Review Article

The Fat-Fed Apolipoprotein E Knockout Mouse Brachiocephalic Artery in the Study of Atherosclerotic Plaque Rupture

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Atherosclerosis has been studied in animals for almost a century, yet the events leading up to the rupture of an atherosclerotic plaque (the underlying cause of the majority of fatal thrombosis formation) have only been studied in the past decade, due in part to the development of a mouse model of spontaneous plaque rupture. Apolipoprotein E knockout mice, when fed a high-fat diet, consistently develop lesions in the brachiocephalic artery that rupture at a known time point. It is therefore now possible to observe the development of lesions to elucidate the mechanisms behind the rupture of plaques. Critics argue that the model does not replicate the appearance of human atherosclerotic plaque ruptures. The purpose of this review is to highlight the reasons why we should be looking to the apolipoprotein E knockout mouse to further our understanding of plaque rupture.

1. Introduction

Despite death rates from cardiovascular disease having fallen in the United Kingdom since the 1970s (40% decrease for people under 75 in the past decade), disease of the circulatory system accounts for approximately 25% of deaths in under 75 year olds, and coronary heart disease is the leading cause of death [1]. The underlying cause of the majority of this disease is atherosclerosis.

Atherosclerosis has been studied in animals since the early 1900s, starting with the work of Ignatowski (reviewed in [2]) despite atherosclerosis not being recognised as a major disease at that time. Due to the recent increasing prevalence of cardiovascular disorders, research into the disease is still extremely important.

In an ideal world, animal models of atherosclerosis would replicate disease exactly as it is found in humans; however, common sense tells us that this is not possible and a different approach is required. A good animal model will reproduce the biochemistry of the disease but does not necessarily need to produce an identical morphology. As long as the processes that lead up to lesion formation are similar between animal and human, and the model exhibits similar features of the disease that make it a clinical risk (plaque rupture, mural thrombosis, intraplaque haemorrhage), the model will yield useful insights. A good example of this is the cholesterol-fed rabbit model. Cholesterol-fed rabbits have been used in atherosclerotic research for over 60 years and this model has provided highly relevant clues as to how lesions develop in humans. This research has been criticised because rabbits are herbivorous and handle cholesterol in a way very unlike humans. This does not mean we should ignore the findings; it means that we should interpret them cautiously, taking into account the interspecies differences.

A good atherosclerosis animal model should enable us to track changes in disease state over time, have similar lesions (but not necessarily identical) to those in humans, and employ animals which consistently develop atherosclerosis in known locations, which breed rapidly, and which are relatively inexpensive.

The general method of choosing an animal model of atherosclerosis, even in present times, has been to use a "top-down" approach, whereby disease features known to be present in humans are required to be present in the animal model. This has led to much sterile debate about whether animal lesions look like human lesions. Anyone who has looked at a good number of human lesions realises that they are extremely heterogeneous, even at similar anatomical sites,

so it is not even possible to prescribe a lesion morphology that the animal model should aspire to. It is time to abandon the top-down approach and apply intellect to the problem. This in essence means using the opposite method, a "bottom-up" approach, to animal model selection. This applies with particular force to animal models of plaque rupture, which have suffered greatly from the narrow strictures of top-down thinkers. A bottom-up philosophy means asking whether a given animal model provides useful information about the human disease; the appearance, genetic makeup, dietary habits, size, and so on of the animal model are all insignificant if the model enables genuine and useful insights.

The top-down approach became adopted for understandable reasons. The only source of information about ruptured plaques was postmortem coronary artery specimens, so all we had available was a snapshot of a disease that may have been developing, even during its final fatal phases, for hours, days, or perhaps weeks. This leads to a number of serious problems of interpretation.

(1) Patients are self-selecting—they have suffered a fatal coronary event—and thus may not be representative of the general population or even of patients with nonfatal unstable plaques. This may bias any analysis towards particular underlying mechanisms.

(2) The elapse of time between symptom onset and specimen retrieval at postmortem will be accompanied by changes in tissue composition which it would be desirable not to reproduce in the animal model.

(3) Within-subject temporal histopathological analysis is impossible, and cohort-based between-subject analysis is extremely difficult.

(4) *Post hoc* interpretation involves speculation that is not backed up by experimental verification.

