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INVITED REVIEW

Acute and persistent platelet and coagulant activities in atherothrombosis

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Summary. The potential relevance of murine atherothrombosis models for understanding human disease has been debated in the past. Despite this, in the last decade, many thrombosis studies with atherogenic *Apoe*$^{-/-}$ mice have been performed, which provide novel insight into the molecular mechanisms by which platelet and coagulation processes accomplish acute thrombus formation after plaque disruption *in vivo*. Support for these mechanisms has come from whole blood flow perfusion studies over plaque material *in vitro*, which are also reviewed in this study. The main plaque-derived triggers for thrombus formation appear to be collagen and tissue factor, next to bioactive mediators such as prostaglandin E2. The atherothrombotic process relies on collagen- and ADP-receptor-induced platelet activation as well as on thrombin/fibrin generation via the extrinsic and intrinsic coagulation pathways. Less is known of the persistent effects of a thrombus on atherosclerosis progression, but evidence suggests roles herein of activated platelets and ongoing thrombin generation.

Keywords: atherosclerotic plaque; blood coagulation; mouse; platelets; rupture; thrombosis.

Introduction

Atherothrombosis, characterized by the acute formation of a thrombus following rupture or erosion of an atherosclerotic plaque, is a major cause of acute coronary syndrome and cardiovascular death. Atherothrombosis is considered to be triggered by a dysfunctional or damaged endothelium causing exposure of subendothelial plaque tissue to the blood stream. This leads to a series of thrombogenic responses, potentially resulting in occlusion of the atherosclerotic artery by a formed thrombus. The reactions leading to thrombus formation after injury of a healthy, non-atherosclerotic artery have been schematised in several reviews, with as main processes platelet activation, thrombin generation, and fibrin clot formation [1,2]. Commonly used schemes start with platelet adhesion to von Willebrand factor (VWF), which is facilitated by the high shear rate at the arterial vessel wall. Subsequent aggregation of platelets is promoted by the release of paracrine mediators, for example, ADP and thromboxane [2]. Thrombin, generated at the surface of a subpopulation of procoagulant platelets and at disrupted (sub)endothelial membranes, activates platelets and cleaves fibrinogen into fibrin. In this way, thrombin promotes thrombus growth, thrombus stabilization, and the massive fibrin-dependent entrapment of red blood cells at downstream sites of lower shear rate [1,3]. This concept of thrombus formation is in agreement with the histology of many thrombotic coronary and carotid arteries obtained from patients during surgery or autopsy, in which thrombi with a white platelet-containing head and a large red tail enriched in fibrin and erythrocytes are demonstrated [4]. It should be taken into account that these schemes are largely based on *in vitro* flow perfusion studies and *in vivo* animal (predominantly mouse) studies where the thrombotic process is investigated in non-atherosclerotic arteries.

Besides the acute process of thrombus formation, there is substantial evidence for a more persistent activity of platelets and coagulation in atherosclerosis and thrombosis. Autopsy material from patients with unstable angina or acute myocardial infarction frequently shows multilayered plaques containing intraplaque fibrin patches, suggestive for repeated episodes of (clinically silent) thrombotic activities followed by lesion growth [4]. Furthermore, arterial thrombi obtained by aspiration from patients with acute myocardial infarction show evidence for prolonged thrombotic activity prior to the thrombotic event [5]. Together, this is suggestive for subacute and likely continuous activity of platelets and coagulation in an atherosclerotic vessel that is prone to thrombogenesis.

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In support of this concept, in many studies, the beneficial effect of prolonged antiplatelet treatment for secondary prevention of atherothrombosis has been demonstrated. However, the currently used antiplatelet agents, blocking cyclooxygenase (aspirin) or $P2Y_{12}$ receptors (clopidogrel, prasugrel), do not completely annul the risk of new thrombotic events [6].

Here, we aim to provide an overview of *in vivo* thrombosis studies using atherogenic $Apoe^{-/-}$ mice and of *in vitro* perfusion studies using isolated plaque material, on the mechanisms by which platelet and coagulation processes contribute to acute thrombus formation on a disrupted plaque. We also propose mechanisms by which persistent activities of the hemostatic system, *that is,* after the formation of a thrombus, can aggravate atherosclerosis.

