Vascular Medicine

Homozygosity for Factor V Leiden Leads to Enhanced Thrombosis and Atherosclerosis in Mice

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Background—Activated protein C resistance due to factor V Leiden (FVL) is a common genetic risk factor for venous thrombosis in humans. Although the impact of FVL on the development of venous thrombosis is well established, its effect on arterial thrombosis and atherosclerosis is controversial.

Methods and Results—To determine the effect of the FVL mutation on arterial thrombosis in the mouse, wild-type (Fv+/+) and heterozygous FVL (FvQ/Q) mice underwent photochemical carotid arterial injury to induce occlusive thrombosis. FvQ/Q mice formed occlusive thromboses 27±3 minutes (n=7) after the onset of injury, which was significantly shorter than that observed for Fv+/+ mice (56±7 minutes, n=9, P<0.01), whereas FvQ/Q mice (41±7 minutes, n=5) were intermediate (P=0.5, compared with Fv+/+). To determine the source of FVL relevant to the enhanced vascular thrombosis, bone marrow transplantation experiments were performed between Fv+/+ and FvQ/Q mice. FvQ/Q mice transplanted with Fv+/+ bone marrow formed occlusive thromboses at 35±5 minutes (n=7, P<0.05 compared with Fv+/+ mice), whereas Fv+/+ mice transplanted with FvQ/Q bone marrow occluded at 59±7 minutes (n=6, P<0.001 compared with FvQ/Q mice). To assess the effect of the FVL mutation on the development of atherosclerosis, FvQ/Q mice were crossed with the atherosclerosis-prone apolipoprotein E (ApoE)–deficient strain (ApoE−/−) to generate FvQ/Q,ApoE−/− mice. By 52 weeks of age, FvQ/Q,ApoE−/− mice (n=8) had developed more aortic atherosclerosis (40±6% lesion area) compared with Fv+/+,ApoE−/− mice (15±3% lesion area; n=12, P<0.02).

Conclusions—in conclusion, homozygosity for the FVL mutation in mice leads to enhanced arterial thrombosis and atherosclerosis. The source of the FVL leading to accelerated thrombosis appears to be circulating, non–platelet-derived plasma FVL. (Circulation. 2005;111:1822-1825.)

Key Words: atherosclerosis ■ coagulation ■ fibrinogen ■ thrombosis

Facto

The relevant source of FVL in vascular thrombosis is also unclear. In both humans and mice, there are distinct platelet and plasma pools of factor V. The origin of platelet factor V in humans appears to result from the plasma compartment, whereas mouse platelet factor V is derived from synthesis within the megakaryocyte. Although the biosynthetic origins are different, the relative sizes of the 2 pools in mice and humans are similar. Thus, the mouse may serve as a particularly useful model to dissect the relative contribution of these pools toward vascular thrombosis.

To test the hypothesis that the FVL mutation will affect arterial thrombosis and atherosclerosis, mice with varying patterns of FVL expression were studied in a carotid artery thrombosis model and crossed with atherosclerosis-prone, apolipoprotein E (ApoE)–deficient mice.

Mice

Generation of mice carrying the murine homologue of the FVL mutation has been previously described. These mice were backcrossed to C57BL/6 mice (Jackson Labs, Bar Harbor, Me) for at least 8 generations before use in thrombosis and atherosclerosis experiments. The ApoE–deficient mice were also obtained from Jackson Labs and were backcrossed to C57BL/6J for >10 generations before crossbreeding to FVL mice. Genotyping for FVL and ApoE was performed by polymerase chain reaction (PCR) analysis of tail DNA with the primers previously described. After weaning, at 3 weeks of age, mice were fed standard chow (laboratory rodent diet No. 5001, TestDiet). At 7 weeks of age, one group of

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mice was switched to Western chow (TD 88137, Teklad) for atherosclerosis experiments.

**Carotid Arterial Thrombosis**

Eight-week-old, male, wild-type (Fv\(^{+/+}\)), FVL heterozygous (Fv\(^{Q/Q}\)), and FVL homozygous (Fv\(^{Q/Q}\)) mice were subjected to photochemical injury of the right carotid artery by the application of rose bengal as previously described. Flow in the vessel was monitored until occlusive thrombosis occurred, defined as cessation of flow for at least 1 minute.