When, many years ago now, all we had to go on was a series of such snapshots, it was reasonable to suggest that an animal model should bear histopathological resemblance to human unstable plaques. However, our ultimate goal is not to make an animal model with perfect miniature plaque ruptures; it is to make an animal model from which we can learn how and why human plaques rupture, and that can be used to test possible interventions to stop it from happening. By this analysis, histopathology is of secondary importance. This has been a hard lesson for some in the field, who continue to resist the force of this logic and demand that mouse lesions should look just like human lesions—only smaller. In this paper, we shall seek to show that an animal model of plaque rupture does exist, that it has provided useful and meaningful insights into human disease, and that top-down thinking continues to dog our attempts to bring experimental pathology in atherosclerosis research up to date.

2. What Is Atherosclerosis?

The term atherosclerosis was coined in 1940 by Félix Marchand [3] as it describes the two constituent regions of plaques very accurately: *athéré* in Greek meaning gruel or porridge and signifying the soft lipid-filled core and *sclerosis* implying hardening and referring to the hard fibrotic cap that separates the blood from the thrombogenic material within the lesion [4]. Interestingly, atherosclerosis is not just a disease of modern civilizations. Egyptian mummies of high priests and priestesses from 1580–527 BC have been dissected and shown to have lesions similar to modern day man. Atherosclerosis in these mummies was described as follows: "the disease being characterised by marked degeneration of the muscular coat and of the endothelium. These diseased patches, discrete at first, fuse together later, and finally form comparatively large areas of degenerated tissue, which may reach the surface and open out into the lumen of the tube" [5].

Atherosclerosis is a chronic disease of the intimal lining of medium to large systemic arteries. In healthy arteries, oxygenated blood can freely travel around the body to the organs and extremities, maintaining a healthy system; however, in atherosclerotic arteries the flow of blood can be seriously compromised due to thickening of the intima. The disease itself does not always cause death. In the earlier stages, the blood vessels can remodel to maintain the luminal area (as shall be discussed later) and counteract the intimal thickening. If the lesion expands at a rate greater than the outward remodelling of the vessel, then the flow of blood will decrease causing, for example, angina (heart pain). However, more significant clinical consequences occur if the lesion develops into a fibrous plaque (advanced lesion) that ruptures. This rupturing brings the blood into contact with the thrombogenic core of the lesion precipitating a thrombus that in itself could severely restrict the lumen [35]. It has been shown that a thrombus over a stenosis in the canine coronary arteries can cause a reduction in vessel resistance (caused by decreased blood flow and/or increased pressure gradient) downstream of the plaque [6]. Another potential consequence of thrombosis formation is the shedding of emboli that break off and become lodged in smaller vessels downstream, completely occluding flow and leading to myocardial infarction, if in a coronary artery, or stroke, if in a carotid. Approximately 75% of the thrombi responsible for acute coronary syndromes are precipitated by rupture of an atherosclerotic plaque [7]. Fortunately, not all plaque ruptures lead to vessel-occluding thrombosis. The rupture can be clinically silent and heal itself by the accumulation of smooth muscle cells at the site of rupture leading to secretion of fibrous extracellular matrix [8, 9], lending a striated appearance to the plaque, so-called buried fibrous caps (Human [10, 11], Mice [12]). Despite healing the rupture, this event can lead to luminal stenosis by increasing plaque growth [9, 11].

3. The Mouse As a Model of Atherosclerotic Plaque Rupture

Mice are highly resistant to atherosclerosis. In the 1960s, a diet-induced mouse model was developed by feeding the C57BL/6 strain a diet containing 30% fat, 5% cholesterol,

and 2% cholic acid leading to the development of atherosclerosis. The downside to this diet was that it was highly toxic and the mice lost weight and tended to develop morbid respiratory infections [13]. This diet was modified in 1990 to contain 15% fat, 1.25% cholesterol, and 0.5% sodium cholate [14] leading to lesion development without the toxic side effects. The same group also determined that there were large differences in strain susceptibility to lesion development, providing strong evidence for genetic composition as a risk factor for atherosclerosis.

