Assessment of Unstable Atherosclerosis in Mice

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Abstract—There is an urgent need for representative animal models where prospective examination of the events leading up to plaque rupture and the rupture process itself can be performed. Recently, reports have begun to emerge that apolipoprotein E and low density lipoprotein receptor knockout mice may spontaneously develop unstable atherosclerosis, with plaques in certain parts of the arterial tree showing features suggestive of plaque rupture. Here we discuss the problems inherent in applying definitions of plaque rupture as seen in human arteries to mice; the anatomic locations in mice where unstable plaques do and do not occur; methods of inducing plaque instability in mice; and how to assess plaque stability in mice. These considerations lead us to a number of general recommendations. (Arterioscler Thromb Vasc Biol. 2007;27:714-720.)

Key Words: plaque rupture ■ animal models ■ apoE knockout mouse ■ vascular histopathology

It is generally accepted that the rupture of an atherosclerotic plaque, with ensuing clot formation, underlies most cases of myocardial infarction. This conclusion is based on extensive studies of postmortem human material. Although these studies are important in identifying histological features of clinical syndromes, they have some limitations:

1. Post-symptom selection of patients may bias toward particular underlying mechanisms.
2. The time span between symptom onset and tissue analysis will be accompanied by changes in tissue composition.
3. Within-subject temporal histopathologic analysis is impossible.
4. Retrospective interpretation cannot be backed up by experimental verification.

Clearly, although valuable in setting end points of disease development and as a reference for validating experimental data, this retrospective strategy has some flaws when studying the pathophysiology of the disease. Consequently, there is an urgent need for representative animal models where prospective examination of the events leading up to plaque rupture and the rupture process itself can be performed.

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Mice do not normally develop atherosclerosis, but can be induced to do so by feeding a diet high in fat. Early studies involved diets containing as much as 50% fat, with the particular susceptibility of the aortic sinus as a noted feature. Paigen and colleagues built on these foundations, establishing a well-tolerated atherogenic high-fat diet and also defining a protocol for standard assessment of lesion severity at the aortic sinus. A major advance in the use of mice for the study of atherosclerosis came with the advent of genetically-modified animals. Mice with targeted deletion of the gene for apolipoprotein E (apoE) or the low-density lipoprotein (LDL) receptor spontaneously develop atherosclerotic lesions at many sites in the arterial tree. In both cases, the situation is exacerbated by feeding a high-fat diet.

More recently, reports have begun to emerge that apoE and LDL receptor knockout mice may spontaneously develop unstable atherosclerosis, with plaques in certain parts of the arterial tree showing features suggestive of plaque rupture.

Defining Plaque Rupture

Plaque rupture in humans has been defined as “Fibroatheroma with cap disruption; luminal thrombus communicates with the underlying necrotic core”. This definition is unusual in that, to be fulfilled, a particular consequence of plaque rupture must occur—namely thrombosis. Does this mean that plaque rupture that does not result in luminal thrombosis is not really a plaque rupture? Such an event would be mechanistically indistinguishable from a plaque rupture that does result in luminal thrombosis, and the plaque structure itself showing a break in the fibrous cap would be identical. In addition, even in human studies, plaque rupture has been identified without thrombus formation: for example, where the presence of old ruptures that have repaired is inferred from buried fibrous caps and layering. Separating the consequences of plaque rupture

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from its genesis in this way is of particular concern when we turn our attention to the mouse.

