Characteristics of Intact and Ruptured Atherosclerotic Plaques in Brachiocephalic Arteries of Apolipoprotein E Knockout Mice

Helen Williams, Jason Lee Johnson, Kevin George Stephen Carson, Christopher Langdale Jackson

Abstract—The brachiocephalic arteries of fat-fed apolipoprotein E knockout mice develop plaques that frequently rupture and form luminal thromboses. The morphological characteristics of plaques without evidence of instability or with healed previous ruptures (intact) and vessels with acutely ruptured plaques (ruptured) have now been defined, to understand the process of plaque destabilization in more detail. Ninety-eight apolipoprotein E knockout mice were fed a diet supplemented with 21% lard and 0.15% cholesterol, for 5 to 59 weeks. Of these 98 mice, 51 had an acutely ruptured plaque in the brachiocephalic artery. Ruptured and intact plaques differed in terms of plaque cross-sectional area (intact, 0.109±0.016 mm²; ruptured, 0.192±0.009 mm²; P=0.0005), luminal occlusion (intact, 35.3±3.3%; ruptured, 57.7±1.9%; P<0.0001), the number of buried caps within the lesion (intact, 1.06±0.12; ruptured, 2.66±0.16; P<0.0001), fibrous cap thickness (intact, 4.7±0.6 μm; ruptured, 2.0±0.3 μm; P=0.0004), and lipid fractional volume (intact, 35.9±3.0%; ruptured, 50.7±2.2%; P=0.0019). This study confirms that plaque rupture is a frequent occurrence in the brachiocephalic arteries of apolipoprotein E knockout mice on a high-fat diet. The data also show that ruptured plaques in these mice show many of the characteristics of vulnerable plaques in humans. This supports the use of this model in studies of the mechanisms and therapy of plaque rupture. (Arterioscler Thromb Vasc Biol. 2002;22:788-792.)

Key Words: plaque ■ rupture ■ apolipoprotein E ■ mouse ■ knockout

Rupture of an atherosclerotic plaque is a primary cause of sudden cardiac death, accounting for 60% of sudden deaths with thrombosis.¹ Study of this phenomenon has, until recently, been hampered by the lack of an adequate animal model of spontaneous plaque destabilization.

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The apolipoprotein E (apoE) knockout mouse has become established as a model of hypercholesterolemia and atherosclerotic lesion development.²⁻⁵ After 6 months on a high-fat diet, advanced fibrous plaques are observed with thin luminal elastin and collagen fibers capping small lipid-rich globular lesions.⁵ Four studies have now reported that the lesions of apoE knockout mice can spontaneously become unstable.⁶⁻⁹ The brachiocephalic (also known as the innominate) artery, which is the first branch from the aortic arch, bifurcating to form the right common carotid artery and the right subclavian artery, is a site of predilection for the development of such vulnerable lesions. It provides a small and well-defined area in which to study the progressive loss of plaque stability and, eventually, rupture.

In a previous study,⁷ a small number of apoE knockout mice were examined. All of these had died suddenly, which meant that it was not possible to perfusion fix their arteries. In the current study, the development and morphology of brachiocephalic artery plaques have been characterized in a cohort of 98 apoE knockout mice. Ruptured and intact lesions have been compared both in animals dying suddenly and in those that were perfusion fixed after scheduled termination.

Methods

Animals

Homozygous apoE knockout mice¹⁰ were obtained from Charles River Laboratories (Manston, Kent, UK). The strain background of these animals was 50% C57BL/6, 50% 129SvJ, as determined by fingerprinting of tail-tip DNA. The housing and care of the animals and all the procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office.

Husbandry

Starting at 6 to 8 weeks of age, 98 apoE knockout mice (51 male) were fed a high-fat rodent diet containing 21% fat from lard and supplemented with 0.15% (wt/wt) cholesterol (Special Diets Services) for a period of 5 to 59 weeks. Cages were checked daily, and any animals that had suffered sudden death were immediately removed and processed for histological examination.

Termination

Animals were anesthetized by intraperitoneal injection of sodium pentobarbitone before exsanguination by arterial perfusion via the...
abdominal aorta with phosphate-buffered saline at a constant pressure of 100 mm Hg, with outflow through the incised jugular veins. This was followed by constant pressure perfusion with either 10% formalin or 4% paraformaldehyde and 10% sucrose, in phosphate-buffered saline.