Changing paradigms on animal models of plaque rupture and atherothrombosis

In 2003, a consensus paper identified a number of restrictions for the use of animal models in the study of atherothrombosis after plaque rupture [7]. Major limitations were seen in the observations that: (i) particularly in mouse and rabbit models, the atherosclerotic lesions are histologically different from the plaques in man in terms of fibrous cap and lipid core; (ii) the size of animal lesions is considerably smaller than in man; (iii) the vulnerability to rupture of animal plaques is quite low when compared to human plaques; and (iv) plaque rupture in animals does only rarely occur without vigorous manipulations, for example, without using a needle or laser. It was stipulated that, for an ideal animal model of plaque rupture, thrombi should be formed via the same mechanisms and be sensitive to the same drug treatments as the thrombi formed in cardiovascular patients.

In spite of this rather negative outlook, in the last decade, a considerable number of animal studies have been published on thrombus formation induced by atherosclerotic plaque disruption, mostly using mice or rabbits. Together, this work provides unexpected new insights into the mechanisms of 'animal' atherothrombosis with clear relevance for the human situation. Most of the mechanistic studies have been carried out in $Apoe^{-/-}$ mice, which are prone to atherosclerosis when fed high-fat diets. Therefore, in this review, we concentrate on published experimental atherothrombosis *in vivo* studies using $Apoe^{-/-}$ mice as well as on *in vitro* whole blood perfusion studies using isolated plaque material.

Novel mouse models of atherothrombosis

In a hallmark paper, Reddick *et al.* [8] described for the first time the formation of a thrombus on a damaged mouse plaque, provoked by clamping the murine atherosclerotic aorta. Since then, a wide range of *in vivo* murine models of atherothrombosis have been developed and exploited predominantly with $Apoe^{-/-}$ mice. Damage of the atherosclerotic vessel wall has been triggered by different approaches, *that is,* from inside the vessel (needle insertion, photochemical, or pharmacological intervention) or from the outside (clamping, ligation, injury by laser light, ultrasound power, or $FeCl_3$ application). The process of thrombus formation is quantified in many papers (Table 1). However, the extent of plaque disruption has in only few occasions been verified by histological analysis and appeared to vary with the methodology applied [9,10]. The formation of either non-occlusive or occlusive thrombi is described, putatively depending on the size and severity of the vascular (plaque) damage or disruption. For instance, the local plaque rupture induced by targeted ultrasound application resulted in non-occlusive thrombus formation [9], whereas the more broad application of $FeCl_3$ or illumination of a photosensitive dye led to full arterial occlusion [11–13]. It should be noted that the latter two techniques primarily cause denudation or disturbance of the endothelium rather than plaque disruption, as seen with ultrasound or inside wire treatment. For a more extensive description of the pros and cons of the various injury models, we like to refer to other reviews [14,15]. Here, we aim to discuss in an integrative way the mechanisms by which thrombi are formed in an atherosclerotic vessel irrespective of the trigger. For the present purpose, we consider all thrombus formation in mouse atherosclerotic arteries as experimental atherothrombosis.

In addition to these *in vivo* models, flow chambers with atherosclerotic plaques have also been used for *in vitro* studies of athero-induced thrombus formation. The first generation flow devices in the seventies and eighties made use of isolated atherosclerotic vessel wall segments that were everted around a central rod [16]. In the last decade, mostly transparent parallel-plate flow chambers have been employed, coated with homogenized atherosclerotic plaque material, and perfused with blood under controlled flow conditions. Advantages of the latter devices are that they require only small blood volumes and that the thrombotic process can be imaged in real time [16]. Table 2 summarizes relevant studies investigating thrombus formation on plaque material using mouse, pig, or human blood.

