq polymerase (ATGC Biotechnologie) in 1× PCR buffer in final volumes of 100 μL.

Plasmid Constructions

Two fragments of the TM gene promoter (spanning nt −294 to +11 or nt −154 to +11) were cloned into the pGL3 Enhancer Vector (Promega). Primers TM PROM F, TM PROM G, and TM PROM H modified to introduce cleavage sites for the restriction enzymes SacI, SacI, and NheI, respectively (New England Biolabs) (Table 1), were used to amplify genomic DNA of both a patient with the G-33A mutation and a normal subject. PCR amplifications and enzymatic digestions were performed as described in Table 3. The SacI NheI fragments were electrophoretically eluted after electrophoresis in a 6% polyacrylamide gel then ligated into the pGL3 Enhancer Vector digested with the same enzymes. The resultant plasmids, pTM-294
TABLE 1. Primers Used in Different Steps of Study

<table>
<thead>
<tr>
<th>Primers Used in Different Steps of Study</th>
<th>Sizes of PCR Products, Base Pair</th>
<th>TM Gene Promoter Region Analyzed, nt</th>
<th>MgCl₂ and DMSO, Final Concentrations</th>
<th>Heteroduplex Formation Cycle</th>
<th>Type of Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC TM PROM A + TM PROM B</td>
<td>387</td>
<td>-293 to 12</td>
<td>1.5 mmol/L (5%)</td>
<td>94°C, 10 min</td>
<td>DGGE</td>
</tr>
<tr>
<td>GC TM PROM A + TM PROM C</td>
<td>303</td>
<td>-293 to -96</td>
<td>1.5 mmol/L (5%)</td>
<td>94°C, 10 min</td>
<td>DGGE</td>
</tr>
<tr>
<td>TM PROM A + TM PROM D</td>
<td>387</td>
<td>-293 to +54</td>
<td>1.0 mmol/L (0%)</td>
<td>no</td>
<td>Sequencing</td>
</tr>
<tr>
<td>TM PROM A + TM PROM Stu</td>
<td>313</td>
<td>-293 to +54</td>
<td>1.5 mmol/L (5%)</td>
<td>no</td>
<td>Restriction site analysis</td>
</tr>
</tbody>
</table>

The thermal profile included 5 minutes of denaturation at 94°C, 35 cycles consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C and extension for 1 minute at 72°C, followed by 7 minutes of final extension at 72°C; heteroduplex formation cycle is specified when used.

mut or pTM-294wt and pTM-154 mut or pTM-154wt, were transfected into Escherichia coli DH5 cells and purified using Qiagen plasmid midi kits (Ceger).

Using the pTM-154wt construct as a template, constructs carrying mutations of the CAAT box -pTMCAATmut- (nt -313 -294) were transfected into Escherichia coli mut or pTM-294wt and pTM-154 mut or pTM-154wt, were transfection experiments to eliminate the possibility of bias originating from DNA preparation.

Cell Culture, Transfection Experiments, and Reporter Gene Assays

The endothelial cell line, EA.hy926, was kindly provided by Dr. C.J.S. Edgell (University of North Carolina, Chapel Hill). Culture conditions were as described by Suggs et al.5 Cells were cotransfected with the pGL3 construct carrying the firefly luciferase as a reporter and a renilla luciferase expression plasmid (pRL-TK, Promega) used as an internal control in transcription efficiency. All the transfection experiments were performed in triplicate. Cells were plated at 5 x 10⁵ cells in 60-mm Petri dishes with 3 mL complete medium and grown overnight at 37°C in 5% CO₂/95% air to obtain 50% to 70% semiconfluent cultures. Cells were then transfected using lipofectin (Gibco-BRL) according to the manufacturer’s instructions with a mixture containing 8 μg of lipofectin reagent; 1 μg of TM promoter firefly luciferase construct or control plasmid, pGL3 enhancer vector, which lacks any promoter, or pGL3 control vector (Promega), which contains an efficient promoter (SV40) upstream of the firefly luciferase gene; and 0.1 μg of pGL-TK renilla luciferase plasmid. The culture medium was changed after incubating for 3 hours at 37°C. Levels of renilla and firefly luciferase activities in cell extracts, prepared 48 hours after transfection, were measured by using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Five to nine independent experiments were performed for each construct.

Results

Characteristics of the Study Population

Demographic data are shown in Table 4. The case and control groups did not differ significantly in terms of age or sex. Most of the cases and controls were born in France. Subjects born outside Europe were similarly distributed between the cases and controls (12% versus 15%).

DVT was recurrent in 27.1% of the cases and pulmonary embolism occurred in 37.6%. Women on oral contraception, a known risk factor for venous thromboembolism (VTE), were more frequent among the cases than the controls. Thrombosis occurred in the absence of acquired risk factors (oral contraceptives, recent surgery, recent trauma, pregnancy or childbirth, and

Statistical Analysis

Mean age in the case and control groups was compared by using the Student’s t test. The statistical significance of the clinical differences between the two groups (sex, oral contraception) was calculated by using a χ² test. FV and prothrombin genotype frequencies were compared between cases and controls by using a χ² test. The nonparametric Wilcoxon’s test was used to compare the promoter activities of the different constructs. Differences with probability value of 0.05 or less were considered significant.
immobilization) in 38.5% of the cases, and 34.9% of the patients were on anticoagulants at the time of blood sampling.