If luminal thrombus is retained as a key diagnostic feature of plaque rupture, animal models of plaque rupture would have to mirror not just the pathophysiological mechanisms of rupture but also display human-like thrombosis. Is such a restrictive approach justified? We believe that, particularly in the mouse, the requirement for luminal thrombus is incorrect. Murine brachiocephalic arteries are approximately 500 μm in diameter whereas human coronary arteries are about 3.5 mm in diameter in their proximal regions, where most plaque ruptures occur. This means that the cross-sectional area of a fully occlusive thrombus is about 50-fold smaller in mice than humans. Furthermore, occlusive thrombi ramify for several millimetres in human coronary arteries but the entire length of a mouse brachiocephalic artery is only about 2 mm, so the volume of even a large thrombus in the mouse is likely to be at least 200-fold less than in humans and its surface area will be about 30-fold less. The fibrinolytic system in mice differs significantly from that in humans, as the plasma level of plasminogen activator inhibitor (PAI)-1 is 5- to 12.5-fold lower in mice than in humans, whereas fibrinogen and tissue-type plasminogen activator (tPA) concentrations are similar. PAI-1 is the major determinant of the rate of lysis of platelet-rich arterial thrombi by pharmacological concentrations of tPA. Furthermore, plasma levels of thrombin-activatable fibrinolysis inhibitor (TAFI) in the mouse are 2- to 7-fold lower than in humans. Activated TAFI can impair fibrinolysis by removing carboxy-terminal lysines from fibrin, which act as binding sites for plasminogen and tPA. Thus, the fibrinolytic balance in mice appears to be shifted toward enhanced lysis. Some human coronary thrombi may persist for months, but even if we assume equal rates of fibrinolysis then mouse plaques will be gone within a few days. If we further assume that the interval between episodes of plaque rupture in mice is of the order of weeks, then the chance of terminating an animal during the period when the thrombus is still present may be as little as 5%, even if luminal thrombus formation is an invariable consequence of murine plaque rupture. It is therefore clear that the presence of luminal thrombosis should not be regarded at present as a defining characteristic for plaque rupture in mice.

As an extension of this assertion, it is also illogical to require evidence of downstream ischemia or infarction to define plaque rupture in mice. Plaque rupture is frequently silent in humans. Ischemia and infarction relate to the size of the clot and the plaque in relation to the size of the vessel, the anatomic site of atherosclerosis, and the extent of collateral circulation. The anatomic location of plaques in mice is very different from that in humans, and in the absence of luminal occlusive thrombosis the lack of end organ infarction in mice is not surprising.

Furthermore, the tear in the fibrous cap will also be much smaller in mice. Our observations of serial sections of ruptured plaques in the mouse brachiocephalic artery suggest that these defects are rarely more than 60 μm in length (an example is shown in Figure 1), whereas in human coronary arteries the average length of a “fracture” in the plaque is 1.9 mm. Given similar rates of healing, the defect in the human fibrous cap will be detectable for 30 times as long.

The foregoing makes it clear that it is not reasonable to apply existing clinical definitions of plaque rupture to mice, simply because their vessels are so much smaller than those in humans. So how can we find, recognize, and quantify plaque rupture in mice?

Anatomic Location of Atherosclerosis in Genetically-Modified Mice

The occurrence and histological appearance of atherosclerosis in apoE and LDL receptor knockout mice has been extensively reviewed elsewhere and we do not intend to recapitulate this work here. However, it is worth pointing out...
that, as in humans, atherosclerosis is a focal disease in these mice, with certain sites of predilection. These include the aortic sinus and the brachiocephalic artery, also known as the innominate artery. In a seminal paper from Russell Ross’s laboratory in 1994,7 the distribution of lesions in fat-fed apoE knockout mice was mapped and the brachiocephalic artery was identified, though no special mention was made of it. The first communication to draw close attention to the brachiocephalic artery came from Rosenfeld et al.9 Using chow-fed animals about one year old, they showed intra-plaque accumulations of material that stained red with Movat’s pentachrome which they identified as intraplaque hemorrhage. During the ensuing 6 years there have been 19 more publications that make mention of the murine brachiocephalic artery,23–33 but 10-fold more that have involved investigation only of the aortic sinus. What are the particular advantages and disadvantages of these two sites?

