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The Hemostasis System in Murine Atherosclerosis

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Abstract

Atherosclerosis is a self-sustaining inflammatory fibroproliferative disease that progresses in discrete stages and involves a number of cell types and effector molecules. The potential importance of the coagulation, anticoagulation, and fibrinolytic systems in atherosclerosis is based on the observation that fibrin deposits and fibrin degradation products are resident in atherosclerotic plaques. A number of investigations have been conducted to probe the relationships between components of the hemostasis system and atherosclerosis; and these types of studies proliferated after the availability of mice genetically manipulated to emphasize the impact of genes of interest. In order to summarize recent progress in this area, this review is focused on mice lacking individual hemostasis genes and their contributions to steps of the atherosclerotic process.

INTRODUCTION

Atherosclerosis is a self-sustaining inflammatory fibroproliferative disease that progresses in discrete stages and involves a number of cell types and effector molecules [1]. Lipid metabolic disorders, principally those dyslipidemias that lead to high LDL and triglyceride levels, and/or low HDL levels, are heavily involved in the genesis and progression of atherosclerosis in humans, and thrombotic complications substantially contribute to the coronary end-stage arterial disease that sometimes accompanies atherosclerosis.

The potential importance of the coagulation, anticoagulation, and fibrinolytic systems in atherosclerosis is based on the observation that fibrin deposits and fibrin degradation products are resident in the atherosclerotic plaque [2]. Patients with coronary atherosclerosis show high levels of plasminogen activator inhibitor-1 (PAI-1) [3, 4] and high levels of PAI-1 have been positively correlated with carotid artery wall thickness [5]. In contrast, plasma levels of plasmin-antiplasmin (PAP) are negatively correlated with the severity of atherosclerosis [6]. These results suggest that high plasmin (Pm) activity attenuates atherosclerosis.

A number of animal models have been utilized to assess the roles of coagulation, anticoagulation, and fibrinolytic systems in the atherosclerotic plaque initiation and/or progression. Among them, gene-deficient mice of the hemostasis system are valuable resources for evaluating the functions of individual genes in plaque pathology. Although there are a number of studies utilizing mouse models of atherosclerosis, strategies and results are quite diverse. Thus, existing knowledge of the role of the hemostasis system in spontaneous atherosclerosis in murine models are summarized in this review.

BASIC MODELS FOR MURINE SPONTANEOUS ATHEROSCLEROSIS

Because of genetic differences in lipid metabolism between mice and humans, low density lipoprotein (LDL) and its precursors, very density lipoprotein (VLDL) and intermediate density lipoprotein (IDL), are rapidly cleared in mice. Thus, differing from humans, the cholesterol present in murine plasma is mostly carried in the atheroprotective high density lipoprotein (HDL) fraction. As a result, wild-type (WT) mice are more resistant than humans to dietary-induced elevation of LDL, and, thus, useful metabolic murine models of human atherosclerosis were not prevalent until recent advances in *in vivo* gene targeting methods. Application of this technology allowed for the generation of genetic strains of mice that possess many characteristics of lipid metabolism of humans. Using the technologies, the low density lipoprotein receptor-deficient (*Ldlr*^{-/-}) [7] and apoE deficient (*Apoe*^{-/-}) [8–10] mouse lines have been generated.

Although *Apoe*^{-/-} mice can develop atherosclerotic plaques on a normal chow diet, *Ldlr*^{-/-} mice do not develop plaques due to the different properties of apoB production in the liver. In order to overcome this limitation in *Ldlr*^{-/-} mice, *Apobec1* gene was ablated [11] and intercrossed to *Ldlr*^{-/-} mice. The resulting doubly deficient mice (*Ldlr*^{-/-}/*Apobec1*^{-/-}) can develop atherosclerotic plaques on a normal chow diet with different lipoprotein profiles found in *Apoe*^{-/-} mice [12].

We have also intercrossed these mice with *Ldlr*^{-/-} mice and generated the *Ldlr*^{-/-}/*Apob*^{100/100} line. This line also represents spontaneous plaque formation with hypercholesterolemia. However, the extent of plaque in the aortas of *Ldlr*^{-/-}/*Apob*^{100/100} mice was much less than that in *Ldlr*^{-/-}/*Apobec1*^{-/-} mice. Although the discrepancies in plaque progression between *Ldlr*^{-/-}/*Apobec1*^{-/-} mice and *Ldlr*^{-/-}/*Apob*^{100/100} mice are quite interesting, this review will not focus on the mechanism of these differences.

