Lethal Perinatal Thrombosis in Mice Resulting From the Interaction of Tissue Factor Pathway Inhibitor Deficiency and Factor V Leiden

Daniel T. Eitzman, MD; Randal J. Westrick, BS; Xiaoming Bi, MD; Sara L. Manning, BS; John E. Wilkinson, MD; George J. Broze, MD; David Ginsburg, MD

Background—Factor V Leiden (FVL) is a common genetic risk factor for thrombosis in humans. The incomplete penetrance of FVL suggests important contributions from other genetic or environmental modifying factors. Variation in the expression of tissue factor pathway inhibitor (TFPI) has also been proposed as a risk factor for venous thrombosis and has been shown to enhance the prothrombotic effect of FVL in vitro.

Methods and Results—To examine the potential in vivo interaction between Tfpi and Fvl, we analyzed crosses between mice carrying FvL and a deficiency of TFPI. The FvQ/Q, Tfpi+/− genotype was nearly completely fatal in the early perinatal period. Increased fibrin deposition was observed in multiple organs from the FvQ/Q, Tfpi+/− fetuses, suggesting disseminated thrombosis.

Conclusions—These observations demonstrate the prothrombotic effect of modest variations in the level of TFPI expression and suggest that TFPI could be an important genetic modifier for the thrombosis associated with FVL in humans. (Circulation. 2002;105:2139-2142.)

Key Words: genetics ■ fibrin ■ gene ■ thrombosis

Complications of venous thrombosis are a common cause of morbidity and mortality. Factor V Leiden (FVL), a missense substitution in the factor V gene resulting in the replacement of arginine at amino acid position 506 by glutamine, is a common polymorphism in European populations and is found in 20% to 50% of patients presenting with venous thrombosis. However, the relative risk of thrombosis and is found in 20% to 50% of patients presenting with venous thrombosis.1–3 However, the incomplete penetrance of FVL suggests important contributions from other genetic or environmental modifying factors. Variation in the expression of tissue factor pathway inhibitor (TFPI) has also been proposed as a risk factor for venous thrombosis and has been shown to enhance the prothrombotic effect of FVL in vitro.

To test the hypothesis that modest reductions in TFPI levels will also enhance factor V Leiden associated thrombosis in vivo, we analyzed mice carrying combined mutations in the Fv and Tfpi genes.

Methods

Mice
Mice carrying the murine homologue of the factor V Leiden mutation (FvQ)13 or the TFPI Kunitz domain deletion14 were generated as previously described. These mice were backcrossed to C57BL/6J mice (Jackson Labs, Bar Harbor, Maine) for 4 to 10 generations for FvL and 2 to 7 generations for Tfpi before crosses were performed. Genotyping for Tfpi14 and FvQ status13 was done by polymerase chain reaction analysis of tail DNA using the oligonucleotide primers previously described. All mice were maintained on normal chow in specific pathogen-free (SPF) facilities. All animal care and experimental procedures complied with the principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.
Tfpi
Fv

3 weeks, and 1 of these died shortly after weaning. This

At embryonic day 18.5, double-heterozygous mice (FvQ/Q, Tfpi−/−) were then crossed to homozygous FvL−/− mice who are also heterozygous or homozygous for Tfpi−/−, and progeny from timed matings were analyzed shortly before birth at 18.5 days after conception. At this late stage in embryonic development, the expected equal mendelian distribution of all potential genotypes was observed. These results indicate that the FvQ/Q, Tfpi−/− mice die sometime between birth and weaning, most likely in the immediate postnatal period.

Because we have reported previously that mouse strain background influences the phenotype of the FvQ/Q mutation, survival analysis of FvQ/Q, Tfpi−/− mice was repeated after FvL and Tfpi−/− mice had been backcrossed to the pure C57BL/6J strain for a total of 10 and 7 generations, respectively (Table). Of 154 offspring analyzed from this cross, no surviving FvQ/Q, Tfpi−/− mice were observed. These results confirm the lethality of the FvQ/Q, Tfpi−/− genotype.

Histology

As shown in Figure 1, extensive fibrin(ogen) staining was detected in the liver, lung, and kidney from FvQ/Q, Tfpi−/− neonates, whereas only scant staining was noted in FvQ/Q, Tfpi−/− neonates. The mean (±SD) fibrin(ogen) staining score for multiple liver, lung, and kidney sections from 5 FvQ/Q, Tfpi−/− neonates was 2.3±0.5 compared with 1.0±0.2 for 4 FvQ/Q, Tfpi−/− neonates (P<0.0001). The sole surviving FvQ/Q, Tfpi−/− adult mouse was found to be circling and gasping in the cage at ≈1 year of age. The mouse was killed, and autopsy revealed an occluded inferior vena cava (gross observation) with dilated accessory venous circulation evident. Histology showed intravascular thrombosis in liver and lung with bilateral renal infarctions (Figure 2).

Discussion

Factor V Leiden (FVL) has a prevalence of 2% to 7% in most European populations1,3,16 and is the major known genetic risk factor for thrombosis in humans. In a small subset of patients, thrombosis is associated with coinheritance of other prothrombotic gene mutations.5–8 However, the potential contribution of additional genetic risk factors in most patients remains unknown. Mice homozygous for the murine FvL mutation exhibit spontaneous thrombosis that is variable depending on the background genetic strain, indicating the presence of strain-dependent genetic modifiers of thrombosis.13 Thus this model is useful for evaluating the impact of potential modifier genes on thrombophilia and recently has been used to unmask the subtle prothrombotic phenotype of mice deficient in protein Z.17 Protein Z–deficient mice appeared indistinguishable from their littermates. However, when crossed with FvL mice, homozygous protein Z–deficient mice who are also heterozygous or homozygous for FvL exhibited a partial or complete perinatal lethal phenotype, respectively. Taken together with the previous protein Z observations, these data suggest that novel interactions of FvL with multiple other genetic loci may be an important determinant of thrombosis penetrance or severity.

