Factor V<sub>Leiden</sub> Inhibits Fibrinolysis In Vivo

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**Background**—Factor V<sub>Leiden</sub> (fV<sub>Leiden</sub>) predisposes to thrombosis by enhancing thrombin formation. This study tested the hypothesis that fV<sub>Leiden</sub> inhibits fibrinolysis in vivo.

**Methods and Results**—Radiolabeled clots were injected into the jugular veins of wild-type mice and mice heterozygous (fV<sup>Q/Q</sup>) or homozygous (fV<sup>Q/Q</sup>) for fV<sub>Leiden</sub>. Mean percent clot lysis 5 hours later was significantly reduced in fV<sup>Q/Q</sup> mice (14.3±3.6%, n=13) compared with wild-type mice (40.2±7.0%, n=17; P<0.01) and intermediate in fV<sup>Q/Q</sup> mice (29.4±8.7%, n=9; P<0.03 versus fV<sup>Q/Q</sup>, P=0.36 versus wild type). The rate of in vitro lysis of plasma clots prepared from fV<sup>Q/Q</sup> or fV<sup>Q/Q</sup> mice was significantly lower than that of wild-type plasma clots, whereas in vitro clot lysis did not differ significantly between groups after inhibiting thrombin-activatable fibrinolysis inhibitor.

**Conclusions**—fV<sub>Leiden</sub> inhibits fibrinolysis in vivo, suggesting an additional pathway by which this mutation promotes thrombosis. (Circulation. 2004;110:3594-3598.)

**Key Words:** fibrin ■ fibrinolysis ■ coagulation ■ thrombosis ■ thrombolysis

The factor V<sub>Leiden</sub> (fV<sub>Leiden</sub>) mutation is the most prevalent genetic risk factor for thrombosis. fV<sub>Leiden</sub> has normal procoagulant activity but is resistant to inactivation by activated protein C (APC) because of an arginine-to-glutamine procarboxypeptidase. By promoting thrombin generation, fV<sub>Leiden</sub> could enhance APC activation and downregulate fibrinolysis, thereby contributing to the prothrombotic phenotype associated with this mutation. In vitro studies demonstrated that fV<sub>Leiden</sub> could enhance TAFI activation and downregulate fibrinolysis, thereby contributing to the prothrombotic phenotype associated with this mutation. In vivo studies demonstrated that fibrin clots prepared from individuals homozygous for fV<sub>Leiden</sub> were resistant to the profibrinolytic effect of APC. However, the effect of fV<sub>Leiden</sub> on fibrinolysis in vivo is unknown. Mice expressing the murine homolog of fV<sub>Leiden</sub> display a prothrombotic phenotype. This study examined the effect of fV<sub>Leiden</sub> on the clearance of thrombi from the pulmonary vasculature of mice.

**Methods**

**Reagents**

Human α-thrombin (Calbiochem), 125I-human fibrinogen (Amersham), human tissue-type Plg activator (t-PA, Genentech), and lepirudin (Berlex) were purchased. Purified potato carboxypeptidase inhibitor (CPI) was a gift from Berlex Biosciences, Richmond, Calif. Mouse thromboplatin was prepared by homogenizing brains in acetone and resuspending the dried pellet in 0.9% NaCl.

**Animals**

Mice heterozygous for the fV<sub>Leiden</sub> mutation (fV<sup>Q/Q</sup>) with a 50%/50% 129X1/SvJ×C57BL/6J genetic background were obtained from Dr David Ginsburg, University of Michigan. Homozygous offspring were designated fV<sup>Q/Q</sup>. For experiments involving Leiden mice with a mixed genetic background, wild-type littersmates were used as controls. The fV<sub>Leiden</sub> mutation also was backcrossed ≥5 generations into the C57BL/6J genetic background. For experiments involving Leiden mice with a C57BL/6J genetic background, controls were C57BL/6J mice from Jackson Laboratory (Bar Harbor, Me). Mean pulmonary clot lysis did not differ significantly between mice with a mixed versus C57BL/6J genetic background.