COAGULATION CASCADE

Intrinsic Pathway

It is well known that whole blood clots upon contact with a glass surface. This occurs not only with artificial negatively charged materials e.g., glass, but also with several physiological and/or pathophysiological materials such as sulfatide, phosphatidyl serine, cholesterol sulfate, heparin, collagen, and endotoxin. This process is included under the contact phase of coagulation. Factor (F) XII, FXI, plasma prekallikrein (PK), and high molecular weight kininogen (HMWK) are also participants in the contact phase of coagulation. FXII, FXI, and PK are serine protease zymogens, and HMWK does not possess enzymatic activity. FXII, alone, can interact with a negatively charged surface. On the other hand, FXI and PK do not demonstrate similar affinities to these surfaces and these proteins bind to HMWK [13]. Once blood is exposed to a negatively charged surface, FXII binds and is activated to FXIIa by activated PK (K) [14]. PK is then further activated by FXIIa. Thus, the activation of FXII and PK is reciprocal and each amplifies the activation of the other. The resulting FXIIa can then activate FXI to FXIa which eventually leads to the generation of thrombin [15]. Unlike other reactions in the coagulation cascade, these initial steps do not require Ca²⁺. Thus, using EDTA or sodium citrate for blood sampling does not attenuate these reactions [16]. After its formation, FXIa/Ca²⁺ catalyzes the activation of FIX which in the presence of phospholipid (PL), Ca²⁺ and the activated cofactor FVIIIa activates FX. FXa mediates the conversion of prothrombin to thrombin, a reaction that proceeds optimally in the presence of Ca²⁺/PL, and the activated cofactor, FVa. There are no components required from outside of blood vessels for initiating blood coagulation *via* this system. Thus, this pathway is called the intrinsic coagulation pathway.

Extrinsic Pathway

Tissue factor (TF) is expressed in a variety of organs such as adipose tissue, intestine, kidney, and brain. On the other hand, its expression is not observed in resting endothelial cells and monocytes [17]. Thus, TF is not normally exposed to the blood. Once blood vessels are injured, blood leaks into subendothelial spaces and endothelial cells are activated, thus exposing TF. FVII in the blood can rapidly bind to TF. Although FVII does not express serine protease activity, preexisting activated FVII (FVIIa) in the blood can also bind to TF and this FVIIa-TF complex can then activate the FVII-TF complex, which comprises extrinsic FXase. Since it was initially believed that TF acts as a nonblood component in initiating this pathway, this pathway was named “extrinsic”. However, recent studies indicate that TF may exist on endothelial cells in a cryptic form and becomes activated, possibly through thiol isomerase activity from activated platelets [18].

Common Pathway

Both extrinsic and intrinsic FXase activate FX to FXa, and activated FX (FXa) in association with phospholipid (PL), Ca^{++} , and FVa (the prothrombinase complex) catalyzes the activation of prothrombin to thrombin (FIIa). In this capacity, FIIa catalyzes the release of the A α -chains and B β -chains of Fg generating fibrin monomer. These fibrin monomers then polymerize and form a polymeric network, which constitutes the major protein component of the blood clot. The clot is covalently stabilized by catalysis with the transglutaminase, activated FXIII (FXIIIa), which generates crosslinks between specific ϵ - NH_2 side-chains of Lys and γ -COOH groups of Glu residues of fibrin monomers [19].

Crosstalk Between the Pathways

Crosstalk exists between these coagulation pathways. For example, FIIa can also directly activate FXI [20]. Indeed, extrinsic FXase can activate FIX more efficiently than FX and now it is believed that the main function of the extrinsic FXase is to activate FIX rather than FX directly [21]. Furthermore, recent studies demonstrated that TF is induced in stimulated endothelial cells, e.g., endotoxemia [22]. Thus, the boundaries of these pathways are not clearly defined and this cascade is controlled in a complex manner (Fig. 1).

FIBRINOLYTIC PATHWAY

Plasminogen (Pg) is a serine protease zymogen produced and secreted by the liver. Its concentration in blood is approximately 1.2–2.0 μM [23]. Human Pg is a 92 kDa single chain glycoprotein and is activated by Pg activators (PA), e.g., tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [24]. PAs catalyze activation of Pg to its active form, Pm, a two-chain serine protease, *via* cleavage of the Arg561-Val562 peptide bond [25]. The N-terminal heavy chain contains five kringle domains that bind to C-terminal lysine residues in fibrin and other proteins [26, 27]. The C-terminal light chain of Pm contains the proteolytic active site which facilitates the hydrolysis of fibrin to fibrin degradation products (FDP). While tPA plays a main role in vascular fibrinolysis and maintenance of vascular patency, uPA has been implicated in cellular functions, e.g., cell migration [28]. The principal endogenous inhibitor of Pm is $\alpha 2$ -antiplasmin (AP). In the fluid phase, Pm is promptly inactivated by AP and the Pm/AP (PAP) complex is an indicator of Pm generation *in vivo*. In contrast, fibrin-bound Pm is inaccessible to AP [27] and cell binding-Pm is protected from AP [29]. Plasminogen activator inhibitor-1 (PAI-1) is an inhibitor of PA and inactivates both tPA and uPA. The expression of PAI-1 occurs in endothelial cells, smooth muscle cells, platelets, and hepatocytes [30]. Interestingly, relatively large amounts of PAI-1 mRNA are found in adipocytes [31]. Pg production regulates these activators and inhibitors in facilitating a balance in fibrinolysis (Fig. 1).

MICE WITH DEFICIENCIES FOR COAGULATION FACTORS

Fg^{-/-} Mice

Two independent groups established *Fg*^{-/-} mouse lines [32, 33]. One team generated *Fga*-deficient mice and our group produced a total targeted *Fgg*-deficiency. Both strains of mice do not contain any detectable Fg in plasma. Thus, both murine models are suitable to investigate the condition of afibrinogenemia *in vivo*. *Fg*^{-/-} mice appear to develop normally in the uteri of *Fg*^{+/-} females and are vaginally delivered. However, after birth, some of the *Fg*^{-/-} mice present with omphalorrhagia, which leads to death from hemorrhage. The frequency of omphalorrhagia is different, depending on the genetic background of the mice [34]. Most of the *Fg*^{-/-} mice are healthy unless they are injured, which can lead to death. This is very similar to afibrinogenemic patients who have an autosomal recessive Fg deficiency.

