Cholesterol metabolism and hematopoiesis interaction in atherothrombosis

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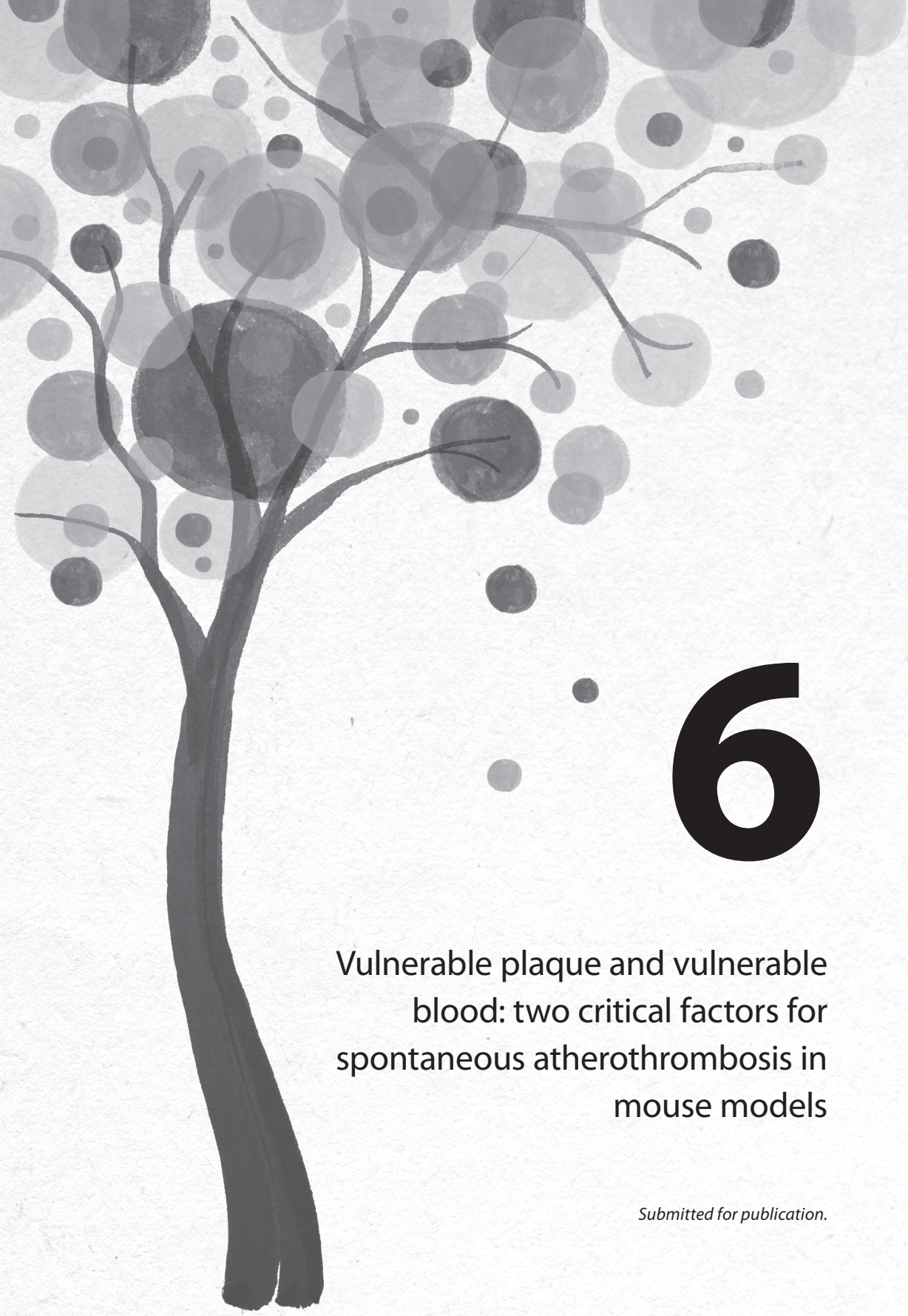

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Vulnerable plaque and vulnerable blood: two critical factors for spontaneous atherothrombosis in mouse models