4. Apolipoprotein E

Apolipoprotein E (ApoE) is a lipoprotein which plays a key protective role in atherosclerosis. All cells and organisms use lipoproteins to move hydrophobic, water-insoluble lipid molecules through the aqueous blood and tissue lymph environment. Lipoproteins consist of apolipoproteins, phospholipids, cholesterol, triacyclglyerols, and cholesteryl esters and it is these that define the particle's buoyant density and enables their separation into four major classes: high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein (HDL, LDL, and VLDL, resp.), and chylomicrons. Discovered in the 1970s [15, 16], apoE is an arginine-rich protein found in chylomicrons, chylomicron remnants, VLDL, and some isotypes of HDL [17]. It is produced in various tissues including brain, spleen, lung, ovaries, adrenal gland, kidney and muscles, but it is in the liver where the majority found in plasma is synthesised [18]. Recognition sites for LDL receptors (LDL-R) and LDL-R related proteins are found in an amino-terminal region of apoE, thus facilitating the hepatic uptake of lipoproteins and maintaining plasma cholesterol homeostasis [19].

ApoE has numerous antiatherogenic roles, predominantly due to its actions on the lipid metabolism pathway (for an in-depth review the reader is directed to [19]). Briefly, it is involved in the hepatic uptake and subsequent degradation of lipoproteins through the LDL-R on parenchymal liver cells, clearance of chylomicron remnants through the action of LDL-R related proteins, the stimulation of hepatic VLDL and TG production, reverse cholesterol transport (the process whereby excess cholesterol is transported via HDL to the liver for excretion in bile) and activation of enzymes, such as hepatic lipase and cholesteryl ester transfer protein, involved in lipoprotein metabolism. ApoE also has antiatherogenic effects not involved directly in lipid metabolism which include inhibition of LDL oxidation [20], platelet aggregation [21], smooth muscle cell proliferation [22], endothelial cell proliferation [23], and inhibition of T-lymphocyte activation and proliferation [24, 25].

5. ApoE Knockout Mouse Model

In humans, decreased expression of apoE caused by some types of genetic disease (Type III hyperlipoproteinaemia associated with familial apolipoprotein E deficiency) leads to an altered lipid profile and an increase in the prevalence of atherosclerosis in these patients [26, 27]. In 1992, an apoE knockout (apoE$^{-/-}$) mouse model of spontaneous atherosclerosis was developed [28] that would enable researchers to track the progression of the disease over a manageable time scale. Crucially there was no effect on fertility or birth weight when compared to normal mice, and they appeared to be healthy. In the study of atherosclerosis, the haemodynamics of the cardiovascular system are important as they have implications for such parameters as the shear stress of the blood flowing over the arterial wall, as regions of lower shear stress are thought to induce lesion development [29–32]. When compared against C57BL/6 wild-type mice, apoE knockout mice have a normal heart rate and blood pressure, but they have been shown to have elevated pulse wave velocity, elevated aortic and mitral flow velocity, alterations in aortic acceleration suggestive of increased pulse wave reflection, decreased haematocrit, and increased heart-to-body weight ratio (decreased body weight, cardiac hypertrophy) [33]. Homozygous apoE knockout mice fed a chow diet have been shown to have plasma cholesterol levels, at 6 weeks of age, that are approximately five times greater than those in normal wild-type mice. However, heterozygous knockouts have cholesterol levels similar to wild-type mice [34].

6. Plaque Vulnerability

6.1. Detection and Quantification of Plaque Rupture. Atherosclerotic plaques can be divided into two distinct types; those that rupture (vulnerable, or unstable, phenotypes) and those that do not (stable phenotypes). Based on retrospective human autopsy studies comparing intact and disrupted plaques, it has been reported that vulnerable plaques are characterised by a large lipid-rich core occupying more than 40% of the plaque's total volume (although 50% has been suggested elsewhere [35]), covered by a thin fibrous cap (<100 μm thick), with extensive macrophage infiltration but very few smooth muscle cells [36].

Human studies have shown that ruptured and vulnerable plaques have the following pathologic features (summarised in [37]):

(1) positive (i.e., outward) remodeling,

(2) a fibrous cap less than 100 μm (and perhaps less than 65 μm) at its minimum thickness,

(3) macrophage infiltration (especially in the thin fibrous cap).

(4) a large lipid/necrotic core often containing haemorrhage and/or calcification,

(5) speckled or diffuse calcification,

(6) abundant intraplaque *vasa vasorum* and/or haemorrhage.