**Histology**

Brachiocephalic arteries were embedded in paraffin or optimum cutting temperature compound (BDH Laboratory Supplies). Sections were cut at 3 µm for paraffin-embedded sections and 6 µm for optimum cutting temperature compound-embedded sections, every 30 µm along the vessel. Sections were stained with hematoxylin and eosin, or Miller’s elastin/van Gieson stain.

**Identification of Plaque Rupture**

Sections stained for elastin were inspected for the presence or absence of plaque rupture, which was defined as a disruption of the fibrous cap accompanied by intrusion of blood products into the plaque itself. Because intraplaque hemorrhage can only occur during life, this definition prevents misinterpretation of post mortem tissue lysis as plaque rupture.

**Plaque Characterization**

Brachiocephalic artery plaques were sampled at the site of acute plaque rupture, where one was present. In most cases, this was within the proximal 10% of the vessel. In vessels without acute ruptures (intact), samples were taken from the proximal 10% also. One vessel cross-section was quantified per mouse. Analysis was performed with a computerized image analysis program (Image Pro Plus, DataCell). The following parameters were measured: plaque cross-sectional area, media cross-sectional area, percentage occlusion of the lumen by plaque, the number of buried fibrous caps, current fibrous cap thickness, and percentage lipid content of the plaque.

**Statistical Analysis**

Mean values for ruptured plaques were compared with those for intact plaques using a t-test, after having performed an F test for homogeneity of variances. When variances were not significantly different, Student’s t test was used; when the variances were different, Welch’s t test was used. The incidence rates for plaque rupture were compared by using the Fisher exact test. Correlations were tested using Pearson’s parametric method. Significance was concluded when the two-tailed P value was less than 0.05. Values are expressed as mean±SEM.

**Results**

**Sudden Death**

Of the 98 mice, 64 died suddenly and 34 underwent scheduled termination. Figure 1 shows the distribution of sudden deaths over time, in the form of a survival curve. The average time of sudden death was 28.9±1.7 weeks of high-fat feeding. There was no difference in the average time of sudden death between animals with intact and animals with ruptured lesions (27.3±2.9 and 30.2±2.0 weeks, respectively, P=0.41).

Morphological characteristics of plaques were compared in the brachiocephalic arteries of animals that died suddenly or underwent scheduled termination. Perhaps surprisingly, there was no difference in the length of the internal elastic lamina (sudden death, 6854±197 µm; termination, 6615±276 µm; P=0.406); indeed, there were no significant differences in any parameter (data not shown), so data from animals that died and animals that were terminated were combined to analyze differences between ruptured and intact plaques.

**Figure 1.** Sudden death in apoE knockout mice. ApoE knockout mice were fed a high-fat diet (21% lard, 0.15% cholesterol) for up to 14 months. The graph is a survival curve for those animals that died suddenly. Of the 98 mice in the study, 64 died suddenly.

**Plaque Rupture**

After histological analysis, 51 of the 98 mice (52%) were found to have an acutely ruptured atherosclerotic plaque in the brachiocephalic artery. Examples of these ruptured lesions are shown in Figure 2. The incidence of rupture did not differ between animals that died suddenly (35/64) and those that had been terminated (16/34, P=0.53, Fisher exact test).

There was no gender difference in the incidence of plaque rupture: 28/51 males and 23/47 females were found to have ruptured plaques (P=0.69, Fisher exact test).

**Thrombus**

All animals that died suddenly had thrombotic material in the lumen that could not be removed by perfusion. The incidence of luminal thrombus in perfused animals with acute ruptures was 73% (11 of 15), but luminal thrombus was not seen in perfused animals that did not have acute ruptures.