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ABSTRACT

Atherothrombotic events such as myocardial infarction and ischemic stroke are a major cause of morbidity and mortality worldwide. Understanding the molecular and cellular mechanisms of atherosclerotic plaque destabilization or erosion, and developing new therapeutics to prevent acute cardiovascular events are important points for vascular biology research and clinical cardiovascular medicine. However, basic research on plaque destabilization, rupture and erosion, is hampered by the lack of appropriate animal models of atherothrombosis. Unprovoked atherothrombosis is very scarce in commonly used mouse models for atherosclerosis, the low-density lipoprotein receptor knockout and apolipoprotein E knockout mice. Therefore, specific interventions are required to induce atherothrombosis in these models. Two strategies can be employed to induce atherothrombosis: 1) plaque destabilization and 2) induction of blood hypercoagulability. Although the individual strategies yield atherothrombosis at low incidence, it appears that the combination of both plaque destabilization and an increase in blood coagulability is the most promising strategy to induce atherothrombosis on a larger scale. In this review we summarize the recent developments on mouse models for the investigation of atherothrombosis.

INTRODUCTION

Acute cardiovascular events, caused by atherosclerosis and subsequent superimposed thrombus formation after plaque rupture or superficial erosion of the overlying endothelium[1,2], are a major cause of morbidity and mortality worldwide. Understanding the molecular and cellular mechanisms of atherosclerotic plaque destabilization or erosion, and developing new therapeutics to prevent acute cardiovascular events are important points for vascular biology research and clinical cardiovascular medicine. However, even after decades of extensive research into the pathophysiology of atherosclerosis, predictability of which plaques will progress to atherothrombosis, and when, still remain largely elusive. Major advances in the prevention of acute cardiovascular events will require early detection of rupture- and erosion-prone plaques. However, basic research on plaque destabilization, rupture and erosion, and superimposed thrombus formation is hampered by the lack of appropriate animal models of spontaneous atherothrombosis. In this review, we summarize the recent development of and use of mouse models for the investigation of plaque vulnerability, atherothrombosis and acute cardiovascular events.

MURINE MODELS OF ATHEROSCLEROSIS

Murine models of atherosclerosis are widely used in basic research to study the molecular mechanisms underlying the development of atherosclerosis and potential therapeutic strategies to inhibit plaque progression and even stimulate plaque regression. Genetically engineered mice with disorders of lipid metabolism, develop spontaneous atherosclerosis, which can be accelerated by feeding the mice a high cholesterol diet. By far the most commonly used mouse models for atherosclerosis are Apolipoprotein E (ApoE) knockout and low-density lipoprotein receptor (LDLr) knockout mice, which were both first described to develop hypercholesterolemia and atherosclerosis in the early 1990s [3–5]. One of the first arterial region where these mice develop atherosclerotic plaques is the tricuspid aortic valve area of the aortic root, and this area is therefore often used as a standard for the quantification of atherosclerotic burden [6]. Moreover, rapid spontaneous atherosclerosis development occurs in the coronary arteries and along the entire aorta at branch points of major arteries in ApoE knockout mice [3,4,7–9]. Another approach to stimulate the onset of atherosclerosis in ApoE or LDLr knockout mice is by placing an external silastic collar around the carotid artery, which results in rapid atherosclerotic plaque development proximal of the collar [10]. The particular advantage of this technique is that, in contrast to the aortic and coronary artery, atherosclerotic plaques develop at a site that is very easily accessible to local manipulation and visualization. The plaques that develop in ApoE and LDLr knockout models are reproducible in size and

composition, and have architectural features reminiscent of human plaques. However, these models were not specifically developed to study atherothrombosis.

ATHEROTHROMBOSIS AND VULNERABILITY OF THE PLAQUE

The concept of the vulnerable plaque and risk for developing atherothrombosis is deduced from early postmortem pathological studies in human cases of fatal coronary atherothrombosis. These studies showed that plaques prone to rupture and superimposed thrombus formation are typically characterized by a thin fibrous cap, a large lipid core, and a relative abundance of inflammatory leukocytes [11]. In these studies, the rupture of these so-called vulnerable plaques was found to be the cause of the acute coronary event in the majority of cases.