A plaque rupture can be defined as a disruption of the fibrous cap accompanied by intrusion of erythrocytes into the plaque itself [12]. This definition enables ruptures to be distinguished from artefactual damage during histological processing. This should not be confused with plaque erosion,

which is defined as loss of endothelium, leading to thrombus formation, without any associated fissure or rupture [38].

From a very young age, lipid permeates into the arterial wall forming what is known as a fatty streak, made up of T-lymphocytes and lipid-rich macrophages. Clinically silent fatty streaks are present in most people, even without the influence of external risk factors, and are thought to be a protective response to potential damage to the endothelium and underlying smooth muscle cells [39]. These early lesions precede the development of intermediate lesions which are made up of further layers of macrophages and smooth muscle cells, which themselves go on to become complex lesions called fibrous plaques [39]. It is these which can go on to rupture with potentially serious consequences. It has been reported that most acute coronary events are precipitated by rupture of an atherosclerotic plaque [40]. However, most plaque ruptures do not result in any symptoms [11]. One possibility for this unusual phenomenon is that plaque ruptures can heal, and the increased lesion size can be accommodated by vessel remodelling [41].

7. Mouse Model of Spontaneous Plaque Rupture

Up until 2001 there were no models of spontaneous plaque rupture in animals. Previous models relied on artificially inducing the plaques to rupture by acute insult. An early example is the cholesterol-fed rabbit injected parenterally with Russell viper venom and histamine, which leads to acute plaque disruption in ~30% of animals [42]. Another approach involves balloon catheter-injured cholesterol-fed rabbits with an implanted balloon catheter in the thoracic aorta [43]. The balloon is left in place for at least 1 month so an atherosclerotic lesion can develop around it, before being inflated thus disrupting the atherosclerotic lesion and precipitating thrombosis. A third model made use of the apoE knockout mouse and involved compression injury using blunt forceps applied to atheromatous lesions in the abdominal aorta [44]. This technique resulted in a third of the animals showing evidence of intraplaque haemorrhage within disrupted plaques and plaque-associated luminal thrombosis. A similar technique was used by Bentzon et al. [45] whereby a microsurgical needle was inserted into the luminal surface of advanced plaques, leading to cap disruption. A further approach has been to use apoE knockout mice with atheromatous lesions induced by an externally placed silastic collar [46], and then to transfect these with an adenovirus expressing the tumour-suppressor protein p53 [47]. At one day posttransfection, increased apoptosis is evident in the cells of the fibrous cap and cap thinning is seen at later time points. These attenuated fibrous caps undergo rupture in 40% of animals after pressor challenge with phenylephrine [47]. Whilst these models are extremely valuable as tools for studying events after rupture, they do not enable us to observe the events leading up to, and triggering, a spontaneous rupture (smooth muscle cell apoptosis, macrophage accumulation, lipid accumulation and loss of extracellular matrix) and are more a model of postrupture thrombosis.

The brachiocephalic, or innominate, artery is a very short, narrow vessel (~2 mm long with a diameter of ~ 0.5 mm in mice) emanating from the top of the aortic arch and branching into the right subclavian and right common carotid arteries. Importantly, it reliably develops complex plaques. Studies of apoE knockout mice aged 42–54 weeks fed a normal chow diet showed a high frequency of intraplaque haemorrhage and fibrotic conversion of necrotic zones and loss of fibrous cap tissue [48]. Although fibrous cap thinning was observed, neither plaque rupture nor thrombosis was identified. The findings of an acellular necrotic core, intraplaque haemorrhage, and erosion of core through to lumen suggest a resemblance to plaque erosion with thrombosis.

The elusive spontaneous plaque rupture was finally observed having fed apoE knockout mice a high-fat diet containing 21% pork lard and 0.15% cholesterol for up to 14 months [49]. Plaque ruptures were found to occur in the brachiocephalic artery. Ruptures occur in the proximal $150\,\mu m$ of the vessel, so there is tight anatomical localisation of the phenomenon making comparison of data across studies straightforward [41]. Observations of serial sections of the brachiocephalic artery suggest that plaque ruptures are rarely more than $60\,\mu m$ in length [50], whereas in human coronary arteries the average tear length is 1.9 mm [51]. Lesions that rupture in the brachiocephalic artery had several common features across mice [49]. They were relatively small, lipid-rich, and globular and were overlying large advanced lesions. The ruptured lesions had intraplaque haemorrhage as evidenced by the presence of erythrocytes and were associated with luminal thrombus formation.