**Plaque Characteristics**

Characteristics of ruptured and intact plaques are summarized in Table 1. The number of buried caps within the lesion was significantly higher in ruptured (2.66±0.16) than in intact lesions (1.96±0.12, P<0.0001). By assuming that each buried cap represents a previous rupture, the rupture frequency can be calculated; this was 0.41±0.04 ruptures/month in ruptured lesions, and 0.21±0.02 ruptures/month in intact lesions (P<0.0001). The fibrous cap was significantly thinner in ruptured lesions (2.0±0.3 µm) than in intact lesions (4.7±0.6 µm, P=0.0004). PLAQUES were significantly larger in ruptured plaques (0.192±0.009 mm²) than in intact plaques (0.109±0.016 mm², P=0.0005). The degree of luminal occlusion was significantly greater in ruptured plaques (57.7±1.9% compared with 35.3±3.3%, P<0.0001). All plaques examined featured a lipid core, but the fractional volume occupied by the core was significantly greater in lesions with ruptures than in those without (50.7±2.2% compared with 35.9±3.0%, P=0.0019).
There was no statistically significant difference between ruptured and intact plaques in terms of media cross-sectional area (ruptured, \(0.079\pm0.006\) mm\(^2\); intact, \(0.076\pm0.007\) mm\(^2\); \(P=0.73\)).

**Discussion**

The current study confirms an earlier finding, that apoE knockout mice fed a high-fat diet develop unstable plaques in the brachiocephalic artery that go on to rupture.\(^7\)

**Sudden Death**

The incidence of brachiocephalic artery plaque rupture did not differ between animals that died suddenly and those that were terminated at scheduled time points with perfusion fixation. This suggests that the occurrence of plaque rupture in this vessel is not the cause of sudden death, a conclusion supported by the finding that animals can survive multiple brachiocephalic plaque ruptures. The cause of sudden death in these mice is as yet unknown but could be related to plaque instability in the coronary and/or cerebral circulations. We have indeed seen myocardial infarcts in some animals (Figure 3), but this is not a consistent finding. It is possible that death is caused by arrhythmias resulting from focal ischemia around cardiac conduction pathways that would not show up on histological analysis.

The presence of ruptured plaques in animals that had been perfusion fixed shows that the disruption of the cap is an

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**Table: Plaque Characteristics in Ruptured and Intact Lesions**

<table>
<thead>
<tr>
<th></th>
<th>Ruptured</th>
<th>Intact</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of buried fibrous caps</td>
<td>2.66±0.16</td>
<td>1.06±0.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ruptures/month</td>
<td>0.41±0.04</td>
<td>0.21±0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean fibrous cap thickness, (\mu m)</td>
<td>2.0±0.3</td>
<td>4.7±0.6</td>
<td>0.0004</td>
</tr>
<tr>
<td>Lipid content, %</td>
<td>50.7±2.2</td>
<td>35.9±3.0</td>
<td>0.0019</td>
</tr>
<tr>
<td>Plaque cross-sectional area, mm(^2)</td>
<td>0.192±0.009</td>
<td>0.109±0.016</td>
<td>0.0005</td>
</tr>
<tr>
<td>Luminal occlusion, %</td>
<td>57.7±1.9</td>
<td>35.3±3.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Media cross-sectional area, mm(^2)</td>
<td>0.079±0.006</td>
<td>0.076±0.007</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Brachiocephalic artery plaques were sampled at the site of plaque rupture, where one was present. In vessels with intact lesions, samples were taken from the proximal 10%, because this is where all of the plaque ruptures were found. Analysis was performed using a computerized image analysis program (Image Pro Plus). Values were compared using an unpaired two-tailed \(t\) test.
in-life phenomenon rather than an artifact caused by postmortem tissue lysis or tissue damage during dissection. This is further supported by analysis of ruptured and intact plaques only in those animals that died suddenly; if the finding of rupture were artifactual, there would be no consistent morphological differences between the two types of plaque. However, ruptured plaques in animals that died suddenly were significantly bigger and more occlusive; they also had thinner fibrous caps and more layers. These data indicate that ruptured and intact plaques develop differently rather than changing at or after death.

Layered Phenotype
The layered appearance of the brachiocephalic artery plaques has also been observed in human coronary arteries. Burke et al. have suggested that the appearance of layers is likely to be the consequence of previous clinically silent ruptures, and it suggests growth of the lesion via rupture, followed by thrombus attachment and subsequent scarring. They noted that this hypothesis is supported by the significant correlation between the number of layers and the degree of luminal occlusion. As shown in Figure 4, a similar highly significant relationship is seen in mice ($r^2=0.252$, $P<0.0001$, Pearson’s parametric correlation test). Of course, this does not prove that the layers are caused by previous ruptures. It is also possible that the lesions may grow episodically with periods of rapid lipid deposition and lesion growth interspersed with periods of relative quiescence. Whatever the mechanism, it appears that there are similarities in the growth of lesions between mouse and man.