FII^{-/-} Mice

Inactivation of the FII gene leads to partial embryonic lethality [35, 36]. Approximately 50% of the *FII*^{-/-} embryos died around E10.5. The explanation for this intrauterine death has been described differently by two groups that independently generated these lines. One reported that malformation of the vessels in yolk sac triggered the lethality [36]. The other team demonstrated that massive bleeding into the yolk sac cavity and varying degrees of tissue necrosis resulted in intrauterine death, although the vascularization in yolk sac was intact [35]. Most of the survivors at this time also expired around E15.5. Some of these mice survived to term and were delivered vaginally, although they rapidly died after birth due to intraabdominal hemorrhage.

TF^{-/-} Mice

Disruption of the TF gene led to embryonic lethality between E9.5-E11.5 [37–39]. This embryonic lethality was initiated by massive hemorrhage and loss of embryonic red cells from both extraembryonic and embryonic vessels, followed by circulatory failure. It was found that the amounts of smooth-muscle α -actin-expressing mesenchymal cells in vitelline vessels from *TF*^{-/-} embryos were heavily reduced. In this case, capillary vessels lacking the smooth muscle layer were connected and fused. This malformation of blood vessels contributed to the bleeding phenotypes in *TF*^{-/-} mice due to lack of resistance to blood pressure. These results clearly indicate that TF plays an important role in vasculogenesis in embryos.

FV^{-/-} and FV^{Q/Q} Mice

Approximately 50% of *FV*^{-/-} embryos from *FV*^{+/-} crosses died between ED11.5 and 18.5 [40]. The other 50% were vaginally delivered, but expired quickly after birth due to massive intraabdominal hemorrhage. Some necrotic vessels in the yolk sacs were observed in the growth-restricted *FV*^{-/-} embryos (40%) at ED9.5, which led to disrupted circulation in the yolk sac and growth restriction. These results indicated that fully functional FV was required not only for normal hemostasis, but also for embryogenesis.

FV Leiden (FVL) originates from a missense mutation of FV at amino acid position 506, at which Arg is replaced by Gln [41]. FVL has normal FV coagulant function but is resistant to inactivation by the Protein C pathway. Therefore, FVL patients present with venous thrombosis [42]. A FVL mouse line (*FV*^{Q/Q}) has been generated [43] in order to investigate the altered functions of the FV resulting from this mutation. Interestingly, the thrombotic phenotypes derived from this mutation were variable in different genetic backgrounds of mice. C57Bl/6 mice bearing this mutation did not show any thrombotic phenotype. In contrast, in a mixed 129Sv/ C57Bl/6 genetic background, the mice developed disseminated

intravascular thrombosis during the perinatal period, resulting in significant mortality shortly after birth.

FVII^{-/-} Mice

A *FVII^{-/-}* mouse line has been generated and characterized [44]. *FVII^{-/-}* embryos from *FVII^{+/-}* mothers developed normally to term and were vaginally delivered. However, *FVII^{-/-}* neonates expired perinatally due to fatal hemorrhage from normal blood vessels. These results indicated that FVII was not required for embryogenesis, but for normal hemostasis. Although FVII and FVIIa are unique ligands for TF, discrepancies were reported in embryogenesis between *TF^{-/-}* and *FVII^{-/-}*. While *TF^{-/-}* embryos had a malfunctional vasculature, *FVII^{-/-}* embryos had intact vasculatures including von Willebrand factor (vWF)-containing endothelial cells and α -smooth muscle actin positive smooth muscle cells. Although it was considered that some maternal transfer of FVII to the deficient embryos can compensate for the mature vasculogenesis, supraphysiological amounts of recombinant FVII administered to the pregnant mice bearing *FVII^{-/-}* embryos could not restore the levels of FVII in the embryos. However, recent studies in our laboratory, utilizing low-expressing FVII mice (*FVII^{TA/TA}*; ~1% normal levels), indicated that embryos from pregnant *FVII^{TA/TA}* females do not develop to term, but embryos from *FVII^{+/-}* female mice with ovaries from *FVII^{TA/TA}* mice survive. This demonstrates that mothers with low maternal circulating FVII cannot support a pregnancy, likely because of diminished FVII that is available for the fetus. This further suggests that maternal-fetal transfer most likely contributed to neonatal delivery in the initial *FVII^{+/-}* crosses.