8. Mice versus Humans

Most human atherosclerosis is the result of a lifetime of multiple risk factors such as tobacco smoking, elevated cholesterol levels, elevated blood pressure, unhealthy diet, age and genetic susceptibility. It is not possible to replicate all of these in a mouse model of atherosclerosis, but does that mean that these models should be ignored?

Atherosclerotic lesions do not occur randomly in the human vasculature but are localised at bends, bifurcations, and T-junctions [31]; these are all regions with altered wall shear stress (the frictional force of the blood flowing across the endothelium). An important feature of atherosclerosis is that it occurs in systemic arteries, but never in veins or pulmonary arteries, suggesting that local haemodynamic factors, wall properties, and pressure play a critical role in lesion formation. Mice develop lesions in a similar manner, suggesting that the arterial wall is affected in a similar manner despite a 20-fold difference in the actual wall shear stress between species [52, 53].

Lesions in mice are located at regions of low or oscillatory shear stress [54] and in particular in the aortic root, brachiocephalic artery, lesser curvature of the aortic arch, and the branch points of the left common carotid and left subclavian arteries, and to a lesser extent in the descending thoracic aorta. Recent research has shown that the geometry of the aortic arch has an effect on where lesions will be

located in mice [55, 56]. Differences in aortic arch geometry are not just a feature of mice and occur in humans as well [57]. The geometry of blood vessels, and in particular the angle that a daughter vessel branches from the parent, has been linked to altered intimal and medial thickness [58].

Perhaps understandably, the mouse as a model of atherosclerotic plaque rupture has been questioned as to how well it replicates the disease in humans due to differences in geometry. If we look at basic physiological properties, there are obvious differences. Mice are about 3000 times smaller than humans. In mammals, cardiac mass varies in direct proportion to body mass (so-called "isometric" scaling) [59] leading to a ventricular stroke volume that also scales isometrically [60]. The heart rate has been shown to scale allometrically and follows a quarter power law (heart beat frequency $\propto$ body mass to the power -0.25) [61]. On top of this, wall shear stresses have been predicted to be 20-fold higher in mice than humans [53]. Whilst human coronary arteries are typically 2.4 mm in radius with a wall thickness of 0.76 mm [62], the mouse brachiocephalic artery is $\sim$0.36 mm in radius with a wall thickness of 0.04 mm [12]. This therefore leads to very different tensile forces on the cap.

Superimposed thrombosis is rarely seen in apoE knockout mice [63]. However, this is not a reason to rule out the mouse as a good plaque rupture model. The lack of thrombus could simply be a consequence of pressure perfusing the mouse vessel before removal of tissue. This could cause any thrombus present to be washed away, preventing it from being seen in subsequent analysis. Perhaps a more likely explanation is that thrombi are cleared very rapidly by the fibrinolytic system in mice. Plasma levels of plasminogen activator inhibitor (PAI-1) are from 5- to 12.5-fold lower in mice than humans, whereas fibrinogen and tissue-type plasminogen activator (tPA) concentrations are similar [64]. This suggests that the fibrinolytic balance in mice is shifted more towards enhanced lysis. The volume and surface area of a thrombus over a human plaque would be roughly 200-fold and 30-fold greater, respectively, than in a mouse. Some human coronary thrombi may be present for months [65], so even if we conservatively assume equal rates of fibrinolysis then mouse thrombi will be resolved within a few days. This suggests that there is only a relatively small time window after a mouse plaque rupture during which a thrombus will be present. Despite superimposed thrombosis rarely being seen in apoE knockout mice, intraplaque haemorrhage is seen in the majority of plaque ruptures [49]. In human coronary plaques that rupture, there are a greater number of neovessels (vasa vasorum) running through the plaque that may lead to intraplaque haemorrhage [66], and this has also been seen in mice [67]. However, haemorrhage has also been observed in plaques without vasa vasorum, including plaques in the brachiocephalic artery, suggesting that luminal blood must have entered via a surface defect [68].