Plaque-derived collagens and platelet collagen receptors in atherothrombosis

Although the fibrillar type I and III collagens are known to stabilize an atherosclerotic plaque, there is considerable evidence that these collagens become platelet-activating upon exposure to the blood stream. Similar to thrombus formation in non-atherosclerotic arteries [17], the platelet collagen receptor, glycoprotein VI (GPVI) has been found to play a key role in thrombus formation on exposed

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Table 1 Literature evidence for involvement of platelet, plasma, and vascular components in atherothrombus formation *in vivo* in *Apoe*$^{-/-}$ mice

Target	Inhibitor, perturbation	*Apoe*$^{-/-}$ vessel: atherothrombosis induction	Effect on thrombus, embolization	Persistent effects (after thrombus*)	References
Platelet components					
ADP receptor $P2Y_{12}$	Cangrelor, clopidogrel, ticagrelor	CA: inside needle, ultrasound, ligation	Thrombus ↓, emboli ↑	Neointima =	[9,10,23,24]
CD40	*Cd40*$^{-/-}$	CA: ultrasound	Thrombus ↓		[9]
Cyclooxygenase 1	Aspirin	CA: ligation	Thrombus =	Neointima =	[24]
Dense granules	*Hps3*$^{-/-}$	CA: $FeCl_3$	Occlusion time ↑	LC infiltration ↓, neointima ↓*	[12]
Glycoprotein VI	JAQ1 Ab	CA: inside needle, ultrasound	Thrombus ↓		[9,10]
Integrin αIIbβ3	Eptifibatide	CA: inside needle, ultrasound	Thrombus ↓		[10]
PGE_2 receptor EP3	*Ptger3*$^{-/-}$, DG-041	CA: inside needle	Thrombus ↓		[26,27]
PI 3-kinase β	TGX-221	CA: ultrasound	Thrombus ↓		[9]
Plasma components					
FII	Hirudin, αNAPAP, FII$^{-/WT}$	CA: $FeCl_3$, inside needle, ultrasound	Thrombus ↓, occlusion time =	Neointima ↓	[9,10,38,55]
FII+FX	EP217609	CA: inside needle	Thrombus ↓		[38]
FVII	FVIIa inhibitor	CA: photochemical, ultrasound	Thrombus ↓, emboli =, occlusion time ↑,		[13,31]
FX	Fondaparinux	CA: inside needle	Thrombus ↓		[38]
FXI	Antisense, anti-FXI Ab	CA: ultrasound, photochemical	Thrombus ↓, emboli ↑, occlusion time =	LC infiltration ↑*	[13,34]
FXII	CTI, rHA-Infestin-4	CA: ultrasound, photochemical	Thrombus ↓, emboli ↑		[13,31]
Interferon-α	IFNα adenovirus	CA: photochemical	Occlusion time ↓	Neointima =	[59]
PAI-1	*Serpine1*$^{-/-}$	CA: $FeCl_3$, photochemical	Thrombus ↓, occlusion time ↑	Neointima ↑*/↓	[40–42]
TFPI	*Tfpi*$^{+/-}$	CA: photochemical	Occlusion time ↓	Neointima ↑	[32]
Tissue factor	Anti-TF Ab	CA: photochemical	Occlusion time ↑		[13]
Vascular components					
CCL2 (JE/MCP-1)	*Ccr2*$^{-/-}$, 2H5 Ab	CA: inside wire		LC adhesion↓, neointima ↓*	[50]
Fibrilin-1	*Fbn1*$^{C1039G+/-}$	AO: spontaneous	Thrombus ↑	Neointima ↑	[45]
Lipoprotein lipase	*Lpl*$^{-/-}$	CA: $FeCl_3$	Thrombus ↑	Neointima ↑*	[43]
Leptin receptors	Leptin	CA: photochemical	Occlusion time ↓	Neointima ↑	[44]
MMP-9	Active MMP-9 expression in MΦ	AO: spontaneous	Thrombus ↑		[46]
Niemann-Pick C1	*Npc1*$^{-/-}$	AO: spontaneous	Thrombus ↑	Neointima ↑	[48]
p53	p53 overexpression in SMC	AO: spontaneous	Thrombus ↑	Neointima =, cap/intima ratio ↓	[47]
Thrombomodulin	TM$^{Pro/Pro}$	CA: $FeCl_3$	Occlusion time ↓	Neointima ↑	[55]