FVIII^{0/-}/FVIII^{-/-} Mice

It is well known that the deficiency of FVIII in humans causes hemophilia A, which is a sex-linked recessive disorder. Similar to humans, the *FVIII* gene is also located on the X chromosome of the mouse genome (Table 1). A *FVIII^{0/-}* (male)/*FVIII^{-/-}* (female) mouse line was generated and the activated partial thromboplastin time (aPTT) was found to be extremely prolonged in these mice [45]. In addition, the amount of blood loss from a tail cut is more prominent in *FVIII^{-/-}* mice, which leads to death in the absence of cauterization. Although the major phenotype of Hemophilia A in humans is bleeding into joints and/or soft tissues, these mice did not develop these phenotypes until 12 weeks of age. Moreover, most of the older deficient mice (around 1 year of age) were healthy without spontaneous bleeding events [46]. The *FVIII^{-/-}* female mice crossed with *FVIII^{0/-}* male mice were able to maintain pregnancy and deliver offspring vaginally, and these neonates also survived to adulthood. These results indicated that FVIII was not necessary for maintaining pregnancy and preventing umbilical bleeding in neonates.

FIX^{0/-}/FIX^{-/-} Mice

Hemophilia B is caused by a deficiency of FIX in humans. Similar to humans, the *FIX* gene is also located on the X chromosome in the mouse genome (Table 1). Thus, the deficiency is also sex-linked, as is the case with FVIII. *FIX^{0/-}* (male)/*FIX^{-/-}* (female) mouse lines were generated and it was observed that these mice present with hemorrhagic swelling on top of the feet or on the footpads [47]. Similar to FVIII deficient mice, the aPTT is prolonged and tail-cut bleeding also results in death without cauterizations. These phenotypes are similar to the phenomena observed in humans and, therefore, this line is a suitable model for Hemophilia B.

FX^{-/-} Mice

A *FX^{-/-}* mouse line has been generated and the genotypic distribution among the offspring from heterozygous breeding pairs suggested that a FX deficiency resulted in a partial

embryonic lethality [48]. The neonatal lethality was triggered by intraabdominal bleeding at an early stage of development, and subcutaneous or intracranial bleeding, or a combination thereof, in later stages. These bleeding tendencies are also observed in severe FX deficient patients, and this strain can serve as good resource for studying FX functions *in vivo*.

FXI^{-/-} Mice

FXI^{-/-} mice have prolonged aPTTs similar to FVIII and FIX deficient mouse lines, although the bleeding tendency is very mild [49]. A deficiency of this gene has also been identified in dogs [50], cows [51], and humans [52], and the phenotypes manifested by a FXI deficiency in these animals are similar to the murine deficiency state.

FXII^{-/-} Mice

Observations of the two *FXII^{-/-}* mouse lines demonstrated that neither line displays overt spontaneous phenotypes, although the aPTT is extremely prolonged [16, 53]. This prolonged aPTT is also observed in FXII-deficient humans and most patients are asymptomatic. Recent studies with these mice demonstrated that arterial thrombus formation was accelerated [54] by the deficiency and bradykinin production was partially diminished [16]. Thus, FXII may play a role in local vascular environments although it is not a critical factor for normal hemostasis.

HMWK^{-/-} and PK^{-/-} Mice

Almost all coagulation factors have been successfully inactivated by a number of investigators utilizing gene targeting technologies, except for PK and HMWK (Table 1). The technical difficulty in making HMWK-knockout mice resides in the fact that there are two active HMWK genes (*Kngr1* and *Kngr2*) in the mouse genome, that are located in tandem on the same chromosome [55]. In contrast, genetic analyses indicate that there are no obvious technical difficulties in making *PK^{-/-}* mice and therefore, it assumed that these mice will be developed in the future.

MICE DEFICIENT FOR FIBRINOLYTIC FACTORS

TPA^{-/-} and UPA^{-/-} Mice

TPA^{-/-} and *UPA^{-/-}* mouse lines have been generated and both *TPA^{-/-}* and *UPA^{-/-}* mice are fertile [56]. There are no significant differences in their litter sizes, the frequency of the litters, and body weights. *TPA^{-/-}* mice do not present with any macroscopic abnormalities up to 14 months. On the other hand, some *UPA^{-/-}* mice develop rectal prolapse (9%) and extensive non-healing ulcerations of the eyelids, the ears around the eartag, and the face (5%). The incidence of rectal prolapse is increased in *TPA^{-/-}/UPA^{-/-}* mice (37%). While *TPA^{-/-}* mice do not present any histological abnormalities, *UPA^{-/-}* mice occasionally display fibrin depositions, which are exacerbated in *TPA^{-/-}/UPA^{-/-}* mice. These results indicated that tPA and uPA are the main Pg activators *in vivo*.

UPAR^{-/-} Mice

The two *UPAR^{-/-}* mouse lines were generated and presented with no apparent phenotypic differences to WT mice [57, 58]. Furthermore, thioglycollate-induced macrophage infiltration into the peritoneal cavity was not affected by this gene deficiency. However, a thrombotic effect, i.e., sinusoidal hepatic fibrin depositions, was observed in one of these lines, coupled with a tPA deficiency [59]. In this case, uPAR is a complementary fibrinolytic factor in mice. Thus, the functions of uPAR in fibrinolysis *in vivo* are very limited.

Pg^{-/-} Mice

In the two *Pg^{-/-}* lines that were generated, systemic fibrin depositions were observed [60, 61]. The lifespans of *Pg^{-/-}* mice are much shorter than those of WT mice. *Pg^{-/-}* mice develop rectal prolapse and are growth-restricted. It was thought that these abnormalities were induced by excessive and persisting fibrin depositions, which was seemingly confirmed since these phenotypes can be attenuated when the deficiency is coupled with a Fg deficiency [62]. These results indicate that Pg plays a central role in fibrinolysis.