The aortic sinus is relatively straightforward to locate for histological processing, and in consequence standardization across studies and laboratories is easy. The lesions that eventually develop at this site in apoE knockout mice are large and lipid-rich, meaning that a simple lipid stain such as oil red O can be used to aid morphometric analysis. On the other hand, the lesions remain as fatty streaks for an extended period, and it is months before a fibrous cap can be discerned. There are no reports of intraplaque hemorrhage or any other signs of plaque disruption at the aortic sinus, even after extended periods of fat-feeding in apoE knockout mice.33 This calls into question the use of the aortic sinus for investigations of plaque rupture. There are many reports of compositional changes in sinus lesions that, if observed at a site where plaques do rupture, would be predicted to be associated with a reduced frequency of rupture. It is not clear though that changes in sinus lesion phenotype can properly be interpreted this way. Indeed, if all investigations of murine plaque instability had been carried out at the sinus, we would be forced to conclude that there are actually no phenotypic markers of vulnerability, which is clearly incorrect. We have to accept that there may be special circumstances—relating perhaps to blood flow or the mechanical properties of the retrovalvular vessel wall—that protect the sinus from rupture, but may still allow the assessment of surrogate parameters of instability. Nevertheless, in studies of plaque vulnerability, the best use of the sinus is as a nonvulnerable comparator site for other parts of the murine arterial tree where ruptures do occur.

The brachiocephalic artery is a site where ruptured plaques have been reported to occur in the apoE knockout mouse.10,24,26,28,33 Lesions develop rapidly at this site, especially under conditions of high-fat-feeding, when advanced plaques are present after as little as 5 weeks.33 Intraplaque hemorrhage is a frequent finding.9 On the negative side, this vessel is very small and consequently difficult to process for histology. This has led to suggestions that so-called plaque disruptions are actually artifacts introduced during postmortem tissue processing.42 The counter-argument is that because formed blood elements, such as erythrocytes, will not be forced into the body of the plaque even with major mechanical trauma, definitions of plaque rupture in mice that require

the presence of blood elements in the plaque are secure. Indeed, we would advocate defining plaque rupture in mice as “a visible defect in the cap . . . accompanied by intrusion of erythrocytes into the plaque below it”.33

**Induced Vulnerable Plaques in Mice**

It is well known that anatomic predilection sites exist for atherosclerosis.44 Numerous studies have associated subtle changes in the velocity field, such as low shear stress, oscillating shear stress, and vortex generation, with atherogenesis at these predilection sites. This observation has been exploited by two independent groups to accelerate lesion formation in mice at a predefined site, the common carotid artery.44,45 von der Thüsen et al.44 generated a low shear region by placing a slightly constrictive silicon collar around this vessel in apoE or LDL receptor knockout mice. Placement of the collar reduced the expression of endothelial nitric oxide synthase and of the established shear sensor, Kruppel-like factor 2,46 and concomitantly increased that of intercellular adhesion molecule-1 (ICAM-1),44 culminating in the formation of complex fibroatheromatous lesions at this site within 6 weeks. Similarly, Cheng et al developed a pericardiotomy device that induced low shear stress proximal and oscillatory shear distal in a straight vascular segment. In apoE knockout mice fed a high-fat, cholesterol-enriched diet for 9 weeks, plaques developed reproducibly in both the low and oscillating shear stress regions.45

These findings are in accordance with studies of the predilection sites for plaque formation, as side branches are often associated with low shear regions and the aortic sinus is a location with shear stress oscillation caused by valve leaflet movement. The morphology of the plaques in the low shear stress region closely mimicked human thin cap fibroatheroma and in both models prolonged angiotensin II infusion induced intraplaque hemorrhage exclusively in plaques with phenotypic features associated with instability.

What is the value of these lesions? Their rapid ontogenesis and ready accessibility render these plaques fit for local luminal or perivascular manipulation and drug or gene administration. Indeed, plaque stability at this site was shown to be affected by focal overexpression of the tumor suppressor gene p53 leading to an increased incidence of intraplaque hemorrhage, fibrous cap erosion, and rupture,47 though the latter was seen to be a rather rare phenomenon. The particular advantage that these models offer is that vulnerable lesions develop at sites that are very accessible to local manipulation and instrumentation, unlike the brachiocephalic artery. However, for systemic intervention studies in plaque stability, the brachiocephalic artery is the preferred site of analysis.

