Role of the Intrinsic Coagulation Pathway in Atherogenesis Assessed in Hemophilic Apolipoprotein E Knockout Mice


Objective—The contribution of thrombosis and coagulation in atherogenesis is largely unknown. We investigated the contribution of the coagulation intrinsic factor VIII (FVIII)–dependent pathway in atherogenesis.

Methods and Results—Apolipoprotein E and FVIII double–deficient mice (E°/FVIII°) were generated. Aortic root lesions were analyzed in 14-week-old and 22-week-old female mice maintained for 8 or 16 weeks, respectively, on a normal chow diet or a hypercholesterolemic diet.

Conclusion—Despite a higher plasma total cholesterol concentration compared with E° mice, E°/FVIII° mice developed dramatically less early-stage atherosclerotic lesions. Whereas early lesions in E° mice contained abundant fibrin(ogen) deposits on which few platelets adhered, lesions in E°/FVIII° were almost devoid of fibrin(ogen), and no platelets could be detected. The genotype effect on development and composition of lesions tended to decrease with time. This study demonstrates that the activation of the intrinsic pathway of coagulation is potently proatherogenic at the early stage of atherogenesis. (Arterioscler Thromb Vasc Biol. 2005;25:e123-e126.)

Key Words: atherosclerosis ■ hemophilia ■ mouse ■ knockout

Complications of atherosclerosis are the main causes of death in industrialized countries. The current paradigm establishes procoagulant state and thrombosis to be the major reason for complications. Consequently, it has been assumed that thrombosis and coagulation would also be involved in lesion formation. For instance, it is known that platelet activation and adhesion occur during the very first steps of the disease, and the presence of the coagulation factor VIII (FVIII) has been reported in the vicinity of macrophages and smooth muscle cells in human atherectomy specimens. Yet, the contribution of coagulation and thrombosis in atherogenesis remains largely unknown. Apolipoprotein E knockout (E°) mice are a reference model for experimental atherosclerosis. As a result of their severe hypercholesterolemia, which can be aggravated by a hypercholesterolemic diet, these mice develop all stages of disease, from early fatty lesions to mature fibrofatty plaques. The present study was aimed at determining the influence of the intrinsic coagulation pathway in atherogenesis by analyzing lesion development in E° mice deficient for the FVIII. We found that the absence of this coagulation factor has a strong impact on the early stage of lesion formation.

Materials and Methods

Generation of E°/FVIII° Mice
E°/FVIII° double–deficient mice were generated by crossing E° and FVIII° single knockout mice. FVIII° mice were provided kindly by Dr Kazazian (University of Pennsylvania, Philadelphia). E°, FVIII°, and E°/FVIII° F2 offspring were used to establish the 3 genotype colonies. The genotyping was performed by polymerase chain reaction on DNA extracted from mouse tails.

Experimental Protocols
Five- to 6-week-old E°/FVIII°, control E°, and FVIII° female mice were placed on a normal chow diet (ND) or a hypercholesterolemic 0.15% cholesterol Western diet (WD) for 8 or 16 weeks (respectively, the “8-week protocol” and the “16-week protocol”). Weights were measured at death. All experiments were approved by our institutional ethical committee.

Plasma Measurements
Citrated blood collected at death was centrifuged at 4°C for 5 minutes at 12 000g, and plasma was stored at −20°C until analysis. Total plasma cholesterol (TC) level was measured using a Boehringer Mannhein kit (France-méthode “CHOD-PAP”). Plasma FVIII activity was measured by a chromogenic assay (Dade-Behring).