PAI-1^{-/-} Mice

The initial characterization of *PAI-1^{-/-}* mice indicated no spontaneous macroscopic or microscopic abnormalities, and *PAI-1^{-/-}* mice were fully viable and fertile [63, 64]. There were no significant differences in the litter sizes between WT and *PAI-1^{-/-}* mice. However, it was recently reported that a PAI-1 deficiency triggers cardiac fibrosis in mice and mice expressing excessive uPA in macrophages also demonstrate a similar phenotype [65]. Results from a number of studies with *PAI-1^{-/-}* mice have been conflicting and controversial. One of the reasons may be that PAI-1 is a pleiotropic protein and belongs to the serine protease inhibitor (Serpins) family. Although PAI-1 is a specific inhibitor of uPA and tPA, the functions of Serpins generally overlap and are seemingly somewhat redundant. As a result, this can compromise the analysis of PAI-1 functions.

COAGULATION FACTORS IN SPONTANEOUS ATHEROSCLEROSIS MODELS

As summarized in Table 2, deficiencies of Fg, TF, and FVIII and FVL were analyzed in *Apoe^{-/-}* mice while a Fg deficiency was also analyzed in *Ldlr^{-/-}/Apobec1^{-/-}* mice.

Apoe^{-/-}/Fg^{-/-}

Apoe^{-/-}/Fg^{-/-} mice in a mixed 129/CF1/C57Bl/6 background were utilized to determine effects of a Fg deficiency in a model of atherosclerosis [66]. Mice were maintained on a normal chow diet and euthanized at 22, 26, and 31 weeks. The plasma total cholesterol (T-C) levels in *Apoe^{-/-}/Fg^{-/-}* mice (806 ± 348 mg/dl, n=68) were significantly lower than those in *Apoe^{-/-}/Fg^{+/+}* mice (966 ± 441 mg/dl, n=72).

Plaque sizes in the aortas were measured using a unique ranking scale, and it was concluded that there were no significant differences between *Apoe^{-/-}* and *Apoe^{-/-}/Fg^{-/-}* mice. Both male and female mice were used in this study. The investigators did not find any significant differences between the genders. Interestingly, in a separate study, reduced plaque formation has been observed in *Apoe^{-/-}* female mice [67] and serum lipid concentrations were found to be much higher in male versus female mice [Adan, 1999 #116]. Moreover, mice in mixed genetic backgrounds possessed large variabilities in this regard, even though littermates were used. This is most likely due to the observation that atherogenic properties depend on the genetic strains [68, 69]. The C57Bl/6 strain is highly susceptible to atherosclerosis and therefore represents the appropriate strain for assessing atherosclerosis development in gene-altered mice.

Ldlr^{-/-}/Apobec1^{-/-}/Fg^{-/-}

This mouse line was generated and characterized by our laboratory [70]. All mice in this study were fully back-crossed into C57Bl/6 strain. Mice were maintained on normal chow diet and euthanized for analyses at 12, 24, 36, and 48 weeks. Cholesterol levels in the various lipoprotein categories, as well as T-C plasma levels, were similar in both *Ldlr^{-/-}/Apobec1^{-/-}* (T-C; 348.4 ± 78.0 mg/dl, N=91) and *Ldlr^{-/-}/Apobec1^{-/-}/Fg^{-/-}* (T-C; 378.0 ±

80.8 mg/dl, N=15) mice. *Ldlr*^{-/-}/*Apobec1*^{-/-}/*Fg*^{-/-} mice had more advanced plaque than *Ldlr*^{-/-}/*Apobec1*^{-/-} mice. The surface area of plaque in the aorta and the size of the plaque in the aortic sinuses were more extensive in *Ldlr*^{-/-}/*Apobec1*^{-/-}/*Fg*^{-/-} mice than that in *Ldlr*^{-/-}/*Apobec1*^{-/-} mice. *Ldlr*^{-/-}/*Apobec1*^{-/-}/*Fg*^{-/-} mice displayed more extended and enlarged plaques at all timepoints in this study, but there was no significant difference in the plaque growth rate between *Ldlr*^{-/-}/*Apobec1*^{-/-} and *Ldlr*^{-/-}/*Apobec1*^{-/-}/*Fg*^{-/-} mice. These results implied that the plaque initiation was accelerated by a Fg deficiency in this model. P-selectin (CD62P) exposure on platelets was more enhanced, vWF:Ag levels were higher, and the levels of its cleaving proteinase, a disintegrin-like and metalloproteinase with thrombospondin type-1 motif 13 (ADMATS13), were significantly lower in *Ldlr*^{-/-}/*Apobec1*^{-/-}/*Fg*^{-/-} mice. These data also support the concept of “earlier initiation of plaque” in *Ldlr*^{-/-}/*Apobec1*^{-/-}/*Fg*^{-/-} mice by platelet activation. Therefore, the effects of a Fg deficiency on atherosclerosis are related to alterations in the expression of multiple genes of the hemostasis network in this model.