Polymorphisms involving human TFPI recently have been proposed to contribute to venous thrombosis risk, although this is controversial.18 Mice homozygous for an inactive Tfpi allele generated by gene targeting exhibit an embryonic lethal phenotype that seems to be attributable to disseminated thrombosis. Even though heterozygous mice exhibit only 50% of functional TFPI activity in the serum compared with wild-type littermates, they develop normally without evi-

<table>
<thead>
<tr>
<th>Progeny of FvL/Tfpi Matings</th>
<th>Matings Pairs</th>
<th>Observed (%)</th>
<th>Expected</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FvQ/Q (n4), Tfpi−/− (n2) × FvQ/Q (n4)</td>
<td>At 3 weeks</td>
<td>FvQ/Q, Tfpi−/−</td>
<td>31 (33)</td>
<td>25</td>
</tr>
<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>2 (2)</td>
<td>25</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>35 (37)</td>
<td>25</td>
<td></td>
<td></td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>26 (28)</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At embryonic day 18.5</td>
<td>FvQ/Q, Tfpi−/−</td>
<td>7 (18)</td>
<td>25</td>
<td></td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>11 (28)</td>
<td>25</td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>13 (33)</td>
<td>25</td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>8 (21)</td>
<td>25</td>
<td></td>
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<tr>
<td>FvQ/Q (n10), Tfpi−/− (n7) × FvQ/Q (n10)</td>
<td>At 3 weeks</td>
<td>FvQ/Q, Tfpi−/−</td>
<td>16 (10)</td>
<td>12.5</td>
</tr>
<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>0 (0)</td>
<td>12.5</td>
<td>&lt;0.005</td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>21 (14)</td>
<td>12.5</td>
<td></td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>24 (16)</td>
<td>12.5</td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>46 (30)</td>
<td>25</td>
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<tr>
<td>FvQ/Q, Tfpi−/−</td>
<td>47 (30)</td>
<td>25</td>
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</table>

Histology

Fetuses were retrieved and fixed as previously described.13 Fibrin(ogen) immunohistochemistry of selected tissues was performed as previously described.13 Tissue fibrin(ogen) staining was graded by a blinded observer using a qualitative scale roughly corresponding to the amount of stainable fibrin(ogen) as a percentage of each section (0, 0%; 1, 1% to 10%; 2, 11% to 50%; and 3, 51% to 100%). Adult mice were perfusion fixed as previously described,11 and multiple organs were sectioned and stained with H&E.

Statistical Analysis

The significance of survival differences between groups was determined using the χ² test, and differences in fibrin(ogen) tissue staining were determined using the Student’s t test.

Results

Effect of Heterozygous Tfpi Deficiency on the FvL Phenotype

To determine the effect of combined mutations in the Fv and Tfpi genes, mice homozygous for FVL (FvQ/Q) (N4) were first crossed with heterozygous TFPI-deficient mice (Tfpi−/−) (N2). Double-heterozygous mice (FvQ/Q, Tfpi−/−) were then crossed to homozygous FvL mice (FvQ/Q, Tfpi−/−) to generate 4 possible genotypes with an expected frequency of 25% each. As shown in the Table, only 2 of 94 offspring with the FvQ/Q, Tfpi−/− genotype were observed at the weaning age of 3 weeks, and 1 of these died shortly after weaning. This marked underrepresentation of the FvQ/Q, Tfpi−/− genotype was significantly different than the expected Mendelian frequency. To determine whether the loss of FvQ/Q, Tfpi−/− mice was occurring in the perinatal period or earlier in embryonic development, progeny from timed matings were analyzed shortly before birth at 18.5 days after conception. At this late stage in embryonic development, the expected equal
cence of spontaneous thrombosis.\textsuperscript{14} Our observation of a synthetic lethal interaction between FVL and heterozygous TFPI deficiency is consistent with a previous in vitro study demonstrating that combination of the FVL mutation and reduced TFPI leads to a marked increase in thrombin generation.\textsuperscript{12} The report of significantly decreased free and total plasma TFPI levels in symptomatic patients with FVL compared with asymptomatic FVL patients\textsuperscript{19} additionally supports the potential relevance of our observations in this murine model system for FVL in humans.

We previously have demonstrated that heterozygous \textit{Tfpi} deficiency in mice affected time to occlusive thrombosis after endothelial injury at the site of an atherosclerotic plaque.\textsuperscript{11} However, no difference in the time to occlusion was noted in the absence of preexisting vascular disease. In the present study, we have demonstrated that heterozygous \textit{Tfpi} deficiency is uniformly lethal in mice that are also homozygous for \textit{FvL}, whereas neither defect alone results in significant mortality. These findings highlight the sensitivity of this genetic cross for detecting prothrombotic gene interactions and suggest that the FVL mouse may constitute a powerful tool for sensitized screening of potential genetic risk factors for thrombosis.

Although the precise cause of death in these animals has not been definitively identified, a disseminated thrombotic process occurring shortly after birth is likely, judging by the widespread increased tissue fibrin deposition observed in neonates. These results demonstrate a critical in vivo interaction between TFPI and FVL in the regulation of thrombin generation. Taken together, these observations suggest that modest variation in TFPI expression could be an important genetic modifier for the thrombosis associated with factor V Leiden in humans.

**Acknowledgments**

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References

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