In the commonly used ApoE knockout mouse model plaque rupture only occurs after prolonged feeding with very high-cholesterol diets. One study that aimed to retrospectively quantify spontaneous atherothrombosis in intervention studies with ApoE knockout mice found aortic plaque rupture and/or thrombi in only 3 out of 82 untreated mice (aged 9-12 months) fed various cholesterol-enriched diets for up to 6 months [12]. Moreover, screening the hearts of 33 older ApoE knockout mice (age 9-20 months) showed extensive atherosclerosis in one or more coronary arteries of 18 animals, 3 of which with blood-filled channels inside the plaques [12]. This suggests the occurrence of previous plaque disruption/thrombotic events followed by recanalization. In the aortic root of these mice, 4 deep plaque ruptures (or erosions reaching necrotic core areas) and a large thrombus originating from the core of a disrupted atherosclerotic plaque were observed [12]. Although this study comprised a limited sample size, it is indicative of the scarcity of spontaneous atherothrombotic events and highlights the need for better mouse models to study this phenomenon.

The brachiocephalic artery is a short communicating vessel originating at the aortic arch, that bifurcates into the right common carotid and right subclavian arteries. In ApoE knockout mice (C57Bl/6 background) aged 42-54 weeks fed a standard chow diet, intraplaque hemorrhage was found in the brachiocephalic artery in 75% of the animals [13]. Moreover, there was fibrotic conversion of necrotic zones and loss of the fibrous cap [13]. However, no plaque rupture, erosion or thrombi were observed. Another study reported that of 11 ApoE knockout mice fed a diet supplemented with 21% lard and 0.15% cholesterol for up to 14 months, 8 mice died after 37–59 weeks of diet feeding. Luminal thrombi associated with atherosclerotic plaque rupture were observed in 7 of these mice, in 6 of which an atherosclerotic plaque rupture was found in the brachiocephalic artery [14]. More recently, this model was developed further by outbreeding the ApoE knockout mice to a mixed C57Bl/6 and 129 background (71%

and 29% respectively). These mice developed pronounced atherosclerotic plaques in the brachiocephalic artery after only 5 weeks challenge with a high-fat/high-cholesterol diet [15]. The plaques displayed a high lipid content and a relatively thin fibrous cap, and were thereby very similar to vulnerable plaques in coronary arteries of human patients suffering from cardiovascular disease. Most importantly, plaques in the brachiocephalic artery of this mixed-background ApoE knockout model showed signs of acute plaque rupture (62% at 8 weeks diet-feeding) with thinning and discontinuity of the fibrous cap and intrusion of blood into the plaque [14,15]. Moreover, the plaques also displayed a high incidence of buried fibrous caps, suggestive of prior silent plaque rupture, making the model specifically pertinent of the human vulnerable plaque [16,17]. However, in none of the models, there is convincing evidence of the formation of platelet and fibrin-rich occlusive thrombi at the site of presumed rupture, which is characteristic of the human plaque rupture leading to coronary heart disease and stroke. Therefore, in order to study this phenomenon in mice, various methods have been explored to provoke this event.

Collar-induced atherosclerotic plaques in the carotid arteries of mice are easily accessible for local manipulation. In one model described by Von der Thüsen et al in 2001 atherosclerotic plaques, induced by placement of a perivascular silastic collar, were locally incubated with a recombinant adenovirus carrying the apoptosis promoting protein p53 into the temporarily ligated artery [18]. One day following transfection, increased apoptosis is evident in the cells of the fibrous cap, while cap thinning was observed at later timepoints. Although thinning of the fibrous caps upon p53 transfection induced plaque rupture in 40% of the animals, upon challenge with the vasopressor phenylephrine, no spontaneous atherothrombosis was observed [18].

Another model used a different type of carotid artery ligation, in which the left common carotid arteries of male ApoE knockout mice were ligated just proximal to their bifurcations [19]. Ligation of the carotid artery in ApoE knockout mice after 4 weeks on a chow diet induced marked intimal hyperplasia, which is a lipid- and collagen-rich plaque that contains a number of macrophages, T lymphocytes, and smooth muscle cells. At 4 weeks after ligation, the mice received polyethylene cuff placement just proximal to the ligated site. Cuff placement evoked intraplaque hemorrhage and plaque rupture, accompanying a decrease in collagen content. At 4 days after cuff placement, plaque rupture associated with fibrin(ogen)-positive thrombus formation in the lumen was detected in 63% of plaques [19]. Together, these studies suggest that initiation of plaque vulnerability may be a good strategy to induce atherothrombosis.