**In Vivo Pulmonary Clot Lysis**

A mixture of 24 μL murine platelet-rich plasma (200 000/μL), 2.1 μL of 1 mol/L CaCl<sub>2</sub>, 1.0 μL 125I-fibrinogen (0.1 μCi), and 4.2 μL thrombin (50 U/mL) was aspirated into an 18-gauge needle. For some experiments, CPI (final concentration, 430 μg/mL) was added to the clotting mixture. After 30 minutes, the clot was washed in 0.9% NaCl and cut to 2-mm length, and its radioactive counts were measured. The radiolabeled clot was injected into the jugular vein of an ~8-week old mouse. Five hours later, the heart and lungs were excised and their radioactive counts were measured. Percent clot lysis was calculated as described. All animal experimental procedures were approved by the University Committee on Use and Care of Animals.

**In Vitro Clot Lysis**

Citrated murine plasma (100 μL) was clotted at 37°C in 96-well Immulon-2 microtiter plates by adding CaCl<sub>2</sub> (12.5 mmol/L), phosphatidylylcholine/phosphatidylserine vesicles (50 μg/mL), t-PA (0.7 nmol/L), and murine thromboplatin (1.2 μg/mL) as described. CPI (100 μg/mL) was added to some reactions. Final volume was

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3594
the decrease in A405, was significantly slower for \(fV^Q\) clots than for wild-type clots (Figure 2A). Mean clot lysis time of \(fV^Q\) mice (77.8±3.1 minutes, \(n=10\)) was significantly longer than that of wild-type mice (49.1±4.5 minutes, \(n=9\); \(P=0.014\)). Clot lysis times were also significantly prolonged in \(fV^Q\) mice (84.8±9.3 minutes, \(n=12\); \(P=0.004\) versus wild type), indicating that \(fV^\text{Leiden}\) produced a dominant-negative effect on fibrinolysis in vitro. However, when clots were formed in the presence of CPI, a TAFI inhibitor, mean clot lysis times were shorter and did not differ significantly between groups (Figure 2B), suggesting that \(fV^\text{Leiden}\) inhibited fibrinolysis in vitro predominantly or solely by promoting TAFI activation. The mean plasma level of activatable TAFI of \(fV^Q\) mice (11.0±0.7 µg/mL, \(n=7\)) did not differ significantly from that of wild-type mice (11.5±1.0 µg/mL, \(n=8\); \(P>0.7\)). Plasma \(fV\) activity did not differ significantly between \(fV^Q\) mice (7.5±0.7 U/mL, \(n=15\)), \(fV^Q\) mice (8.7±1.0 U/mL, \(n=13\)), and littermate wild-type mice (7.4±0.5 U/mL, \(n=13\); \(P>0.15\) versus other 2 groups).