***Apoe*^{-/-}/*TF*^{+/-} and *Apoe*^{-/-}/*TF*^{-/-}/*hTF*⁺**

Since *TF*^{-/-} mice are embryonic-lethal, the role of TF in atherosclerosis was studied using *TF*^{+/-} mice or low TF expressers [71], the latter of which contains a hTF minigene expressed in the *TF*^{-/-} background (*TF*^{-/-}/*hTF*⁺) [72]. All mice were fully backcrossed into the C57Bl/6 strain for the studies. There were no significant differences in the extent of plaque formation and T-C levels between *Apoe*^{-/-} and *Apoe*^{-/-}/*TF*^{+/-} mice at 34 weeks on a normal chow diet. However, the 50% reduction of TF expression might not be sufficient to affect the phenotype. Therefore, efforts were made to generate *Apoe*^{-/-}/*TF*^{-/-}/*hTF*⁺ mice. These mice were generated, but all died prematurely before atherosclerotic analysis. Characterization of this sudden and premature death was not performed, but it was speculated that the mice died due to destabilized plaques followed by plaque rupture. However, it should be noted that *TF*^{-/-}/*hTF*⁺ mice are highly susceptible to bleeding which could lead to death. Thus, any other bleeding events associated with plaque formation can serve to accelerate the lethality.

Apoe*^{-/-}/*FVQ/Q

For these studies, mice were fully backcrossed into the C57Bl/6 strain [73]. Both high fat and normal chow diets were utilized, and the plaque covering the entire area of the aorta was measured at various time points. Plaque-covered areas in the aortas were similar between *Apoe*^{-/-} and *Apoe*^{-/-}/*FVQ/Q* mice at 19 weeks on a high fat diet and 34 weeks on a normal chow diet. However, the plaque area in *Apoe*^{-/-}/*FVQ/Q* mice became significantly larger than that in *Apoe*^{-/-} mice at 52 weeks. The extent of fibrin deposition in the plaques in the aortic sinus was measured and it was determined that enhanced fibrin deposits were found in *Apoe*^{-/-}/*FVQ/Q* mice at 52 weeks. However, as stated, plaque progression in *Apoe*^{-/-}/*Fg*^{-/-} mice showed no significant differences compared to that in *Apoe*^{-/-} mice [66].

***Apoe*^{-/-}/*FVIII*^{-/-}**

These mice were generated in order to study the role of the intrinsic pathway in atherosclerosis [74]. Female mice were used but it is unknown whether these mice were in the atherosclerosis-susceptible strain, C57Bl/6. Gender differences have been reported in atherogenesis, in which the female mice are highly affected by the estrous cycle.

Apoe^{-/-}/*FVIII*^{-/-} mice were significantly heavier and contained higher T-C levels than *Apoe*^{-/-} mice. It was speculated that the lack of FVIII production by hepatocytes may increase cholesterol biosynthesis, which could contribute to higher T-C levels as well as increased body weight. This hypothesis is supported by other studies. A FVIII deficiency can upregulate Fg production by hepatocytes [75]. Fg over-expression can enhance apoB

secretion from hepatocytes, which could lead to hypercholesterolemia [76, 77]. While the *Apoe*^{-/-}/*FVIII*^{-/-} mice had some phenotypes of atherosclerosis, the size of the plaques was significantly smaller than in *Apoe*^{-/-} mice at early stages. These studies measured the plaque covered area of the aortic root (aortic sinus) sections. The aortic root is the site that first develops atherosclerosis, so these studies suggested an effect of FVIII deficiency on lesion initiation. These studies also included the analyses of cellular components. Adhesion molecules and extracellular matrix genes were more highly expressed in *Apoe*^{-/-} than in *Apoe*^{-/-}/*FVIII*^{-/-} mice at the early stage but was similar in both groups at the late stage of the disease.

FIBRINOLYTIC FACTORS IN SPONTANEOUS ATHEROSCLEROSIS MODELS

As indicated in Table 2, deficiencies of Pg and PAI-1 were analyzed in *Apoe*^{-/-} mice.

Apoe^{-/-}/*Pg*^{-/-}

Apoe^{-/-}/*Pg*^{-/-} mice in a mixed background of 129/NIH Black Swiss/C57Bl/6 were utilized in order to study the impact of a Pg deficiency in atherosclerosis [78]. The mice were maintained on a normal chow diet and euthanized at 18, 22, and 25 weeks. Plasma T-C levels in *Apoe*^{-/-}/*Pg*^{-/-} mice (870 ± 390 mg/dl, n=17) were equivalent to those in *Apoe*^{-/-}/*Pg*^{+/-} mice (860 ± 350 mg/dl, n=44). Interestingly, the plasma HDL-C levels in *Apoe*^{-/-}/*Pg*^{-/-} mice (10 ± 5 mg/dl, n=12) were significantly lower than those in *Apoe*^{-/-}/*Pg*^{+/-} mice (30 ± 10 mg/dl, n=44). The investigators speculated that these differences might be due to the smaller size of *PG*^{-/-} mice. Total and HDL-C levels in *Apoe*^{+/-}/*Pg*^{+/-} and *Apoe*^{+/-}/*Pg*^{-/-} mice were also measured. There were no significant differences in T-C levels in both genotypes (120 ± 20 mg/dl, n=17 and 100 ± 20 mg/dl, n=6, respectively). However, HDL-C levels were affected by Pg deficiency (90 ± 20 mg/dl, n=14 and 50 ± 20 mg/dl, n=4, respectively). Therefore one can speculate that a Pg deficiency may affect lipid metabolism, although further investigations were not pursued.