As indicated above, the concept of the vulnerable plaque is widely used in the cardiovascular field. However, this concept is largely based on findings in postmortem pathological studies, which are essentially retrospective. It is important to be aware that these studies did not consider the amount of plaques with morphological characteristics associated with vulnerability that did not cause fatal cardiovascular events. Plaques

with vulnerability characteristics often are asymptomatic, and seldom cause events due to rupture, debating the inevitable nature of the vulnerable plaque-concept [20,21].

Fatal cardiovascular events can also be the consequence of superficial erosion of the plaque [11,22]. In contrast to plaques associated with plaque rupture, eroding plaques with superimposed thrombi do not have thin fibrous caps, large lipid pools and abundant inflammatory cells [23]. Interestingly, over the past decade, there has been a shift in the morphology of human atherosclerotic plaques. Plaques obtained from patients with symptomatic carotid artery disease in recent years show significantly more fibrous, non-inflammatory characteristics. This trend is also visible in asymptomatic patients [24–26]. Possibly, this shift in plaque characteristics could lead to a subsequent shift in plaque rupture versus erosion occurrence [27,28]. More studies that delve into the causes of human atherothrombotic events are therefore warranted and of vital importance to the development of a representative spontaneous animal model.

ATHEROTHROMBOSIS AND VULNERABILITY OF THE BLOOD

Species differences in anticoagulation could contribute to the low atherothrombosis susceptibility of mouse models. The half-life of active coagulation factor IIa in mouse plasma is significantly shorter as compared to its human counterpart, pointing towards more potent natural anticoagulation in mice[29]. Therefore, besides manipulation of plaque stability and/or erosion, another strategy to induce spontaneous atherothrombosis is by increasing the coagulability of the blood.

Mice carrying a null mutation of a gene regulating intracellular cholesterol transport (the Niemann-Pick C1 (Npc1) gene, BALB background) that were crossed with ApoE knockout (C57BL/6J background) mice developed large, protruding thrombi associated with the plaques in 24% (6 of 26) of the mice [30]. These thrombi were formed primarily on the surface of atherosclerotic plaques with loss of the endothelial monolayer at the plaque-thrombus interface. Genetic studies suggested that the ~25% BALB background was permissive for plaque complications compared with the commonly used C57BL/6J background. Moreover, increased thrombin generation was indicated by a significant elevation in mean thrombin-antithrombin levels in the double-mutant mice compared with controls. Together, these data suggested that the double-mutant mice harbor a procoagulant state. Moreover, the combination of the underlying prothrombotic state and Npc1 deficiency-related plaque vulnerability led to atherothrombosis in the double-mutant mice, albeit at low incidence [30].

We have recently published a proof-of-principle study in which we showed that transient (7 days) siRNA mediated lowering of the natural anticoagulant protein C in atherosclerotic ApoE knockout mice induced large, organized, and fibrin- and leukocyte-rich

thrombi on top of advanced atherosclerotic plaques in the aortic root [31]. Although the incidence of atherothrombosis was low (14%), it is the first report of provoked spontaneous atherothrombosis in the aortic root. In a second study, we investigated if plaque characteristics or circulating factors could be linked to atherothrombosis susceptibility. Moreover, we aimed to increase the incidence of atherothrombosis by transiently increasing blood pressure, and to direct atherothrombosis to an additional predefined vascular site by placement of a perivascular silastic collar around the carotid artery [32]. Spontaneous atherothrombosis in the aortic root of protein C-targeted siRNA-treated ApoE knockout mice was reproduced and occurred at an incidence of 23%, while the incidence of collar-induced atherothrombosis in the carotid artery was only 2.6%. Treatment with phenylephrine, to transiently increase blood pressure, did not increase the incidence of atherothrombosis in the aortic root of the ApoE knockout mice, nor in the carotid arteries with collars. Plaques in the aortic root with an associated thrombus were lower in collagen and macrophage content, and mice with atherothrombosis had significantly more circulating platelets. Remarkably, our data revealed that thrombus formation preferentially occurred on plaques in the right coronary sinus of the aortic root. The identification of factors that drive the predilection for the right coronary sinus may provide novel clues on factors that drive atherothrombosis in mice and humans.