It was previously assumed that smooth muscle cells found in plaques were derived from local medial or intimal smooth muscle cells within the vessel wall. However, a study looking at postmortem human tissue found that $\sim$20% of smooth muscle cells originated from bone marrow (which had been transplanted for haematological disease), suggesting that plaques get some of their smooth muscle cells from the circulating blood [69]. In mice, the original theory appears to stand, in that tracking marker-labelled smooth muscle cells were not found in the plaques, only smooth muscle cells from the local wall [70]. It is currently unclear whether these are true differences between species or whether there are simply methodological differences which could lead to the different outcomes.

Should these differences stop us using the mouse as a model of atherosclerosis? Perhaps somewhat surprisingly there are also important similarities between mice and men. Murine systolic and diastolic blood pressures (125 and 90 mmHg, resp.) are similar to those found in the human coronary arteries [71]. Doppler ultrasound studies show that the average peak aortic root blood velocities are 1.04 m/s in mice [72] and 1.03 m/s in humans [73]. The location of atherosclerotic lesions appears fairly uniform across all species, implying a role of haemodynamic properties, and in particular implicating regions of lower shear stress resulting in increased lipid deposition [29–32].

9. Vessel Remodelling

It is widely accepted that arterial dimensions change throughout life, even in the absence of disease, as part of a homeostatic system that maintains a constant flow of blood through the vessels. In the presence of atherosclerotic lesions, it has been observed that radial enlargement of vessels (outward remodelling) can compensate for the obstructive bulk of the lesion, thus reducing the degree of flow-reducing stenosis in the vessel [74]. However, in some cases the lumen cross-sectional area can be decreased without an increase in lesion size (inward remodelling), suggesting that the arterial wall actually shrinks, increasing the degree of stenosis [75, 76].

The degree of stenosis caused by a plaque is an important factor when looking at the mechanics of plaque rupture, in part shown by Poiseuille's equations and Laplace's law. There are three main factors that determine resistance to blood flow within a vessel: blood viscosity, vessel length, and most importantly physiologically, vessel diameter (or radius). Poiseuille found that the resistance to flow between two points is inversely proportional to the tube radius raised to the fourth power, r^4 [77] showing that small decreases in lumen diameter caused by, for example, an atherosclerotic plaque, will greatly increase the resistance and frictional forces of the blood flowing over the plaque. However, using Laplace's law, it can be shown that for two vessels each with fibrous caps of the same tensile strength, caps covering mildly or moderately stenotic plaques compared to more severe stenotic plaques, the former will be exposed to a greater circumferential strain and as such be more prone to rupture [78]. Laplace's law shows the relationship between circumferential wall stress, the radial wall stress, the radius of the vessel, and the thickness of the wall and suggests that high circumferential stress can develop in thin fibrous caps, possibly causing the mechanical failure of the plaque [79].

A recent study using human computationally simulated cross-sectional plaque morphologies (acquired by IVUS), mimicking different stages and variations in atherosclerotic lesion growth, looked at the effects of anatomical plaque features on peak cap stress, a known predictor of rupture in human lesions [80]. It was found that at the early stages of positive remodelling, lesions were more prone to rupture and that, in addition to fibrous cap thickness, necrotic core thickness rather than area, was critical in determining plaque stability.

A study by Jackson [41] observing lesions in the brachiocephalic arteries of apoE knockout mice fed high-fat diet for up to a year showed that an increase in plaque area leads to an increase in vessel area, as the vessels remodel to accommodate the increased plaque burden (the vessels expansively remodel), as is the widely held view. Strain-matched, wild-type mice fed the same high-fat diet did not develop atherosclerosis and did not exhibit the same expansive remodelling showing that this is not simply a phenomenon associated with ageing and growth. The plaques were subsequently analysed based on their vulnerability (stable versus unstable) and a different story emerged. Stable plaques increased in area, but the vessel area remained the same, leading to a decreased lumen size. Unstable plaques also increased in area at the same rate as in the stable lesions, but interestingly the vessel area increased and the vessel expansively remodelled. Therefore, plaque growth itself cannot be causing the increased rate of vessel expansion in the brachiocephalic artery, supporting the finding that there is vessel expansion even in the absence of plaque (there were no differences in vessel expansion between male and female mice) [41]. The question is whether plaque rupture is causing expansive remodelling, or whether expansive remodelling is causing the rupture. The finding that even in the absence of plaques remodelling still occurs supports the latter explanation. When the vessel expansively remodels, the fibrous caps of plaques are placed under tension and when this force overcomes the cap strength a rupture ensues. The actual rupture event, if it were not for the potentially detrimental thrombotic consequences, could be seen as beneficial to the patient as it allows the lumen to be restored and maintained, and this could explain why plaque rupture can be asymptomatic.