**Bone Marrow Transplantation**

Bone marrow transplantations were performed similarly to previously described methods. Six-week-old, male Fv\(^{+/+}\) and Fv\(^{Q/Q}\) mice were irradiated with 1300 rads followed by tail vein injection of RPMI containing 5×10\(^6\) fetal liver cells derived from Fv\(^{+/+}\) or Fv\(^{Q/Q}\) donors. Control mice were irradiated but received only tail vein injection with medium. These control mice died 1 to 2 weeks after irradiation, consistent with engraftment in the surviving mice. Using an identical transplantation protocol in similar mice with the same genetic background, we have consistently observed complete engraftment by PCR analysis.

**Analysis of Atherosclerotic Lesions in ApoE-Deficient Mice**

At 19, 34, and 52 weeks of age, mice were perfusion fixed with zinc formalin under intraperitoneal pentobarbital anesthesia (100 mg/kg). For quantification of surface area occupied by atherosclerosis, the aorta and carotid arteries were stained with oil red O and then subjected to quantitative morphometry as previously described.

**Fibrin Immunostaining**

Fibrinogen immunostaining was performed with a goat anti-mouse fibrinogen polyclonal antibody (Accurate Chemical and Scientific Corp). The primary antibody was detected with a biotin-labeled rabbit anti-goat IgG (Chemicon International, Inc). Aortic atherosclerotic lesions were graded for fibrin deposition by an observer who was blinded to the genotype of the mice by using a scale from 1 to 10, with each number representing a percent area of lesion staining for fibrinogen (ie, 1=1% to 10%, 2=11% to 20%, 3=21% to 30%, etc).

**Cholesterol Measurement**

Serum obtained from retro-orbital bleeding was used to measure cholesterol with a commercial cholesterol kit from Wako Chemicals Inc.

**Statistical Analysis**

The statistical significance of differences in time to occlusion and atherosclerosis surface area between the various groups was determined by the Student 2-tailed t test when only 2 groups were being compared and by 1-way ANOVA (followed by pairwise post hoc comparison) when >2 experimental groups were included in the analysis. A probability value <0.05 was considered significant.

**Results**

**Effect of FVL on Development of Occlusive Thrombosis After Carotid Injury**

To examine the contribution of FVL to the development of occlusive thrombosis after carotid injury, 8-week-old mice were subjected to photochemical injury of the right middle common carotid artery. The mean time to occlusion in Fv\(^{Q/Q}\) mice (n=7) was significantly shorter than that observed in Fv\(^{+/+}\) (n=9) mice (Figure 1). Times to occlusion in Fv\(^{Q/Q}\) mice (n=5) were intermediate but not significantly different from those for Fv\(^{+/+}\) or Fv\(^{Q/Q}\) mice.

To determine whether the source of the FVL affecting vascular thrombosis after arterial injury was derived from platelets or plasma, bone marrow transplantation was performed between Fv\(^{+/+}\) and Fv\(^{Q/Q}\) mice. Fv\(^{+/+}\) mice (n=6) that received marrow from Fv\(^{Q/Q}\) donors formed occlusive thrombi in a time period similar to that of Fv\(^{+/+}\) nontransplanted mice and significantly longer than in Fv\(^{Q/Q}\) mice. Fv\(^{Q/Q}\) mice (n=7) that received marrow from Fv\(^{+/+}\) mice occluded in a time period similar to Fv\(^{Q/Q}\) nontransplanted mice but significantly shorter than in Fv\(^{+/+}\) mice (Figure 1).