The population was screened for known genetic risk factors for thrombosis. The FV R506Q mutation was observed in 19.5% of cases and 3.5% of controls (P < 0.001). The prothrombin gene G20210A mutation was observed in 10.2% of cases and 2.8% of controls (P < 0.001).

**TABLE 3. PCR and Enzymatic Digestion Conditions Used in Plasmid Constructions Experiments**

<table>
<thead>
<tr>
<th>TM Gene Promoter Regions Studied, nt</th>
<th>Template</th>
<th>Primers, 50 pmoles</th>
<th>MgCl₂ and DMSO, Final Concentrations</th>
<th>Annealing Temperature</th>
<th>Digestion Conditions for 90 μL of PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>−293 to +217</td>
<td>1 μg genomic DNA</td>
<td>TM PROM A + TM PROM E</td>
<td>1 mmol/L (0%)</td>
<td>58°C</td>
<td>20 U Nhe I + 20 U Sac I Nhe I buffer + BSA 3 h, 37°C</td>
</tr>
<tr>
<td>−284 to +3</td>
<td>2 μL of the −313/+237 PCR product</td>
<td>TM PROM F + TM PROM H</td>
<td>2.5 mmol/L (0%)</td>
<td>66°C</td>
<td>20 U Sac I Nhe I buffer + BSA 3 h, 37°C</td>
</tr>
<tr>
<td>−143 to +3</td>
<td>1 μg genomic DNA</td>
<td>TM PROM G + TM PROM H</td>
<td>1.5 mmol/L (5%)</td>
<td>60°C</td>
<td>20 U Nhe I + 20 U Sac I Nhe I buffer + BSA 3 h, 37°C</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 200 μmol/L dNTP, 1 × PCR buffer with 2.5 U of Tag polymerase (Perkin Elmer Cetus Instruments) in a final volume of 100 μL. The thermal profile included 5 minutes of denaturation at 94°C and 30 cycles consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at annealing temperature (as indicated in the table), and extension for 90 s at 72°C, followed by 7 minutes of final extension at 72°C.

**TABLE 4. Characteristics of the Study Population**

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 205)</th>
<th>Controls (n = 394)</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women, %</td>
<td>55.1 (3.5)</td>
<td>50.0 (2.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age, y</td>
<td>42.2 (11.1)</td>
<td>42.9 (9.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Oral contraception in women, %</td>
<td>32.7 (4.4)</td>
<td>18.6 (2.8)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Spontaneous VT, %</td>
<td>38.5 (3.4)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Recurrent VT, %</td>
<td>27.1 (3.1)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Pulmonary embolism, %</td>
<td>37.6 (3.4)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Mean age at first VT, y</td>
<td>38.1 (12.0)</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are means (SD). VT indicates venous thrombosis; NS, not significant.

**Effect of the G-33A Mutation on Gene Expression**

Transient transfection experiments were performed in EA.hy926 cells with the TM promoter constructs described above. These constructs contained either fragments of the normal promoter (wt) or of the promoter bearing the G-33A mutation (mut), a fragment of the promoter bearing a CAAT box mutation previously demonstrated to decrease the TM promoter activity, or a fragment of the promoter bearing a TATA box mutation (the effects of the CAAT box and TATA box mutations were studied to validate the expression system). The results are reported in Figure 4. The pGL3 enhancer vector, which does not contain promoter sequences, was used as a negative control and displayed a very low activity (2.5 ± 0.7%). As expected, the activity of the construct bearing the CAAT box mutation was reduced, to less than half the activity of the wild-type promoter (51.0 ± 7.5% versus 117.4 ± 25.7%, respectively; P = 0.04). The activity of the construct bearing the TATA box mutation was also significantly reduced (37.1 ± 4.9%, P = 0.04). The activity of the constructs bearing the G-33A mutation was slightly lower than the activity of the wild-type constructs (181.9 ± 24.5% versus 198.4 ± 27% for pTM-294 and 98.9 ± 27.5% versus 117.4 ± 25.7% for pTM-154). The difference was significant for the pTM-154 constructs (P = 0.015) but not for the pTM-294 constructs.

**Figure 2.** DGGE patterns of the amplified 387-bp TM gene fragment. Lanes 1 and 3 indicate normal subjects; Lane 2, the patient heterozygous for the G-33A mutation.
Discussion

Polymorphisms in the TM proximal promoter region could be responsible for an impaired expression of TM contributing to thrombophilia, as previously described for polymorphisms or mutations in the PC gene promoter.52–56 We have therefore investigated 205 patients with VTE and 394 healthy subjects of similar age and sex distribution for the presence of such gene variations. The patient and control populations were not different from other European populations in term of genetic risk factors for thrombosis such as the FV R506Q mutation4 and the prothrombin gene G20210A mutation.57– 61