Only studies providing quantitative data are cited. Ab, antibody; CA, carotid artery; AO, aorta; LC, leukocyte; = unchanged; ↑ increased; ↓ decreased.

plaque collagens. Accordingly, blockage of GPVI with a monoclonal antibody abolished the thrombotic process in *Apoe*$^{-/-}$ mice *in vivo* triggered by ultrasound-induced plaque rupture (Table 1) [9,10]. Interestingly, atherothrombosis induced by needle insertion appeared to be less sensitive to GPVI blockage than the ultrasound model, whereas the thrombotic process in both models was suppressed by thrombin inhibition [10]. *In vitro*, blockage of GPVI abrogated plaque-induced human thrombus formation in a flow chamber (Table 2) [18–20]. In such a flow chamber system, no role of the other platelet collagen receptor, integrin $\alpha_2\beta_1$, could be demonstrated [19]. Consistent with a platelet-activating role for type I and III collagens in atherothrombosis is the observation that, in serial cross sections of human atherosclerotic plaques, the areas of high platelet deposition are enriched in these collagens [21].

Roles of other platelet signaling proteins in atherothrombosis

Given the key platelet-activating role of GPVI in experimental atherothrombosis, one can assume that also other platelet signaling proteins with known involvement in

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Table 2 Literature evidence for involvement of platelet and plasma components in atherothrombus formation in flow perfusion studies *in vitro*

Target	Blood origin	Inhibitor, perturbation	Flow device: plaque origin	Effect on thrombus, embolization	References
Platelet components					
ADP receptor $P2Y_1$	H	MRS-2179	PP: carotid plaque (H)	Thrombus ↓	[18,22]
ADP receptor $P2Y_{12}$	H	Cangrelor, ticagrelor	PP: carotid plaque (H)	Thrombus ↓, emboli ↑	[18,22,23]
ADP receptor $P2Y_{12}$	M	$P2ry12^{-/-}$	PP: aortic plaque (M)	Thrombus ↓, emboli ↑	[23]
Cyclooxygenase-1	H	Aspirin	PP: carotid plaque (H)	Thrombus =	[22]
Glycoprotein Ib-V-IX	H	6B4 Ab	PP: carotid plaque (H)	Thrombus ↓	[22]
Glycoprotein VI	H	GPVI-Fc protein; 10B12, 9O12 Ab	PP: carotid plaque (H)	Thrombus ↓	[18–20]
Glycoprotein VI	M	JAQ1 Ab	PP: carotid artery (H)	Thrombus ↓	[19]
Integrin $\alpha2\beta1$	H	6F1 Ab	PP: carotid plaque (H)	Thrombus =	[19]
Integrin $\alpha2\beta1$	M	$Itg\alpha2^{-/-}$	PP: carotid plaque (H)	Thrombus =	[19]
PGE_2 receptor EP3	H	AE5-599, AE3-240	PP: carotid plaque (H)	Thrombus =	[28]
Serotonin receptor $5HT_{2A}$	H	Ketanserin, R-96544	PP: arterial plaque (H)	Thrombus =	[60]
Plasma components					
FVII	H	FVIIai	PP: carotid plaque (H)	Thrombus ↓	[31]
FVII	M	FVIIai	PP: aortic plaque (M)	Thrombus ↓	[31]
FXII	H	CTI, FXII-deficient patient	PP: carotid plaque (H)	Thrombus ↓/=	[20,31]
FXI, FXII	M	$F11^{-/-}$, $F12^{-/-}$, $F11^{-/-}/F12^{-/-}$	PP: aortic plaque (M)	Thrombus ↓	[31]
Lipoproteins (HDL ↑ LDL ↓)	P	Gemfibrozil	TB: carotid plaque (P)	Thrombus =	[61]
Tissue factor	P	Anti-TF Ab, TFPI	TB: arterial plaque (H)	Thrombus ↓/=, emboli ↓	[20,30]
Platelet membranes	H	Microparticles	TB: aortic plaque (H)	Thrombus ↑, emboli ↑	[62]