Further evidence for this controversial hypothesis can be found in studies using HMG-CoA reductase inhibitors. Statins are used to treat humans as they have been shown to lower plasma cholesterol levels and cause regression of atheroma [81]. Their effects include improving endothelial function, modulating inflammatory responses, preventing thrombus formation, and improving plaque stability [82]. This potential effect on plaque stability in humans led to statins being investigated in mice. High-fat diet-fed mice treated with pravastatin showed a reduced incidence of plaque rupture coinciding with a 5-fold increase in fibrous cap thickness. However, it did not influence overall rates of vessel remodelling, but significantly increased the amount of vessel expansion and the time between plaque ruptures [41].

10. Where Does the ApoE Knockout Mouse Model of Plaque Rupture Stand Today?

There has been some resistance to the notion that mice suffer spontaneous rupture of atherosclerotic plaques. This has been articulated in press primarily by a small group of dissenting scientists [68, 83]. Their criticisms centre on the following points.

(1) Definitions of plaque rupture as used in clinical histopathology are not satisfied by murine plaque ruptures.

(2) Murine plaque ruptures could be the result of damage during postmortem tissue processing.

(3) The structures described as healed plaque ruptures in mice are in fact nothing more than layering of the plaque resulting from episodic growth.

Let us dispose of these one by one—and, we hope, this time once and for all. They have already been carefully considered and disproved [50, 84], but it appears that the message is failing to be appreciated and the standard view of mouse models of spontaneous plaque rupture is that they are controversial. They are not controversial at all. Here is why.

(1) Definitions of plaque rupture as used in clinical histopathology are not satisfied by murine plaque ruptures.
The issue of definition is a classic example of "top-down" thinking, which proposes that mouse plaque ruptures cannot be useful unless they look just like human plaque ruptures. One key part of this was the insistence for a long time that mouse plaque ruptures had to have an overlying thrombus to even be considered as genuine ruptures. We have pointed out many times how confused this idea is. Thrombosis is the secondary consequence of rupture, not the rupture itself, so there is no *a priori* reason why it should be faithfully modelled; furthermore, murine thrombi are tiny but their fibrinolytic systems are very effective, so thrombi may form but be lysed before the vessel is harvested. We note that in studies by the authors of these dissenting articles that in their own studies of disrupted murine plaques, they do not see persistent thrombosis [45]. Perhaps this is why the formerly inflexible requirement for thrombosis over disrupted murine plaques has now been dropped [68]. This is the correct position, because overlying thrombus is not a cardinal feature of murine plaque rupture.

(2) Murine plaque ruptures could be the result of damage during postmortem tissue processing.
This is a testable hypothesis because it leads to predictions that can be addressed experimentally. The first prediction is that defects in the fibrous cap will not be accompanied by ingress of blood cells into the plaque. The operating definition of acute