Genetic manipulation of smooth muscle cells within the vessel wall inducing apoptosis is sufficient to induce multiple features of vulnerability in several vascular beds, including the aortic root and brachiocephalic artery. These latter features include thinning of the fibrous cap, expansion of the necrotic core, loss of collagen, and extracellular matrix, and widespread inflammation.48

**Assessing Plaque Rupture in Mice**

If it is true that there is rapid lysis of thrombi and healing of tears in the fibrous caps of mice, acute plaque ruptures of the
type usually shown to illustrate the phenomenon in humans would only occasionally be seen in mice. What is more, clinical pathologists often investigate plaques from patients that are known to have suffered an acute cardiac event, further increasing the likelihood that a ruptured culprit plaque will be seen. This situation does not obtain in mice. Nonetheless, acute plaque ruptures are sometimes seen in mice, and an example in a brachiocephalic artery plaque is shown in Figure 1. Our calculations suggest that it is more likely that a murine plaque will be interrogated after a rupture has occurred and the healing process has already started, than during the period when the acute rupture is still present. How can a previous, now healing or healed, plaque rupture be recognized?

Buried Fibrous Caps

We see laminar structures in the intima of advanced murine lesions that are rich in elastin and are populated by strongly α-smooth muscle actin-positive cells, taken to be smooth muscle cells, as shown in Figure 2. These appearances are highly suggestive of remnants of previous fibrous caps that have ruptured and have been incorporated into the growing lesion as it develops. When such buried fibrous caps are seen in humans, they are interpreted as indicative of previous, nonfatal, healed plaque rupture. A number of lines of evidence support a similar interpretation in mice.

The first involves consideration of the stability of plaques at the aortic sinus: at this site, where plaque ruptures (defined as a visible defect in the cap accompanied by intrusion of erythrocytes into the plaque below it) are not observed, buried fibrous caps do not occur. When aortic sinus lesions were examined in a series of 28 animals with ruptured plaques in the brachiocephalic artery, none had acute plaque ruptures or buried fibrous caps. We can assert with confidence that buried fibrous caps do not appear at the sinus, but do at a nearby site that is prone to plaque rupture. This refutes the argument that buried fibrous caps are normal in murine lesions unless we accept that aortic sinus lesions are abnormal structures that develop differently from all other murine plaques: we have seen acutely ruptured plaques and buried fibrous caps at other sites in the mouse, including the aortoiliac bifurcation and the left and right common carotid bifurcations. It is certainly possible that aortic sinus lesions are unrepresentative of murine lesions in general—although this would cast yet more doubt on the use of this anatomic site for studies of atherosclerosis—so what other lines of evidence can we adduce to support the link between acute plaque rupture and buried fibrous caps?

If buried fibrous caps are caused by the healing of a plaque rupture, they will be associated with fibrin (until it has been fully lysed). This would not be the case if the buried caps arose by another mechanism. The goat polyclonal antibody used to detect fibrin in these studies (a kind gift from Dr Douglas Thompson, Department of Pathology, University of Aberdeen, UK) was unreactive with mouse fibrinogen at any dilution, but incubation of the fibrinogen with thrombin to generate fibrin resulted in immunodetection at dilutions in excess of 1 in 500. The antibody also bound to clotted mouse blood, but was unresponsive to thrombin alone. We therefore conclude that this antibody specifically detects fibrin and, as can be seen in Figure 4 of Johnson et al., a clear association of fibrin and a buried cap was noted. In this figure, a plaque with a single necrotic core and a relatively thick fibrous cap is devoid of fibrin immunopositivity, but a plaque with two buried fibrous caps has clear immunofluorescent evidence of fibrin deposition in and around the most recent of them. These data provide further support for the idea that buried fibrous caps are signs of previous plaque ruptures.