Analysis of Atherosclerotic Lesions
After vascular perfusion with sterile PBS, the heart and ascending aorta were dissected. Lesions were quantitated in the aortic sinus as described.
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carotid and femoral arteries10 or significantly reduced in the patients with hemophilia was either minimally reduced in the compared with healthy controls, the extent of intimal lesions in metabolic potential more efficiently for the cholesterol biosynthesis. which hepatocytes do not produce FVIII, could use their hepatic production of coagulation factors that can be partially rescued on inhibition of cholesterol biosynthesis.9 Thus, hemophilic mice, in between the hepatic synthesis of FVIII and cholesterol metabolism. FVIII° mice on ND or a WD displayed a significantly higher TC regardless of the genotype (Figure 1A). Surprisingly, E°/FVIII° mice had an increased body weight compared with single-deficient mice (Figure 1A). The type of diet did not modify the activity (Figure 1A) was indistinguishable from background levels in FVIII° and E°/FVIII° mice. The plasma FVIII activity was confirmed that the residual plasma FVIII were performed as described in the Figure 2 legend and the Table. Results and Discussion The chromogenic assay confirmed that the residual plasma FVIII activity (Figure 1A) was indistinguishable from background levels in FVIII° and E°/FVIII° mice. The plasma FVIII activity was increased by the WD in E° mice, thus suggesting that the intrinsic coagulation pathway is solicited during the disease process. E°/FVIII° mice had an increased body weight compared with single-deficient mice (Figure 1A). The type of diet did not modify the weight. As expected, E° mice had a significantly higher TC compared with FVIII° mice. The WD resulted in a further increase in TC regardless of the genotype (Figure 1A). Surprisingly, E°/FVIII° mice on ND or a WD displayed a significantly higher TC compared with E° mice. This might be suggestive of a competition between the hepatic synthesis of FVIII and cholesterol metabolism. Indeed, partial hepatectomy results, among other effects, in reduced production of coagulation factors that can be partially rescued on inhibition of cholesterol biosynthesis.14 Thus, hemophiliic mice, in which hepatocytes do not produce FVIII, could use their hepatic metabolic potential more efficiently for the cholesterol biosynthesis. Recent studies by B-mode ultrasonography showed that, compared with healthy controls, the extent of intimal lesions in patients with hemophilia was either minimally reduced in the carotid and femoral arteries10 or significantly reduced in the abdominal aorta and leg arteries.11 The discrepancies between these clinical studies might be attributable to the small number of patients available and heterogeneity of treatments (frequency of FVIII infusion, plasma-derived or recombinant FVIII, on-demand or prophylactic treatment). It is important to note that up to 90% of severe hemophilic patients are nowadays under prophylactic FVIII treatment.12 These restraints may hamper the analysis of the impact of FVIII deficiency on atherosclerosis in hemophilia. The mouse model developed in the present study may provide an adequate system to address this issue. We found that regardless of the diet, FVIII° mice developed only small fatty streaks. Despite of higher TC, E°/FVIII° exhibited reduced lesions compared with E° mice (Figure 1B). This effect was dramatic in the 8-week protocol and declined after 16 weeks. Indeed, lesions were respectively 79.0% (ND) and 67.2% (WD) smaller in E°/FVIII° compared with E° mice in the 8-week protocol. In the 16-week protocol, lesions were 26.5% (ND) and 29.8% (WD) smaller in E°/FVIII°. These results show a proatherogenic contribution of the FVIII-dependent intrinsic coagulation pathway, which is prominent during the early stage of atherogenesis and which declines with time. Next, we aimed at correlating the major difference in lesion size observed in the 8-week protocol between E°/FVIII° and E° mice with differences in plaquelet content, fibrin(ogen) deposition, and inflammation. Resting and activated platelets were analyzed using antibodies that bind to both. Expression of vascular cell adhesion molecule-1 (VCAM-1) and MAC-3 was considered an index of endothelium activation and macrophage infiltration. In lesions of the double-knockout mice (consistently of the early type), traces of fibrin(ogen) deposition but not platelet adhesion was detected (Figure 2). These lesions contained few macrophages and expressed almost undetectable levels of VCAM-1 (Figure 2; Table). Proteoglycan and collagen deposition in the intimal lesions analyzed by the Periodic acid–Schiff/Alcian blue and Sirius red histological staining were not detected in E°/FVIII° mice in the 8-week protocol (Table). In E° mice, fibrin(ogen) deposition was abundant and platelets were found adherent to the lesions (Figure 2). Lesions were also infiltrated by numerous macrophages. VCAM-1 expression was intense (Figure 2; Table) but not restricted to endothelial cells, as reported.13 Therefore, in the 8-week protocol, hypercholesterolemic mice deficient for FVIII developed lesions that were not only smaller but also strikingly different from the FVIII-competent mice. Plaque composition was studied in the 16-week protocol, a stage in which difference in plaque size between E° and E°/FVIII° mice tended to reduce. VCAM-1, MAC-3, platelet glycoprotein (GP) Iα+Iβ, and fibrin(ogen) immunolabeling

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### Table: Changes in plaque composition

<table>
<thead>
<tr>
<th>8-Week Protocol</th>
<th>16 Week Protocol</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E°</td>
</tr>
<tr>
<td><strong>MAC-3</strong></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>13 351±3550</td>
</tr>
<tr>
<td>WD</td>
<td>21 646±7059</td>
</tr>
<tr>
<td><strong>VCAM-1</strong></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>2586±1019</td>
</tr>
<tr>
<td>WD</td>
<td>6430±1470</td>
</tr>
<tr>
<td><strong>Acidic PG</strong></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>33 221±1150</td>
</tr>
<tr>
<td>WD</td>
<td>10 504±3406</td>
</tr>
<tr>
<td><strong>Neutral PG</strong></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>723±653</td>
</tr>
<tr>
<td>WD</td>
<td>1493±422</td>
</tr>
<tr>
<td><strong>Collagen</strong></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>68±19</td>
</tr>
<tr>
<td>WD</td>
<td>672±197</td>
</tr>
</tbody>
</table>

Computer-assisted morphometry was performed on aortic root sections immunolabeled for MAC-3 and VCAM-1, stained by periodic acid–Schiff/Alcian blue (acidic and neutral proteoglycans were identified as magenta-colored and blue-colored areas, respectively), and stained by Sirius red (collagen). Measures were performed on sections from E° and E°/FVIII° mice maintained for 8 or 16 weeks on ND or WD. Values are expressed as mean surface area (μm²) ±SEM measured within the lesions.