murine plaque rupture that we have always used is "a visible defect in the cap accompanied by intrusion of erythrocytes into the plaque below it" [12]. We have reported on the presence of ruptures that fulfil this definition in the proximal brachiocephalic arteries of male apoE knockout mice from 5 weeks of fat-feeding onwards [85]. After 8 weeks of fat-feeding, 62% of 173 animals exhibited acute plaque rupture in the brachiocephalic artery. Therefore, the hypothesis that handling artifacts may be misinterpreted as plaque disruptions occurring during life can be shown not to hold true, as dozens of ruptures have been shown to be accompanied by the insudation of formed elements of blood, which can only happen during life. A second prediction is that the rate of incidence of defects in the fibrous cap will be similar at all time points once plaques have developed, because handling injuries are not related to the stage of development of the plaque. As described above, published data show that the incidence of defects in the fibrous cap varies markedly and significantly at different time points. For example, the incidence falls from 62% to 30% within the course of a week ($P = .0005$). Therefore, this prediction also fails to hold true: defects in the fibrous cap occur at a rate that is different at different time points. The third prediction based on the handling artifact hypothesis is that the rate of incidence of defects in the fibrous cap will be independent of drug treatment, because treatments administered to mice during life cannot influence the infliction of postmortem handling damage on plaques. However, the incidence of acute plaque rupture is statistically very highly significantly reduced by treatment with pravastatin: after 40 weeks of high-fat feeding, continuous treatment with pravastatin reduced the incidence of acute plaque rupture by 86% ($P < .0001$) [85]. Even when treatment was delayed until 16 weeks of high-fat feeding had already elapsed, acute plaque ruptures occurred 56% less frequently ($P < .0001$). It is particularly notable that the latter effect was achieved in the absence of any significant effect on plaque size (-6%). Therefore, this third prediction also fails, because defects in the fibrous cap occur at a rate that can be influenced by pharmacological treatment and this can be achieved independently of any effect on plaque size.

(3) The structures described as healed plaque ruptures in mice are in fact nothing more than layering of the plaque resulting from episodic growth.

This is another testable hypothesis. The first prediction is that buried fibrous caps should occur with equal frequency at all anatomical sites that develop sufficiently large plaques. This is because a manifestation of standard plaque expansion should not be distributed in any anatomically dependent fashion and should not be related to the presence or absence of acute plaque rupture at that site. Acute plaque ruptures are not observed, in our very long experience, in the retrovalvular lesions of the apoE knockout mouse aortic sinus. However, these plaques are very large—about twice the size of the plaques in the nearby proximal brachiocephalic artery after 9 months of fat-feeding. The incidence of buried fibrous caps in plaques in the aortic sinus in a series of 28 fat-fed apoE knockout mice was zero. In the brachiocephalic artery plaques of the same 28 mice, there were 35 such structures ($P < .0001$). Therefore, the incidence of buried fibrous layers is significantly related to the occurrence of acute plaque rupture but not to plaque size, and the prediction is not borne out by the available data. The second prediction is that the presence of fibrin in the plaque should be independent of the presence of a buried fibrous cap, because fibrin would be a sign of thrombosis whereas buried fibrous caps are hypothesised to be part of normal plaque development. We have shown that immunoreactive fibrin is present at the sites of buried fibrous caps in apoE knockout mouse brachiocephalic arteries, using a goat polyclonal antibody that is not reactive with fibrinogen. In a series of 50 male apoE knockout mice that had been fat-fed for 8 weeks, the presence of fibrin (as assessed by bright red staining with Masson's trichrome) was significantly associated with the presence of a buried fibrous cap. Of the 50 mice, 23 had buried fibrous caps at this site, and 19 of these 23 plaques were positive for fibrin. Amongst the 27 mice with no buried fibrous caps, 4 were stained positively for fibrin ($P = .000001$). We conclude that fibrin accumulation at sites of buried fibrous caps is verified by specific antibody staining, and by tinctorial criteria it is statistically very highly significantly associated with the presence of buried fibrous caps. This means that the second prediction is also contradicted by the data. The third prediction that flows from the hypothesis that buried fibrous caps are part of normal plaque development in mice is that their incidence should covary with plaque size. Studies in apoE knockout animals treated with pravastatin or with an additional null mutation to the cathepsin S gene [86] show that plaque size and the number of buried fibrous caps can be modulated independently. When pravastatin treatment commenced after advanced plaques had already developed, the formation of buried fibrous caps was reduced by 36% ($P < .0001$) but there was no significant effect on plaque size (-6%). In the case of cathepsin S, the incidence of buried cap formation normalised to plaque size was reduced by 37% ($P = .044$) in the double knockouts. Thus the incidence of buried fibrous caps can be modulated independently of any effect on plaque size, and the data are once again at variance with the prediction.

Thus the objections to a mouse model of atherosclerotic plaque rupture have all been tested and can be discarded with extraordinarily high levels of statistical confidence.

11. Conclusion

The fat-fed apoE knockout mouse proximal brachiocephalic artery model is an excellent test-bed for potential therapies for plaque rupture and should yield useful insights into the pathophysiology of this phenomenon for many years to come.

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