The third line of evidence that suggests that buried fibrous caps represent healed plaque ruptures is that they usually come from intervention studies. Studies in apoE knockout animals treated with pravastatin, or with an additional null mutation to the cathepsin S gene, show that plaque size and plaque stability can be modulated independently. When pravastatin treatment commenced after advanced plaques had already developed, the formation of buried fibrous caps was significantly inhibited by 36% but there was no effect on plaque size (there was a nonsignificant 6% decrease). In the case of cathepsin S, the incidence of buried cap formation normalized to plaque size was significantly reduced by 72% in the double knockouts. These data are irreconcilable with the idea that buried fibrous caps are part of normal plaque growth.

In summary, buried fibrous caps form only at sites where plaque ruptures occur; are associated with fibrin deposition; and can be modulated independently of changes in plaque size. This suggests that buried fibrous caps either represent sites of previous plaque rupture in mice or occur in parallel with plaque rupture. It is not a tenable argument to suggest that buried fibrous caps arise as part of normal plaque development in mice, because this hypothesis cannot be reconciled with their patterns of occurrence, their significant association with thrombus remnants, and the fact that they can be modulated independently of plaque size.

Intraplaque Hemorrhage

In our definition of murine plaque rupture, we require to see formed blood elements within the plaque: “a visible defect in the fibrous cap... accompanied by intrusion of erythrocytes
into the plaque below it.33 This stipulation is made to exclude the possibility of postmortem damage being misinterpreted as plaque rupture. However, we must consider the possibility that intraplaque hemorrhage could occur in the same vessel as postmortem traumatic damage to the cap, leading to misidentification of a plaque rupture. Does intraplaque hemorrhage ever occur in mice in the absence of plaque rupture?

Intraplaque hemorrhage in the spontaneous atheromata of apoE knockout mice has been shown by two groups,9,10 and the phenomenon can also be provoked by some interventions.50–54 For intraplaque hemorrhage to occur in the absence of plaque disruption, we would have to postulate bleeding from intraplaque microvessels. The presence of such vessels is disputed: despite one report of their occurrence in the aortas of apoE/LDL receptor double knockout mice,55 we have not observed them in brachiocephalic arteries and there are no literature reports describing them at this site (though it must be acknowledged that this may simply reflect technical problems with detecting such vessels: absence of evidence is not evidence of absence). An important issue with intraplaque hemorrhagic masses is that they can ramify some distance within the plaque away from the site of rupture. Figure 1 shows serial sections of a mouse brachiocephalic artery taken at 30-μm intervals. The sections at 30, 60, and 90 μm contain what appear to be intraplaque hemorraghes, but the 120-μm section clearly reveals their origin in fact to be a plaque rupture. Figure 1 also nicely illustrates the way in which luminal thrombus can be completely lysed and washed away but accumulations of erythrocytes within the plaque are trapped and remain to bear witness to rupture. Regardless of their source, these extravasated erythrocytes may contribute independently to plaque instability as they promote oxidative stress and cholesterol accumulation.56,57

We must conclude that the jury is still out on the matter of intraplaque hemorrhage in the brachiocephalic arteries of mice. The safest course, when intraplaque hemorrhage is seen, is to make a careful survey of the vessel by multiple serial sectioning to exclude the possibility that there is a plaque rupture in the vicinity.

Indirect Indicators of Plaque Instability

Some phenotypic characteristics of plaques, such as collagen content, necrotic core size, smooth muscle cell:macrophage ratio, and fibrous cap thickness, have been widely used as indirect indicators of their stability. As discussed above, the relevance of these indirect indicators in the aortic sinus may be rather limited, as plaques at this site rarely, if ever, evolve into lesions with overt evidence of plaque rupture. When applied at sites of established unstable plaque formation such as the brachiocephalic artery or induced vulnerable plaques in the carotid artery, they may help to identify pathways underlying destabilisation. In addition, they may serve as early sensitive markers of end point disease alongside intraplaque hemorrhage and fibrous cap defects. If it can be shown that changes in surrogate parameters in the aortic sinus do correlate with similar changes at other anatomic sites such as the brachiocephalic artery, it will be possible to use indirect indicators in the aortic sinus with more confidence.

