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 FvL(Tfpi<sup>−/−</sup>) genotype was nearly completely fatal in the early perinatal period. Increased fibrin deposition was observed in multiple organs from the FvL(Tfpi<sup>−/−</sup>) 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 is only 2.7 in FVL carriers. This incomplete penetrance is similar to that of other known genetic risk factors for thrombosis. Several reports have documented enhanced thrombosis risk associated with FVL and the cosegregation of other known prothrombotic genetic risk factors, such as mutations in protein C, protein S, antithrombin III, and prothrombin.

Tissue factor, the primary initiator of blood coagulation, is regulated by tissue factor pathway inhibitor (TFPI). TFPI is a Kunitz-type proteinase inhibitor that regulates extrinsic pathway initiation of coagulation by producing factor Xa-mediated feedback inhibition of the factor VIIa/tissue factor (TF) catalytic complex. Animal studies have demonstrated an important in vivo regulatory role of TFPI in vascular thrombosis. Variations in TFPI expression may be particularly important in the setting of other prothrombotic risk factors. In a synthetic in vitro assay of thrombin generation, reduction of TFPI concentration by 50% resulted in a slight increase in thrombin generation. However, when combined with factor V Leiden, the same reduction in TFPI concentration led to a marked increase in thrombin generation similar to that observed in the complete absence of the protein C pathway.

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 mouse homologue of the factor V Leiden mutation (FvQ<sup>−/−</sup>) or the TFPI Kunitz domain deletion were generated as previously described. These mice were backcrossed to C57BL/6J mice before crosses were performed. Genotyping for Tfpi<sup>−/−</sup> and FvQ status 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.
Progeny of FvL/Tfpi Matings

<table>
<thead>
<tr>
<th>Matings</th>
<th>Observed (%)</th>
<th>Expected</th>
<th>P</th>
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<tr>
<td>Fv&lt;sup&gt;Q&lt;/sup&gt;(n4), Tfpi&lt;sup&gt;++&lt;/sup&gt;(n2) × Fv&lt;sup&gt;Q&lt;/sup&gt;(n4)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>At 3 weeks</td>
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<tr>
<td>Fv&lt;sup&gt;Q&lt;/sup&gt;, Tfpi&lt;sup&gt;++&lt;/sup&gt;</td>
<td>31 (33)</td>
<td>25</td>
<td></td>
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<tr>
<td>Fv&lt;sup&gt;Q&lt;/sup&gt;, Tfpi&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2 (2)</td>
<td>25</td>
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<tr>
<td>Fv&lt;sup&gt;Q&lt;/sup&gt;, Tfpi&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Fv&lt;sup&gt;Q&lt;/sup&gt;, Tfpi&lt;sup&gt;-&lt;/sup&gt;</td>
<td>26 (28)</td>
<td>25</td>
<td></td>
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<tr>
<td>At embryonic day 18.5</td>
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<tr>
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<tr>
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<td>8 (21)</td>
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<td>Fv&lt;sup&gt;Q&lt;/sup&gt;(n10), Tfpi&lt;sup&gt;++&lt;/sup&gt;(n7) × Fv&lt;sup&gt;Q&lt;/sup&gt;(n10)</td>
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<td>At 3 weeks</td>
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<tr>
<td>Fv&lt;sup&gt;Q&lt;/sup&gt;, Tfpi&lt;sup&gt;++&lt;/sup&gt;</td>
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<td>12.5</td>
<td>&lt;0.005</td>
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<td>47 (30)</td>
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Histology

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

Statistical Analysis

The significance of survival differences between groups was determined using the χ<sup>2</sup> test, and differences in fibrinogen 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 (Fv<sup>Q</sup>) (N4) were first crossed with heterozygous TFPI-deficient mice (Tfpi<sup>++</sup>) (N2). Double-heterozygous mouse (Fv<sup>Q</sup>, Tfpi<sup>++</sup>) were then crossed to homozygous FvL mice (Fv<sup>Q</sup>, Tfpi<sup>++</sup>) to generate 4 possible genotypes with an expected frequency of 25% each. As shown in the Table, only 2 of 94 offspring with the Fv<sup>Q</sup>, Tfpi<sup>++</sup>- genotype were observed at the weaning age of 3 weeks, and 1 of these died shortly after weaning. This marked underrepresentation of the Fv<sup>Q</sup>, Tfpi<sup>++</sup>- genotype was significantly different than the expected Mendelian frequency. To determine whether the loss of Fv<sup>Q</sup>, Tfpi<sup>++</sup>- 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 mendelian distribution of all potential genotypes was observed. These results indicate that the Fv<sup>Q</sup>, Tfpi<sup>++</sup>- 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 Fv<sup>Q</sup> mutation, survival analysis of Fv<sup>Q</sup>, Tfpi<sup>++</sup> mice was repeated after FvL and Tfpi<sup>++</sup> 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 Fv<sup>Q</sup>, Tfpi<sup>++</sup> mice were observed. These results confirm the lethality of the Fv<sup>Q</sup>, Tfpi<sup>++</sup>- genotype.

Histology

As shown in Figure 1, extensive fibrinogen staining was detected in the liver, lung, and kidney from Fv<sup>Q</sup>, Tfpi<sup>++</sup>- neonates, whereas only scant staining was noted in Fv<sup>Q</sup>, Tfpi<sup>++</sup>- neonates. The mean (±SD) fibrinogen staining score for multiple liver, lung, and kidney sections from 5 Fv<sup>Q</sup>, Tfpi<sup>++</sup>- neonates was 2.3±0.5 compared with 1.0±0 for 4 Fv<sup>Q</sup>, Tfpi<sup>++</sup>- neonates (P<0.0001). The sole surviving Fv<sup>Q</sup>, Tfpi<sup>++</sup>- adult mouse was found to be circling and gasping in the cage at ≈1 year of age. The mouse was killed, and autopsies 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 populations 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. 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. 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. 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.
dence of spontaneous thrombosis. 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. The report of significantly decreased free and total plasma TFPI levels in symptomatic patients with FVL compared with asymptomatic FVL patients additionally supports the potential relevance of our observations in this murine model system for FVL in humans.

We previously have demonstrated that heterozygous Tfpi deficiency in mice affected time to occlusive thrombosis after endothelial injury at the site of an atherosclerotic plaque. 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 Tfpi deficiency is uniformly lethal in mice that are also homozygous for 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**

This work was supported by National Institutes of Health grants HL-35989 and PO1 HL5734 (to Dr Eitzman), HL 34462 (to Dr Eitzman et al Tissue Factor Pathway Inhibitor and Factor V Leiden 2141

\[ Fv\textsuperscript{0/0},Tfpi^{+/+} \quad Fv\textsuperscript{0/0},Tfpi^{+/-} \]

\[ \text{liver} \quad \text{lung} \quad \text{kidney} \]

\[ \text{Figure 1. Antifibrin(ogen) staining of tissues from neonates.} \]

\[ \text{Figure 2. H&E-stained tissues from surviving adult Fv\textsuperscript{0/0}/Tfpi^{+/-} mouse demonstrating evidence of thrombosis in liver (A) and lung (B) along with old infarction in kidney (C).} \]
Broze), and PO1 HL5734 (to Dr Ginsburg). Dr Ginsburg is a Howard Hughes Medical Institute investigator.

References
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_Circulation_. 2002;105:2139-2142; originally published online April 15, 2002; doi: 10.1161/01.CIR.0000017361.39256.82

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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