Using a unique ranking scale, it was found that *Apoe*^{-/-}/*Pg*^{-/-} mice had significantly larger plaque covered areas than those in *Apoe*^{-/-} mice. The size of the plaques in the aortic sinuses were also significantly enlarged in *Apoe*^{-/-}/*Pg*^{-/-} mice (8600 μm²) relative to that in *Apoe*^{-/-} mice (1200 μm²) at 20 weeks old of age. Thus, the authors concluded that a Pg deficiency accelerates vessel wall remodeling in *Apoe*^{-/-} mice.

Apoe^{-/-}/*PAI-1*^{-/-}

Apoe^{-/-}/*PAI-1*^{-/-} mouse lines were generated and characterized by several groups [79–82]. In two of these studies [80, 81], mice were fully backcrossed into the C57Bl/6 strain. Other studies utilized mice in a mixed background of C57Bl/6 and 129/Sv [79, 82]. Zhu *et al* indicated that the levels of T-C in *Apoe*^{-/-}/*PAI-1*^{-/-} mice (1478 ± 176 mg/dl) were significantly higher than those in *Apoe*^{-/-} mice (1088 ± 49 mg/dl) at 10–18 weeks old of age on high fat diet [81]. However, this difference was not observed by Sjoland *et al* [79] (*Apoe*^{-/-}/*PAI-1*^{-/-} mice; 1707 ± 379 mg/dl, *Apoe*^{-/-} mice; 1932 ± 164 mg/dl) at 10 weeks old of age on high fat diet. More extensive investigations have not been conducted, thus, the lipid metabolism in *Apoe*^{-/-} mice with a PAI-1 deficiency is still unresolved.

The results from studies conducted by Sjoland *et al* [79], Eitzman *et al*. [80], and Luttun *et al*. [82] in *Apoe*^{-/-} and *Apoe*^{-/-}/*PAI-1*^{-/-} mice are summarized as follows. Sjoland *et al*. maintained mice on a high fat diet for 6, 15, and 30 weeks and compared the sizes of the plaques in the aortic sinuses. It was concluded that there were no significant differences in plaque sizes between *Apoe*^{-/-} and *Apoe*^{-/-}/*PAI-1*^{-/-} mice. Eitzman *et al*. [80] who

maintained the mice on a normal chow diet for 18, 30, and 52 weeks found that lesions sizes were smaller in both the aortic arch and carotid artery of *Apoe*^{-/-}/*PAI-1*^{-/-} mice relative to *Apoe*^{-/-} mice. On the other hand, Luttun *et al.* [82] maintained the mice on a high fat diet or normal chow diet for 25 weeks and analyzed various aortic segments such as aortic valves, aortic arch, thoracic and abdominal aorta, carotid artery, and brachiocephalic artery. It was found that more advanced plaque formation occurred in *Apoe*^{-/-}/*PAI-1*^{-/-} mice. Thus, there is still considerable controversy involving the role of PAI-1 in atherosclerosis utilizing these models.

CONCLUSIONS

Most of the spontaneous atherosclerosis murine models to evaluate the functions of specific genes in coagulation and fibrinolysis were performed in an *Apoe*^{-/-} background. This is predominantly a VLDL and IDL model of atherogenesis. Although certain features of atherosclerotic plaques are generally similar in both VLDL- and LDL-induced atherosclerosis models, pathways of atherosclerosis may differ. VLDL can bind to FII, VII, IX, and X, which are all Vitamin K-dependent proteins and contain Gla domains which facilitate binding to phospholipids. However, LDL binding to these proteins has not been observed [83]. Although TF-independ-ent FVII activation can be equally induced by LDL and VLDL after normalizing amounts of phospholipids [84], more prothrombinase activity is obtained with VLDL [85]. These observations indicate that alterations of coagulation and fibrinolytic systems induced by hyperlipidemia are dependent on the source of lipids, and, therefore, it is important to consider these effects on atherosclerosis when using various murine models. While the *Ldlr*^{-/-} models emphasize LDL as the predominant lipoprotein, more studies would be particularly rewarding with the *Ldlr*^{-/-}/*Apobec1*^{-/-} model.

Another complication in these analyses is that different laboratories have investigated, and/or are investigating, functions of hemostasis genes using animal models under different conditions, *i.e.*, gender, diet, age, genetic strain background. Although these different conditions can provide important data on the character of plaques at various stages under different conditions in a standard model, such as *Apoe*^{-/-} mice, the process is not ideal. Thus, the characterization of these mice, including the experimental regimens, needs to be standardized, and comparisons need to be made under conditions as closely matched as possible.

Despite the passage of 15 years since the generation of *Apoe*^{-/-} mice, and 9 years from the generation of *Ldlr*^{-/-}/*Apobec1*^{-/-} mice, a number of components of coagulation and fibrinolytic system still have not been investigated in atherosclerotic models. Although the role of coagulation and fibrinolytic systems in atherosclerosis is complex, utilizing individual gene deficiencies, as well as gene-modified mice, could facilitate the roles of these pathways in this disease.