Another strategy focused on disruption of the protein C pathway was described by Borissoff *et al* in 2013. They used a model in which ApoE knockout mice had a mutation in the thrombomodulin gene, resulting in diminished trombomodulin-dependent generation of activated protein C[33]. The collar-induced carotid artery plaques of these mice had large necrotic cores and a decreased collagen content, resulting in thin fibrous caps. Moreover, carotid artery plaques from these mice tended to rupture and dissect in both a nonocclusive phase, leading to non-occlusive thrombus formation, as well as in more advanced stages, leading to full occlusion of the artery by thrombosis. Also, strong evidence of buried caps was found, indicating multiple consecutive plaque ruptures, similar as in the human situation. Unfortunately, the percentage of plaques that progressed to atherothrombosis in the carotid artery, nor indications of atherothrombosis in the aorta or aortic root were mentioned in the paper.

In a study by Liu and colleagues published in 2015, it was shown that the best strategy to induce atherothrombosis is to use a combination of factors that destabilize the plaque together with augmentation of blood coagulability. In this study ApoE knockout mice that overexpress thrombin were exposed to long term restraint stress, which led to atherothrombosis in collar-induced carotid artery plaques of 70% of the animals [34]. Moreover, pathohistological examination revealed that many of the atherothrombotic plaques were associated with both fresh and organized thrombi, indicating that they had undergone plaque disruption more than once, thereby showing considerable similarity to human coronary plaques. Finally, thrombosis was substantially reduced by

dual antiplatelet therapy, suggesting that this model may be useful in preclinical drug development.

CONCLUSIONS AND PERSPECTIVES

In summary, two strategies can be employed to induce atherothrombosis: plaque destabilization and/or erosion and induction of blood hypercoagulability. Although the single use of both strategies yields atherothrombosis at low incidence, it appears that a combination of plaque destabilization and an increase in blood coagulability is the most promising strategy to induce atherothrombosis on a larger scale (Table 1). Efforts in developing new models for atherothrombosis should therefore be aimed at targeting both parameters. Importantly, caution is warranted in the interpretation of studies that describe loss of collagen and increase in plaque macrophages as markers for plaque vulnerability, in the context of models that do not develop spontaneous atherothrombosis. Further studies in mouse models that do develop atherothrombosis should provide the proof for the predictive power of these vulnerability markers in mice.

Table 1: Mouse models of spontaneous atherothrombosis.

Model	Location of atherothrombosis	Plaque destabilization	Modification of coagulation	Atherothrombotic plaques (%)
Von der Thüsen et al. [18]	Carotid artery (collar induced)	p53 overexpression and phenylephrine	-	40%
Sasaki et al. [19]	Carotid artery (ligation induced)	Artery ligation	-	63%
Ouweneel & Heestermans et al. [31]	Aortic root	-	siRNA silencing of Protein C	14%
Heestermans & Ouweneel et al. [32]	Aortic root and carotid artery (collar induced)	-	siRNA silencing of Protein C	Aortic root: 21% Carotid artery: 2.6%
Heestermans & Ouweneel et al. [32]	Aortic root and carotid artery (collar induced)	Phenylephrine	siRNA silencing of Protein C	Aortic root: 20% Carotid artery: 0%
Borissoff et al. [33]	Carotid artery (collar induced)	-	Thrombomodulin mutation	Unknown
Liu et al. [34]	Carotid artery (collar induced)	Restraint stress	-	14%
Liu et al. [34]	Carotid artery (collar induced)	-	Thrombin overexpression	21%
Liu et al. [34]	Carotid artery (collar induced)	Restraint stress	Thrombin overexpression	70%
Welch et al. [30]	Proximal aorta	-	Npc1 knockout	24%

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