Luminal Occlusion
The degree of occlusion of the vessel lumen was significantly greater in the ruptured group. This is again similar to the situation in human plaques, in which the degree of occlusion of the vessel is greater in acute ruptures than at the site of healed ruptures.

Lipid Core
An increase in lipid core size has been linked to vulnerability of human plaques. Davies et al. reported an average core fractional volume of 56.7% in acutely ruptured plaques associated with thrombosis. This corresponds well with the lipid contents determined in the current study, in which ruptured plaques in mouse brachiocephalic arteries had a lipid content of 50.7±2.2%, which was significantly greater than the 35.9±3.0% lipid content of intact plaques.

Fibrous Cap
The mean fibrous cap thickness in ruptured lesions in this study, 2.0±0.3 μm, is much less than the 23±19 μm reported for human ruptured coronary artery lesions. It is difficult to compare directly across species because of differences in arterial lumen size and wall thickness and, hence, the tension exerted on the cap. Human coronary arteries are typically 2.4 mm in radius with a wall thickness of 0.76 mm, whereas the mouse brachiocephalic artery is approximately 0.36 mm in radius with a wall thickness of 0.04 mm. Assuming equal arterial blood pressures, this gives an averaged value of 50.5 kPa for human coronary artery wall stress and 130.8 kPa for the mouse brachiocephalic artery. It appears that fibrous caps in mouse brachiocephalic artery plaques, as well as being thinner than those overlying human coronary plaques, are subject to stresses that are greater than those encountered by rupturing human coronary artery plaques. This may account for the high frequency of plaque rupture in mouse brachiocephalic arteries.

Plaque Development
Of the plaques characterized as intact in this study, 79% had a history of previous rupture (as evidenced by the presence of one or more buried caps). This suggests that the morphological distinction made here between intact and ruptured plaques is really a distinction between recently ruptured plaques and sites where the most recent rupture has healed. The fact that such developmental measures as the number of previous ruptures and the frequency of silent rupture are significantly greater in the ruptured group suggests that the key underlying variable may be the inter-rupture interval. Animals with shorter inter-rupture intervals would presumably have larger plaques through the process of thrombus incorporation, and they may have thinner caps because the time available for matrix synthesis within the cap is reduced. Whether predisposition to frequent rupture is a risk factor for coronary artery disease in humans is not known, but healed ruptures are more frequent in patients who have survived a myocardial infarction.

Strain Differences
The presence of attached thrombus at the point of plaque rupture and an extensive layered phenotype of the lesions indicating a high incidence of rupture are features that are not commonly reported in fat-fed apoE knockout mice. It is possible that the genetic background of the mice is a key determinant of rupture susceptibility. The mice used in this study have a mixed background of C57BL/6 and 129SvJ, whereas most published studies make use of animals back-
crossed through at least 10 generations onto a C57BL/6 background. The importance of strain differences to phenotypic expression has been highlighted in reports by Dansky et al. and Lominska et al. These studies compared apoE knockout mice on a C57BL/6 background with those on an FVB background. The extent of atherosclerosis was 7- to 9-fold greater in C57BL/6 mice than in FVB, despite lower total plasma cholesterol levels. Furthermore, 129Sv mice absorb dietary cholesterol more effectively than C57BL/6 mice.

Possibly an even more telling difference between these mouse strains is the platelet content of transforming growth factor β-1 (TGF-β1), which is 4-fold higher in C57BL/6 mice than in 129Sv mice. It is possible that TGF-β1 protects against rupture by increasing matrix synthesis and decreasing the degree of inflammation within the plaque, and indeed, inhibition of TGF-β1 at the level of its receptor produces an unstable phenotype in the lesions of apoE knockout mice on a C57BL/6 background.

This study confirms that the fat-fed apoE knockout mouse is a reliable and reproducible model of atherosclerotic plaque rupture and that the lesion characteristics in the brachiocephalic artery are similar to those associated with plaque instability in humans.

Acknowledgments

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References


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