Only studies providing quantitative data are cited. H, human; M, mouse; P, pig; Ab, antibody; PP, parallel plate; TB, tubular; = unchanged; ↑ increased; ↓ decreased.

collagen-dependent thrombus formation [1,2] can contribute to the assembly of thrombi on a damaged atherosclerotic plaque. This has been demonstrated for the GPIb-V-IX complex, interacting with collagen-bound VWF, which was mediated initial platelet adhesion to plaque material at high, but not at low wall-shear rates [22]. Another contributor is the platelet ADP receptor $P2Y_{12}$, which was found to act by stabilizing thrombi formed on disrupted plaques of $Apoe^{-/-}$ mice *in vivo* as well as in flow devices *in vitro* [23] (Fig. 1). In contrast, the other platelet ADP receptor, $P2Y_1$, does not seem to have a major role in thrombus formation [18,22]. Finally, atherothrombus formation *in vivo* was inhibited by blocking phosphoinositide 3-kinase β or integrin $\alpha_{IIb}\beta_3$ [9,10], which are implicated in platelet aggregation. *In vitro* and *in vivo* studies indicated that blockage of thromboxane formation with aspirin did not impair thrombus formation on atherosclerotic plaques [22,24]. The latter findings are in line with data from *in vitro* perfusion studies over purified collagen in which either no effect or reduced thrombus formation was observed after aspirin addition or treatment [16]. An interesting observation in this regard is that aspirin treatment of patients with peripheral artery disease, in whom platelet reactivity is generally increased, was found to normalize thrombus formation under flow [25]. This suggests that the net effect of aspirin on thrombus formation is influenced by the basal platelet activation state.

Some controversy exists on a role of the platelet EP3 receptor for prostaglandin E2 in atherothrombosis models. This prostaglandin can be produced in murine and human atherosclerotic plaques, and reports exist that blockage or absence of the EP3 receptor reduces thrombus formation in $Apoe^{-/-}$ mice [26,27]. However, another report describes no effect of EP3 receptor blockers on plaque-induced human thrombus formation *in vitro* [28]. A possible explanation for the latter observation is that plaque-derived prostaglandin E2 is reported to be only shortly active after plaque dissection [27]. Yet, additional research is needed to resolve this matter.

The extrinsic coagulation pathway in acute atherothrombosis

In the atherosclerotic vessel wall, several cell types express tissue factor (TF), especially macrophages and smooth muscle cells. Upon exposure to the blood stream, de-encrypted TF forms a complex with coagulation factor FVII(a), which activates FIX, FX, and prothrombin, in proteolytic reactions that are potently stimulated by the tenase and prothrombinase complexes [2]. These complexes are efficiently formed on phosphatidylserine-exposing, procoagulant membranes, including GPVI-stimulated platelets, injured vascular cells, and microparticles [2]. *In vitro* flow chamber and thrombin generation studies point