Modeling Plaque Rupture in Mice

There has been much debate about how closely mouse plaque ruptures resemble those seen in humans. Implicit in this has been the consideration that mouse models will not be useful unless there is good histopathologic correspondence with human ruptures, and that the same processes that cause plaque rupture in humans (assuming we know them) cause it in mice. There is a deep conceptual problem with this argument, however. This can be illustrated by means of a thought experiment. Let us imagine that we somehow know all of the processes that take place in a human plaque in the period leading up to rupture—their sequence, durations, and all other details—and can then apply them to a hitherto stable mouse plaque. What would be the histopathologic appearance of that mouse plaque once all the relevant processes had taken place? We do not know the answer to this question. Perhaps the mouse plaque would look normal; perhaps it would be utterly destroyed; perhaps it would look very much like a human ruptured plaque; perhaps it would have a small defect in the cap with ingress of some erythrocytes; perhaps small variations in the structure and physiology of the plaque before beginning all the rupture processes would result in considerable variations in the resultant histopathology. Regardless, because we do not know what the plaque would look like, we cannot use histopathology as an absolute criterion for judging the fitness of a mouse model. We believe that it is proper at present to be very careful not to discard mouse models for incorrect reasons, and simply to say that if the mouse has a plaque with a defect in its cap and-ingress of erythrocytes then it might provide useful evidence about the processes of rupture in humans. A model that does generate persistent thrombus at the site of rupture is still awaited, though one option may be to inhibit fibrinolytic activity in existing mouse models of plaque rupture.

General Conclusions

1. The current clinical definition of plaque rupture, “Fibroatheroma with cap disruption; luminal thrombus communicates with the underlying necrotic core”,12 is flawed in terms of its application to nonhuman lesions because it includes induced thrombosis as a diagnostic feature. Indeed, the requirement for luminal thrombus is ignored when defining old ruptures in humans. The consequences of plaque rupture are of course vitally important, but should not be regarded as integral to the pathophysiology of the generation of the unstable plaque.

2. This definition of plaque rupture should not be applied to murine lesions. The difference in size between humans and mice means that fibrous cap defects sufficient to induce luminal thrombus in mice would be trivial in the context of a human plaque, and plaque ruptures in mice are likely to heal very rapidly. Furthermore, the small thrombi that result from the rupture of a murine fibrous cap are likely to be lysed in a very short space of time. It is reasonable to require plaques to contain erythrocytes as part of the definition of murine plaque rupture as this avoids the inclusion of vessels damaged by postmortem mechanical trauma, though this probably means that murine acute plaque ruptures
are undercounted. Acute plaque rupture can accordingly be defined in mice as “a visible defect in the fibrous cap accompanied by intrusion of erythrocytes into the plaque below it.”

3. Buried fibrous caps in murine lesions may represent old plaque ruptures that have healed, as they do in humans. If so, the presence and numbers of these structures could be used as a surrogate marker of plaque rupture. These structures do not arise as part of normal plaque development.

4. Plaque rupture, defined as a visible defect in the fibrous cap accompanied by intrusion of erythrocytes into the plaque below it, occurs at several anatomic locations in apoE knockout mice but not at the aortic sinus. In studies of plaque vulnerability it makes more sense to use the aortic sinus as a stable comparator site for other locations within the same animal that generate genuinely unstable lesions.

5. The anatomic site in mice that is best characterized currently in terms of the production of unstable lesions is the proximal part of the brachiocephalic artery.

6. Mouse models of mouse plaque rupture exist, but we are not sure if there are mouse models of human plaque rupture. Such a model would have to mimic the processes that occur during the rupture of a human plaque, but we do not know what the histopathologic consequences of this set of processes would be in a mouse. Therefore it is not currently possible to reject mouse models of plaque rupture on the basis of histopathology alone.

Disclosures
None.

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