**Discussion**

Our study demonstrates that mice expressing \(fV^\text{Leiden}\) exhibit impaired pulmonary clot lysis and suggests that the prothrombotic phenotype produced by \(fV^\text{Leiden}\) arises not only from enhanced fibrin formation but also from impaired fibrinolysis. This is the first study to demonstrate that \(fV^\text{Leiden}\) exerts a negative effect on fibrinolysis in vivo in a pathophysiologically relevant model of human disease. Our results confirm the significance of an alternative pathway by which \(fV^\text{Leiden}\) promotes vascular fibrin deposition and suggest that strategies aimed at enhancing fibrinolysis could prove useful in treating patients with \(fV^\text{Leiden}\) and thrombosis. Our studies do not establish the mechanism(s) by which \(fV^\text{Leiden}\) inhibits fibrinolysis in vivo. One potential mechanism is enhanced TAFI activation, resulting from enhanced thrombin formation. We tested this hypothesis by incorporating CPI, a TAFI inhibitor, into clots that embolized to the lungs of \(fV^Q\) mice. This intervention did not significantly enhance clot lysis in vivo. A previous animal study showed that systemic administration of CPI by 2 intravenous injections significantly enhanced clot lysis in vivo, whereas incorporation of CPI into the clot during its formation in vitro did not produce a statistically significant profibrinolytic effect in vivo.\(^{14}\) Those were 90-minute experiments in which thrombolysis was driven by pharmacological administration of t-PA. We studied endogenously mediated clot lysis for 5 hours, during which clot-incorporated CPI would have an even greater opportunity to lose activity or diffuse away from the clot. The half-life of CPI in vivo is \(\approx25\) minutes.\(^{15}\) Therefore, the failure of CPI, as used in our experiments, to restore the extent of pulmonary clot lysis in \(fV^Q\) mice to levels observed in wild-type mice does not exclude the possibility that TAFI is an important mediator of \(fV^\text{Leiden}\) in vivo antifibrinolytic effect. Future studies involving crosses between \(fV^\text{Leiden}\) mice and TAFI-deficient mice should prove useful in testing this hypothesis.\(^{16,17}\) Additional studies are also necessary to examine other potential mechanisms by which \(fV^\text{Leiden}\) produces an antifibrinolytic effect in vivo. For example, by promoting thrombin formation, \(fV^\text{Leiden}\) could enhance the release of plasminogen activator inhibitor-1.
from vascular wall cells. Enhanced thrombin generation produced by fVLeiden could also alter the structure of fibrin, potentially affecting its sensitivity to lysis.

Fibrin formation can continue in vivo as clot lysis proceeds because of exposure of clot-bound thrombin and continued activation of the blood coagulation pathways. The reduced clearance of radiolabeled fibrin in mice expressing fVLeiden could be mediated by a decreased rate of fibrinolysis and/or by increased formation of unlabeled thrombus on the surface of an injected clot, which could shield it from fibrinolytic factors. The latter process, ie, enhanced clot extension, would not represent a true fibrinolytic defect. Our in vitro studies demonstrated that the actual rate of clot lysis was attenuated by fVLeiden and that clot extension did not differ between wild-type mice and fVQ/Q mice, suggesting that the decreased clearance of radiolabeled clots from the pulmonary vasculature of fVLeiden mice was due to a fibrinolytic defect and not simply to enhanced thrombus formation on the surface of injected clots after they lodged in the lung. Nevertheless, enhanced fibrin formation as fibrinolysis proceeds could also contribute to the enhanced stability of thrombi in mice expressing fVLeiden. Measurement of other indices of in vivo clot lysis (eg, the kinetics of appearance of plasma fibrin degradation products), which was not performed in our

Figure 2. Effect of fVLeiden and CPI on in vitro clot lysis. A, Wild-type (WT) and fVQ/Q (QQ) plasmas were clotted in absence or presence of t-PA. A405 of reaction mixtures was monitored as index of clot formation and lysis. B, Mean lysis times of pooled plasma clots prepared in absence or presence of CPI. Data represent mean of 3 or 4 experiments. Numbers of mice in each group are shown. *P<0.3 vs WT without CPI; †P<0.02 vs WT without CPI; ‡P<0.002 vs same genotype without CPI. All other abbreviations are as defined in text.
experiments, could prove useful in more precisely defining the relative roles of the blood coagulation and fibrinolysis pathways in modulating the effect of $\text{F}^{\text{VLeiden}}$ on clot stability in vivo.