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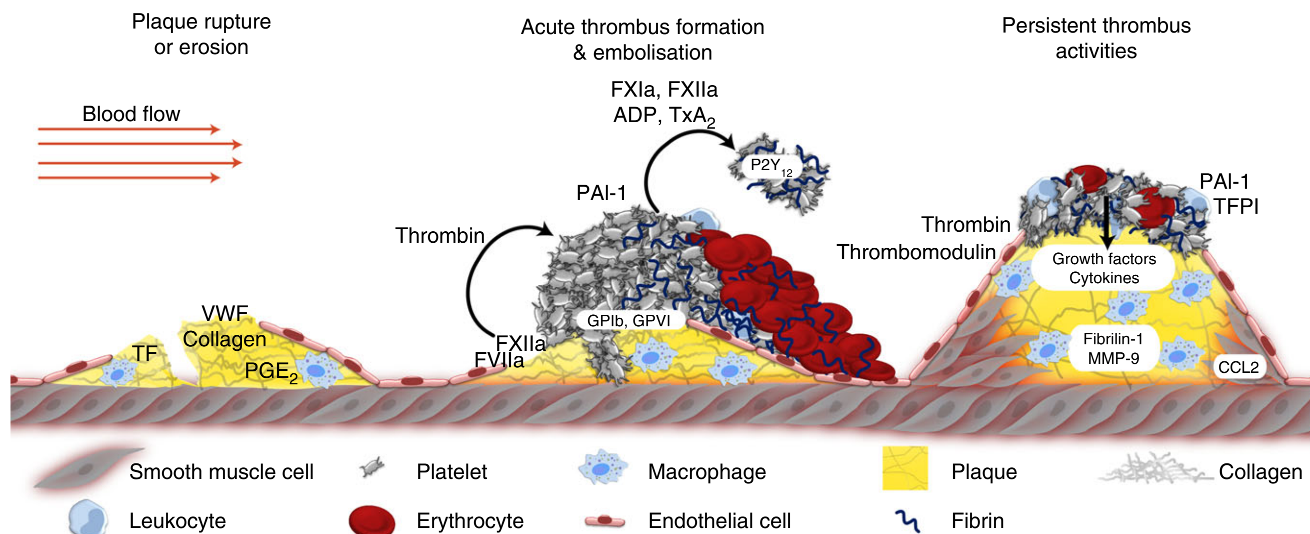

Fig. 1. Schematic overview of factors involved in thrombus formation at a site of plaque disruption. A ruptured or eroded atherosclerotic plaque rapidly recruits circulating platelets, which interact with thrombogenic substances such as collagen and PGE2 of the plaque interior. Collagen also binds and activates FXII, which results in sequential activation of FXI and FIX, and subsequent thrombin generation. Thrombin is also generated via plaque-derived tissue factor in complex with FVIIa. Thrombin activates platelets and cleaves fibrinogen into fibrin, which acts in thrombus stabilization and accomplishes massive trapping of red blood cells. Activated platelets secrete secondary mediators ADP and TxA_2 to recruit other platelets and form a stable platelet aggregate. Furthermore, they release growth factors and cytokines, which in interplay with coagulant (thrombin), anticoagulant (thrombomodulin), and antifibrinolytic (PAI-1, TFPI) factors exert persistent vessel wall directed activities.

to a high thrombogenicity of the TF-enriched, inner atheromatous plaque core [29]. Using *Apoe*$^{-/-}$ mice, subjected to ultrasound plaque rupture or photochemical injury, it was shown that inhibition of the TF/FVIIa complex diminishes the process of thrombus formation, pointing to a key role of the extrinsic coagulation pathway in experimental atherothrombosis [9,13]. A role of TF has also been observed *in vitro* in flow perfusion studies using human plaque material [20,30,31]. In this case, inhibition of TF did not affect platelet aggregation, but markedly delayed fibrin formation [20]. In line with these data, heterozygous deficiency in tissue factor pathway inhibitor (TFPI), the major TF antagonist, was found to stimulate thrombus formation in *Apoe*$^{-/-}$ mice [32].

Roles of the intrinsic coagulation pathway

The intrinsic coagulation pathway, which according to its name can operate with blood factors only, is triggered by contact of FXII with negatively charged surfaces, through its cleavage by high molecular weight kininogen and kallikrein. It has been shown that fibrillar collagens such as present in plaques can bind FXII and support the activation to FXIIa [33]. Like thrombin, activated FXII cleaves FXI, which in turn cleaves FIX. Several observations point to a unique role of this pathway in experimental atherothrombosis (Fig. 1). In an *in vivo* model of ultrasound-induced plaque rupture, inhibition of FXIIa or treatment with FXI antisense oligonucleotides caused a substantial suppression of thrombus formation [31,34]. This corresponds well to findings with damaged healthy (non-atherosclerotic) arteries, where thrombus formation was markedly impaired and destabilized in mice lacking FXII or FXI [35,36]. On the other hand, in an atherothrombosis model of $FeCl_3$ application, no prominent role of FXII could be observed [13]. In *in vitro* studies where blood was flowed over plaque material, inhibition of FXIIa with corn trypsin inhibitor (human) or the absence of FXII or FXI (mouse) led to unstable thrombus formation [31]. Interestingly, immunostaining indicated that some FXIIa was located at collagen fibers in the plaque, but that the bulk of FXIIa co-localized with fibrin fibers. As the majority of this work points to a clear role of the FXII pathway in atherothrombus formation without affecting hemostasis, the intrinsic coagulation pathway may be an attractive target for antithrombotic drugs with low risk of bleeding. Evidence for this comes from a recent study, in which a lowered FXI level by antisense oligonucleotide treatment was shown to reduce the occurrence of postoperative venous thromboembolism in patients undergoing elective primary unilateral total knee arthroplasty [37].