*P<0.05 E°/FVIII° vs aged-matched E° mice.
densities were equivalent in E° and E°/FVIII° mice (Table; data not shown). The proteoglycan and collagen contents were equivalent in both groups (Figure 2; Table), indicating that they are not dependent on an operative intrinsic pathway of coagulation at this stage of the disease. Given that it has been proposed recently that the accumulation of erythrocyte membranes within an atherosclerotic plaque may represent a potent atherogenic stimulus and that such events were expected to be more frequent in hemophilic atherosclerosis-prone mice, hemosiderin was searched for by the Perl’s iron method as an indicator of previous hemorrhage. An increased frequency of intraplaque hemorrhages in E°/FVIII° mice would have been expected to contribute to a more severe atherosclerosis according to the concept proposed recently by Kolodgie et al. We rather observed a more limited disease. Although iron was deposited in the perivascular myocardium of E°/FVIII° mice, it was not detected in the lesions of the aortic root (data not shown). This indicates that erythrocyte accumulation in the plaque does not contribute to lesion formation in this model. Thus, at later stage of lesion development, the lack of FVIII still exerts an effect on lesion size, but the lesions have a composition proportionately similar in E° and E°/FVIII° mice. These results are in agreement with those reported in atherosclerosis-prone mice deficient for the fibrinogen. Fibrinogen deficiency was reported to decrease the early lesion development in apolipoprotein(a)-transgenic mice as well as in E° mice. However, in the latter study, the effect is less patent because the earliest time point at which lesions were evaluated is 22 weeks of age.

In conclusion, this study shows that the intrinsic pathway of coagulation is critically involved in the early phase of atherogenesis. The activation of this pathway is clearly proatherogenic at the stage of fatty streak. It has been proposed that platelets that adhere to the arterial wall in vivo in the absence of endothelial cell denudation can locally release preformed inflammatory mediators on GPIIb-IIIa signaling. Such mediators alter the inflammatory phenotype of the endothelium by enhancing its expression of adhesion receptors and of chemokines that initiate monocyte recruitment. At the early stage of lesion development in hemophilic hypercholesterolemic mice, we did not find platelets adherent to the vessel wall, and VCAM-1 expression was significantly low. Therefore, we may speculate at this stage that platelet adhesion is predominantly dependent on the intrinsic pathway of coagulation. Consequently, FVIII deficiency resulted in a limited inflammation in the vessel wall. Further studies on atherosclerosis-prone mice deficient for a factor of the extrinsic pathway will be necessary to verify that this coagulation pathway is not involved in the early stage of lesion formation. At a later stage, the impact of the intrinsic pathway seems to decline, and the absence of FVIII does not lead to a difference in lesion composition but rather induces a delay in the progression of the disease. This might be an indication that limited but chronic inflammation and tissue

Figure 1. A, FVIII activity, TC, and weight were measured from plasma collected at death on E°/FVIII°, E°, and FVIII° mice maintained for 8 or 16 weeks on ND or WD. FVIII activity was measured by a chromogenic assay in which thrombin-activated FVIII acts as a cofactor for Factor Xa in the conversion of Factor X to Factor Xa, using plasma from wild-type C57BL/6 mice as a standard. Results are expressed as arbitrary units (au). TC level was measured using a Boehringer Mannheim kit (France-méthode “CHOD-PAP”). B, Extent of lesion development was analyzed by computer-assisted morphometry on oil red-O stained and hematoxylin counterstained sections of the aortic root from E°/FVIII°, E°, and FVIII° mice maintained for 8 or 16 weeks on ND or WD. Serial cryostat sections were cut from the proximal 1 mm of the aortic root. The lesion density (surface area of lesions/surface area of vessel; %) was determined in each aortic root on 4 hematoxylin/oil red-O stained sections cut at 220, 400, 600, and 800 μm from the appearance of the first cusp. (Results are expressed as means±SEM.) Nonparametric Mann–Whitney tests were performed using Statview 5.0 software (SAS Institute Inc.). Differences between groups were considered significant if P<0.05, and significant differences shown for lesion densities refer to differences compiled from all sections. †E°/FVIII° vs E°; §E°/FVIII° vs FVIII°; ¶E° vs FVIII°. Four symbols P<0.0001; 3 symbols P<0.001; 2 symbols P<0.01; 1 symbol P<0.05.
damage with the release of tissue factor can ultimately activate the extrinsic pathway in hemophilic mice (FVIII deficiency lowers clotting efficiency but does not neutralize clot formation). Once activated, the extrinsic pathway might trigger platelet activation intricately associated with a chronic inflammation, which is then independent of the intrinsic pathway.

Acknowledgments

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