Our in vitro clot lysis experiments suggested that TAFI is the dominant mediator of the effect of $\text{F}^{\text{VLeiden}}$ on fibrinolysis, because inhibition of TAFI completely relieved the fibrinolytic defect. Plasma clots formed in vitro from humans homozygous for the $\text{F}^{\text{VLeiden}}$ mutation are resistant to the profibrinolytic effects of added APC, and APC does not promote fibrinolysis of TAFI-depleted clots. Our studies demonstrate that even in the absence of exogenous APC, $\text{F}^{\text{VLeiden}}$ inhibits fibrinolysis. It is likely that in a static, in vitro system, complete TAFI inhibition can be achieved much more readily than in vivo, which may account for the differences in the effect of CPI in our in vitro and in vivo experiments, although other factors could be involved. Our in vitro experiments also show that heterozygous $\text{F}^{\text{VLeiden}}$ expression inhibits fibrinolysis, results consistent with a study demonstrating impaired plasma clot lysis in vitro in women with $\text{F}^{\text{VLeiden}}$ and APC resistance who developed deep venous thrombosis. The antifibrinolytic effect of heterozygous, murine $\text{F}^{\text{VLeiden}}$ was as potent in vitro as that produced by homozygosity for the mutation. In contrast, heterozygosity for $\text{F}^{\text{VLeiden}}$ did not statistically significantly inhibit clot lysis in vivo. We cannot explain these differences, but they are probably related to factors, such as blood flow and products of the vascular endothelium (eg, thrombomodulin), that are not recapitulated in vitro. $\text{F}^{\text{VLeiden}}$ can potentially modulate thrombin production and fibrinolysis by different mechanisms, including enhanced $\text{F}$ procoagulant activity and diminished capacity to serve as a cofactor for the inactivation of $\text{FVIIa}$ by APC. Differences in the relative impact of these processes in a static, in vitro system versus the pulmonary vasculature of a living mouse may contribute to the differences between our in vitro and in vivo assays in regard to the fibrinolytic defect produced by heterozygosity for $\text{F}^{\text{VLeiden}}$. Effects of sample size must also be considered. Although our in vivo experiments suggest that the antifibrinolytic effect of heterozygous $\text{F}^{\text{VLeiden}}$ is less potent than that produced by homozygous $\text{F}^{\text{VLeiden}}$, it is possible that with analysis of larger numbers of mice the reduced clot lysis in heterozygotes may have achieved statistical significance compared with wild-type mice.

A recent study found that mice completely lacking plasma carboxypeptidase B (pCPB, or TAFI) did not exhibit accelerated pulmonary clot lysis compared with controls. However, in the presence of heterozygous Plg deficiency, mice lacking pCPB (ie, $\text{Plg}^{+/+}, \text{pCPB}^{-/-}$) exhibited accelerated pulmonary clot lysis compared with mice with normal TAFI expression (ie, $\text{Plg}^{+/-}, \text{pCPB}^{++}$ mice). The authors hypothesized that the ratio of TAFI to Plg in plasma plays a key role in regulating fibrinolysis and that TAFI inhibited fibrinolysis in vivo when the ratio was increased by partial Plg deficiency. By promoting TAFI activation, $\text{F}^{\text{VLeiden}}$ provides an alternate route to increasing the active TAFI-to-Plg ratio in plasma, which we hypothesize may enable TAFI to regulate pulmonary thrombolysis in mice with normal Plg expression. Methods are not currently readily available to measure plasma levels of activated TAFI in mice. We found no significant difference between mean plasma levels of activatable TAFI of $\text{F}^{\text{QQ}}$ mice versus wild-type mice, although the number of mice in each group was modest. In contrast, a human study involving a greater number of subjects observed slightly lower levels in individuals expressing $\text{F}^{\text{VLeiden}}$ compared with controls, which the authors hypothesized was due to enhanced TAFI activation in vivo, resulting in reduced plasma levels of TAFI zymogen capable of activation in vitro.

In conclusion, we have demonstrated that $\text{F}^{\text{VLeiden}}$ inhibits fibrinolysis in vivo. Further studies are indicated to define the mechanism(s) underlying this effect and its in vivo significance in the setting of heterozygous $\text{F}^{\text{VLeiden}}$. However, our in vitro data and those of others suggest that TAFI may play a key role in mediating the antifibrinolytic effect of $\text{F}^{\text{VLeiden}}$. The inhibition of fibrinolysis by $\text{F}^{\text{VLeiden}}$ has significant clinical implications. For example, strategies aimed at enhancing fibrinolysis may prove useful in treating or preventing thrombosis in patients with $\text{F}^{\text{VLeiden}}$. Conversely, it is possible that pharmacological thrombolysis could be attenuated by $\text{F}^{\text{VLeiden}}$. Additional studies are necessary to address these issues.

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References


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