Other genetic atherosclerosis crosses of interest would be those with FVII and FXII. This would facilitate investigations on the relationships between the initiation of coagulation and atherosclerosis. Although FXII-deficient mice are viable, FVII-deficient mice are neonatal lethal. However, *FVII*^{TA/TA} mice, which express very low amounts of FVII [86], are being used. Therefore, individually eliminating both pathways of coagulation initiated by FXII and FVII in mice genetically prone to atherosclerosis could be achieved through intercrosses of these lines with *Apoe*^{-/-} and/or *Ldlr*^{-/-}/*Apobec1*^{-/-} mice. Recent studies indicate that uPA, uPAR, and PAI-1 are involved in binding to the LDL receptor-related protein (LRP), and induce cell signalling. Therefore, uPA and uPAR are also important target genes for determining the role of plasminogen activation in plaque progression and potentially lipid metabolism.

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Fig. 1.
The hemostasis pathway. Boxed objects, except Fg and its derivatives, indicate zymogens. Oval and hatched components indicate active forms. Green, blue, and orange objects belong to the intrinsic, extrinsic, and common blood coagulation pathways, respectively, and pink objects are part of the fibrinolytic pathway. Black arrows indicate the catalytic conversion from zymogens to active forms, except Fg and its derivatives. Blue arrows indicate the enhancement of conversions and red arrows indicate their inhibition. Ca²⁺; calcium. PL; phospholipid.

Table 1

Gene Altered Mice for the Study of Lipid Metabolism and Hemostasis

Gene	Protein	Chromosome	Location	Embryonic/Neonatal Lethality	Reference
Lipid metabolism					
<i>Ldlr</i>	LDLr	9	5.0 cM	No	[7]
<i>ApoBec1</i>	APOBEC1	6	54.5 cM	No	[11]
<i>ApoB</i>	apoB-48,100	12	A1.1	Yes	[87, 88]
<i>ApoB¹⁰⁰</i>	apoB-100 only	12	A1.1	No	[89]
<i>ApoE</i>	apoE	7	4.0 cM	No	[8–10]
Coagulation factors					
<i>Fga</i>	Fg Aα chain	3	44.8 cM	No	[32]
<i>Fgb</i>	Fg Bβ chain	3	48.2 cM	Unknown	NA
<i>Fgg</i>	Fg γ chain	3	41.3 cM	No	[33]
<i>F2</i>	FII	2	47.5 cM	Yes	[35, 36]
<i>F3</i>	TF	3	50.3 cM	Yes	[37–39]
<i>F5</i>	FV	1	86.6 cM	Yes	[40]
<i>F5Q</i>	FVL	1	86.6 cM	No	[43]
<i>F7</i>	FVII	8	7.0 cM	Yes	[44]
<i>F8</i>	FVIII	X	30.5 cM	No	[45]
<i>F9</i>	FIX	X	22.0 cM	No	[47]
<i>F10</i>	FX	8	7.0 cM	Yes	[48]
<i>F11</i>	FXI	8	25.0 cM	No	[49]
<i>F12</i>	FXII	13	B2	No	[16, 53]
<i>F13a1</i>	FXIII a1 subunit	13	A3.3	No	[90]
<i>Kngr1.2</i>	HMWK	16	B1	Unknown	NA
<i>Klkb1</i>	PK	8	26.0 cM	Unknown	NA
Fibrinolytic factors					
<i>Plat</i>	tPA	8	9.0 cM	No	[56]
<i>Plau</i>	uPA	14	2.5 cM	No	[56]
<i>Plaur</i>	uPAR	7	A3	No	[57, 58]

Gene	Protein	Chromosome	Location	Embryonic/Neonatal Lethality	Reference
<i>Plg</i>	Pg	17	7.3 cM	No	[60, 61]
<i>Seppin1</i>	PAI-1	5	G2	No	[63]

Low density lipoprotein receptor (LDLR), apolipoprotein B (apoB), apolipoprotein E (apoE), apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1), high molecular weight kininogen (HMWK), plasma prekallikrein (PK), coagulation factor 12, 11, 10, 9, 8, 7, and 5 (FXII, XI, X, IX, VIII, VII, and V, respectively), tissue factor (TF), prothrombin (FII), fibrinogen (Fg), tissue type plasminogen activator (tPA), urokinase type plasminogen activator (uPA), plasminogen (Pg), and plasminogen activator inhibitor-1 (PAI-1).

Table 2

Atherosclerosis models with Altered Hemostasis Genes

Gene	<i>Apoε</i> ^{-/-}	<i>Ldlr</i> ^{-/-}	<i>Ldlr</i> ^{-/-} / <i>ApoBc1</i> ^{-/-}	
Coagulation Factors				
	Effect	Ref.	Effect	Ref.
<i>Fga</i>	→	[66]	N/A	N/A
<i>Fgg</i>	N/A		N/A	↑ [70]
<i>F3</i>	↓	[71]	↓	[71]
<i>F5Q</i>	↑	[73]	N/A	N/A
<i>F8</i>	↓	[74]	N/A	N/A
Fibrinolytic Factors				
<i>Plg</i>	↑	[78]	N/A	N/A
<i>Serpine1</i>	↑→↓	[79-82]	N/A	N/A

↑: Enhanced, →: No effect, ↓: Decreased, N/A: Not available.