**Effect of FVL on the Development of Atherosclerosis**

To determine the effect of FVL on the development of atherosclerosis, groups of Fv\(^{+/+}\), ApoE\(^{-/-}\), and Fv\(^{Q/Q}\), ApoE\(^{-/-}\), and Fv\(^{Q/Q}\), ApoE\(^{-/-}\) mice were euthanized at different ages. Group 1 (Fv\(^{+/+}\), ApoE\(^{-/-}\) [n=4], Fv\(^{Q/Q}\), ApoE\(^{-/-}\) [n=6], and Fv\(^{Q/Q}\), ApoE\(^{-/-}\) [n=4]) was started on a Western chow diet at 7 weeks of age and then euthanized at 19 weeks. With this protocol, significant differences in atherosclerosis were observed among the 3 genotypes (Figure 2a). Because high-fat feeding greatly accelerates the progression of atherosclerosis, it is possible that relevant genetic modifiers of atherosclerosis could be overwhelmed under these circumstances. Therefore, additional experiments were performed with mice fed normal chow. Mice in group 2 (Fv\(^{+/+}\), ApoE\(^{-/-}\) [n=9], Fv\(^{Q/Q}\), ApoE\(^{-/-}\) [n=9], and Fv\(^{Q/Q}\), ApoE\(^{-/-}\) [n=9]) were maintained on normal chow and euthanized at 34 weeks of age. As in group 1, there was no significant difference in aortic lesion area among the 3 different groups of mice (Figure 2a).

Because alterations in coagulation might have a chronic, low-grade effect on atherosclerosis and only be apparent after a prolonged period, group 3 (Fv\(^{+/+}\), ApoE\(^{-/-}\) [n=12], Fv\(^{Q/Q}\), ApoE\(^{-/-}\) [n=12], and Fv\(^{Q/Q}\), ApoE\(^{-/-}\) [n=8]) was maintained on normal chow and euthanized at 52 weeks of age. In group 3, Fv\(^{Q/Q}\), ApoE\(^{-/-}\) mice displayed significantly more aortic...
atherosclerosis than \( Fv^{+/-}, ApoE^{-/-} \) mice, whereas \( Fv^{+/-}, ApoE^{-/-} \) mice had an intermediate phenotype (Figure 2a). Aortic plaques from \( Fv^{0/0}, ApoE^{-/-} \) mice (n=6) in group 3 also showed significantly more fibrin deposition compared with \( Fv^{+/-}, ApoE^{-/-} \) mice (n=10) (Figure 2b). A similar trend for fibrin deposition was also seen in group 2 between \( Fv^{0/0}, ApoE^{-/-} \) (n=8) and \( Fv^{+/-}, ApoE^{-/-} \) mice (n=6), but this difference did not reach statistical significance (Figure 2b). No differences in carotid artery atherosclerosis were noted among the groups (data not shown). There were no differences in cholesterol among the various genotypes in group 3 (\( Fv^{+/-}, ApoE^{-/-} = 403 \pm 44, Fv^{+/-}, ApoE^{-/-} = 406 \pm 58, Fv^{0/0}, ApoE^{-/-} = 449 \pm 61 \text{ mg/dL} \)).

**Discussion**

FVL has a prevalence of 2% to 7% in most European populations and is the major known genetic risk factor for venous thrombosis in humans. The risk of arterial thrombosis and atherosclerotic vascular disease associated with the FVL muta-

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bed–specific effects of various factors involved in coagulation and fibrinolysis, depending on gene expression patterns. This enhanced late atherosclerosis was associated with more fibrinogen deposition in FvQ/Q mice, with many FvQ0 mice showing intense focal fibrin deposits. This observation may relate to the importance of enhanced thrombin formation with fibrin deposition during the later stages of atherosclerosis, when thrombotic complications are observed in humans. It may also take a long time for the effects of alterations in coagulation on atherosclerosis to become apparent. Fibrin has been shown to affect the development of atherosclerosis in mice overexpressing an ap(a) transgene; however, another study that analyzed mice with combined fibrinogen and ApoE deficiency demonstrated that fibrin is not required for the development of atherosclerosis. Thus, the mechanism for the increased atherosclerosis observed in the current study may also relate to fibrin-independent activities of thrombin. Additional experiments will be required to clearly define the underlying mechanisms.

These studies indicate that homozygosity for the FVL mutation in the mouse leads to enhanced arterial thrombosis and atherosclerosis. In addition, the prothrombotic phenotype observed after arterial injury appears to result from plasma-derived FVL. This may have important implications with regard to potential therapies designed to reverse the prothrombotic phenotype of patients with the FVL mutation. For example, it may be possible to reduce thrombophilia due to FVL by selectively targeting plasma factor V while still maintaining physiological hemostasis.

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