Thrombus embolization

Clinical studies indicate that embolization is a frequent phenomenon during and after arterial thrombosis, especially in the carotid arteries, although it often remains clinically silent. At present, embolization has only limitedly been quantified in experimental atherothrombosis

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models. The few reports on thrombus stability point to increased embolus formation upon blockage of the platelet $P2Y_{12}$ receptors or the coagulation factors FXI or FXII in *Apoe*$^{-/-}$ mice [10,13,38]. Similar embolizing effects by $P2Y_{12}$ or FXII blockage have been described for thrombi that are formed on injured, non-atherosclerotic arteries [39]. This supports the idea that the stability of thrombi is similarly regulated after disruption of an atherosclerotic or a healthy artery.

Role for PAI-1 in atherothrombus dissolution

In a few thrombosis studies, a role of the fibrinolytic pathway has been investigated, with primary focus on involvement of the fibrinolysis suppressor, plasminogen activator inhibitor-1 (PAI-1). In PAI-1-deficient mice, atherothrombus formation was shown to be repressed, while the time to vascular occlusion was increased [40–42]. Other support for a role of PAI-1 is the finding that the increased thrombus formation in *Apoe*$^{-/-}$ mice caused a high-fat diet is accompanied by a higher arterial expression of PAI-1 [40,42,43]. Although much is known of the dynamics of the thrombus-forming process [1–3,39], our insight into the mechanisms of thrombus dissolution is still limited.

Roles of vascular proteins in acute atherothrombosis

There is evidence for a role of vascular proteins other than TF and collagen in atherothrombus formation. In *Apoe*$^{-/-}$ mice, it has been reported that lipoprotein lipase and leptin receptors in the vessel wall have a suppressive effect on atherothrombus formation, via mechanisms that are unknown [43,44]. Also unexplored is how vessel wall proteins facilitate 'spontaneous' or induced thrombosis in different atherosclerotic vascular beds of *Apoe*$^{-/-}$ mice. So far, unprovoked thrombus formation has been examined in mice with: (i) mutations in elastin-fragmenting fibrilin-1 [45]; (ii) overexpression of activated matrix-degrading matrix metalloproteinase-9 in the plaque macrophages [46]; (iii) overexpression of the tumor suppressor gene p53 in smooth muscle cells when triggering with phenylephrine [47]; and (iv) deficiency in the Niemann-Pick C1 protein, although it should be remarked here that *Npc1*-deficient mice display shortened coagulation times in plasma [48]. Congruous for these cases of spontaneous plaque disruption and thrombosis are intraplaque hemorrhages, increased fibrin deposition, increased matrix degradation, and increased neointima formation, which are jointly indicative for a vulnerable plaque phenotype [4].

Persistent platelet activities in atherothrombosis

Atherosclerosis and atherothrombosis have aptly been described as an 'insidious cycle of acute inflammation and thrombosis,' often remaining clinically silent until the development of a catastrophic cardiovascular event [1]. Indeed, in *Apoe*$^{-/-}$ mice, persistent interactions of platelets with the atherosclerotic vessel wall have been observed, which may contribute to the progression of plaque formation [49]. Smooth muscle cells may promote plaque formation by the release of CC chemokine ligand 2 (CCL2), which binds to the platelet CCR2 receptors and facilitates platelet–monocyte interaction. The CCL2–CCR2 axis appears to promote neointima formation, also after inside wire-induced atherothrombosis [50]. Platelets themselves contain large pools of bioactive proteins, including many growth factors and cytokines, in their storage granules [51]. These platelet-derived biomediators, released at the inflamed or damaged vessel wall, are likely to play a role in the propagation of atherosclerosis [49,52]. Support for this concept comes from experimental atherothrombosis studies with *Apoe*$^{-/-}$/*Hps3*$^{-/-}$ mice, which lack platelet-dense granules. After provoked thrombosis, deficiency in HPS3 led to a reduction in platelet–leukocyte aggregates and to a diminished neointima formation [12].