First, we screened this TM promoter region in 125 patients by PCR-DGGE, a scanning strategy previously shown to be highly sensitive for the detection of point mutations in other genes.62,63 No polymorphisms and only 1 point mutation (G-33A) were found, confirming data showing that sequence variations of the proximal TM promoter are not frequent in patients with VTE.45,46

Later, the G-33A mutation, searched for in the other 80 patients and in the controls by restriction site analysis, was found in another patient and in 1 control. The prevalence of the mutation was slightly higher in the whole patient group (2/205) than in the control group (1/394) (0.97% versus 0.25%, respectively) suggesting that this rare mutation might be a risk factor for VTE. Unfortunately, because of the low prevalence of the mutation, statistical analysis of the results could not be performed to confirm this hypothesis. It must however be pointed out that in both patients carrying the mutation, the first thrombotic event had occurred before age 30, that both had suffered from recurrent thrombotic episodes, and that one of them had a strong family history of thrombosis. Taken together, these data suggest the possible presence of genetic risk factors for thrombosis in both patients.

The G-33A mutation is located 7 nt upstream of the TATA box, within a promoter region important for basal TM gene transcriptional activity12,13,43 and very near the putative TNF-α and heat shock responsive sequences.12,13,35 Moreover, efficient transcription initiation of a human protein-encoding gene requires assembly on the promoter DNA of a multiprotein complex containing RNA polymerase II and 6 general transcription factors, and a consensus sequence (G/C-G/C-G/A-CGCC) located immediately upstream of the TATA element has recently been shown to affect the ability of one of these general transcription factors to enter transcription complexes and support transcription initiation.64 Being located in this consensus sequence, the G-33A mutation might therefore induce a down-regulation of the TM promoter. This hypothesis was studied in vitro in an experimental system consisting of EA.hy926 cells transfected with promoter TM gene fragments (nt −294 to +11 or nt −154 to +11) cloned into a luciferase reporter vector. The EA.hy926 cell line, which results from the fusion of human umbilical vein endothelial cells with the lung cancer A549 cell line,65 has been previously demonstrated to express the TM gene.66,67

Figure 3. A 6% polyacrylamide gel electrophoresis of the PCR-amplified fragment TM PROM A-TM PROM Stu digested with StuI. Lane 1 indicates basepair marker, FX174/HaeIII (Gibco BRL); Lane 2, normal subject; Lanes 3 to 5, subjects heterozygous for the G-33A mutation.

Figure 4. Luciferase activities of various TM promoter constructs in EA.hy926 cells. Known regulatory elements in the 5' end sequence of the TM gene are schematically represented. The star (*) represents the G-33A mutation. Mutations of the CAAT box and the TATA box are represented in bold and the base changes are indicated. The pGL3 Enhancer Vector was used as a negative control. Relative luciferase activities (firefly/renilla luciferase units) are expressed as percentages relative to the activity of the pGL3 Control Vector. The mean results of 5 to 9 independent experiments ±1 SEM are reported.
The position of the TM promoter fragments was chosen according to the findings of Tazawa et al., who had previously demonstrated in transient transfection assays that the activity of a construct containing the nt −290 to +145 region is maximal and that a construct containing the nt −181 to +145 smaller region, thus lacking 2 Sp1 binding sites, displays about half of the peak activity. Our results showing that the pTM-294wt and pTM-154wt constructs exhibit 198% and 117%, respectively, of the activity of the pG3L control vector are in accordance with these previous findings.

We have verified that the transient transfection assay system used in the present study was able to recognize an impaired transcription. For this purpose, the activity of pTM-154 constructs bearing a mutation of the TATA box or a mutation of the CAAT box was studied. As expected, the activity of these constructs was low (41.6% and 30.5% of the activity of the normal construct, respectively). The effect of the CAAT box mutation was similar to the effect previously described for a CAAT box mutation introduced in a −374 to +145 construct. The TATA box mutation also decreased TM promoter activity to less than half the activity of the wild-type promoter, demonstrating that the TATA box plays a critical role in transcriptional activity of the TM promoter.

Mean promoter activities of the constructs that bore the G-33A mutation were slightly lower than activities of the wild-type constructs (92% and 84% for pTM-294 mut and pTM-154 mut, respectively). The difference was significant only for the pTM-154 construct (P=0.015). An explanation for this finding could be the difference in the size of the promoter fragments. The pTM-294 fragment is 140 nt longer than the pTM-154 fragment. It has a stronger transcriptional activity and comprises more regulatory elements, particularly 2 additional Sp1 binding sites. The effect of one or several regulatory elements located in this region could have offset the effect of the G-33A mutation on promoter activity. On the whole, these results do not clearly support the hypothesis of the G-33A mutation being a risk factor for venous TED by lowering the expression of the TM gene on vascular endothelial cells. However, it must be pointed out that the effect of the G-33A mutation was studied only in conditions of basal transcription. Different modulators, for example, interleukin-1, TNF, or endotoxin, can downregulate TM expression while in a concomitant manner favor tissue factor expression and thereby induce a hypercoagulable state. Being localized near the region responsive to TNF-α (nt −76 to −56), the G-33A mutation might modify the response of the promoter in stimulated endothelial cells.

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References


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