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 through 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 12 000 g, and plasma was stored at −20°C until analysis. Total plasma cholesterol (TC) level was measured using a Boehringer Mannheim 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...
carotid and femoral arteries or significantly reduced in the patients with hemophilia was either minimally reduced in the patients available and heterogeneity of treatments (frequency of these clinical studies might be attributable to the small number of patients) previously.

Lesion morphology analysis and immunohistochemistry 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 in E° mice, thus suggesting that the intrinsic 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 platelet 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 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 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 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 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 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 inflammation.
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...
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.

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