Considering that ADP is a main constituent of the dense granules and that platelet-derived ADP greatly supports thrombus formation, it may be expected that ADP also contributes to atheroprogression. However, in *Apoe*$^{-/-}$ mice, blockage of the main ADP receptor $P2Y_{12}$ or blockage of thromboxane formation with aspirin was without effect on platelet–vessel wall interaction or neointima formation [24]. Along the same line, two studies indicate that neither clopidogrel nor aspirin affects vascular stiffness in patients with coronary artery disease [53,54]. This suggests that, although clopidogrel and aspirin effectively antagonise incorporation of new platelets into a thrombus, the effects of these drugs on platelet adhesion-dependent atheroprogression are limited. However, further research is required to definitively assess the effect of these antiplatelet drugs on the suppression of platelet activities once a thrombus is formed.

Persistent coagulant and fibrinolytic activities in atherothrombosis

Impaired generation of thrombin, such as in mice with a gene-targeted 50% reduction in prothrombin, attenuated atherosclerotic progression [55]. Conversely, hypercoagulability in mice with inactive thrombomodulin promoted atherothrombosis as well as atherosclerosis progression [55]. Similarly, other studies indicate that thrombin inhibition can have a long-term stabilizing effect on atherosclerotic plaques [56]. Whether the fibrinolysis process is also active at later stages is less clear. One paper suggests that, in the presence of a fibrin-containing thrombus, the fibrinolysis inhibitor PAI-1 stimulates neointima formation [42]. Other studies indicate that, in the absence of an overt thrombotic trigger, deficiency in PAI-1 leads to either increased [41] or site-specific decreased [40] neointima formation. A site-specific effect has also been reported for TFPI, which may act to

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suppress atherosclerosis particularly at sites of turbulent flow, *that is,* in the carotid bifurcation [32]. Together, this suggests that the long-term vascular effects of hemostatic factors on the vessel wall rely on the presence of an appropriate thrombotic trigger and specific biorheologic conditions. Absence of a thrombus may also explain why a 50% reduction of TF was without effect on the development of murine atherosclerotic lesions [57].

Conclusions

In contrast to perhaps what was expected in 2003 [7], recent work on mouse models of provoked thrombosis in atherosclerotic arteries *in vivo* has provided considerable insight into the molecular mechanisms underlying the process of atherothrombosis. Further support for these mechanisms comes from *in vitro* studies where mouse or human whole blood is perfused over isolated atherosclerotic plaque material. Current data indicate that the main plaque-derived factors triggering acute thrombus formation are collagen and TF, next to bioactive mediators like prostaglandin E2 produced by smooth muscle cells and macrophages, which can to attract platelets. Jointly, these studies indicate that the atherothrombotic process is regulated by similar mechanisms as the process of thrombus formation in damaged non-atherosclerotic vessels. Key event herein are collagen- and ADP-receptor-dependent platelet activation and thrombin/fibrin generation via the extrinsic and intrinsic coagulation pathways. Less is known of the persistent effects of a thrombus on neointima formation and atherosclerosis progression, but the available evidence suggests a role herein of both activated platelets and ongoing thrombin generation. The clinical evidence of this is already becoming clear, because combined inhibition of platelets (aspirin plus a thienopyridine) and coagulation (FXa inhibitor rivaroxaban) can lead to an additional decrease in in-stent thrombosis and mortality in patients with acute myocardial infarction, when compared to dual antiplatelet therapy alone [58].

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Disclosure Conflict of Interests

The authors state that they have no conflict of interest.

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