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...
абдоминальная аорта с фосфатом, руку стерильную солевой водой в комнате, в которой температура воздуха поднимается до 100 mm Hg, с последующим перфузии с введением 10% формальдегида или 4% параформальдегида и 10% сахарозы в фосфат-буферную солевую воду.

**Histology**

Брахимедиастинальные артерии были помещены в парафин или оптимальный температурный компонент (BDH Laboratory Supplies). Сечения были вырезаны на 3 μм для парафиновых срезов и 6 μм для оптимальных температурных компонентов. Каждое 30 μм было взято по всей артерии. Сечения были окрашены эозином и эластином, или Miller’s elastin/van Gieson став.

**Identification of Plaque Rupture**

Сечения, окрашенные эластином, были проверены на присутствие или отсутствие тромбозов, так как это означает разрыв диска хондроцитов. Благодаря внутривенной геморрагии, только в определенные периоды время жизни, этот определение предотвращает ошибочное толкование ткани посмертно.

**Plaque Characterization**

Брахимедиастинальные артерии с атеросклеротическими плаками, где был один, были взяты. В большинстве случаев, это было в пределах 10% от общего объема артерии. В артериях, которые не подверглись резким изменениям (интактные), были взяты сечения от 10% наружной стенки. Один срез биометрии был измерен на перфузируемой мыши. Анализ был проведен с помощью компьютерной программы Image Pro Plus. (DataCell). Следующие параметры были измерены: площадь сечения, медиа-область сечения, процентная оCLUSия, и процентная жировая часть плаки.

**Statistical Analysis**

Предполагаемые значения для интактных и резорбтивных плак изучены с помощью t теста для оценки нормальности распределения. Когда вариации были не одинаковы, Стьюдентов-уравнение t теста было использовано; при помощи среднего теста анализировалась корреляция. Сигнificance was concluded when the two-tailed П value was less than 0.05. Values are expressed as mean±SEM.

**Results**

**Sudden Death**

Исследовали 98 мышей, 64 из них погибли внезапно, а 34 были подвергнуты запланированной смерти. На рисунке 1 показаны распределения внезапных смертей во времени, в форме кривой выживаемости. Средний срок внезапной смерти составил 28.9±1.7 недель после высокой жирной диеты. В артериях без внезапного разрыва (интактные), вариации не были значимо выше. Стьюдентов-уравнение t теста погибло в интактных плаках (2.66±0.6 μm), чем в интактных плаках (1.49±0.12 μm, P<0.0001). По предполагаемой высокой репрезентативности, внезапный разрыв был небольшим присутствие вне плаки, где смертность составила 28/51 (52%), а внезапная смертность составила 35/64 (55%). Стьюдентов-уравнение t теста погибло в интактных плаках (2.66±0.6 μm), чем в интактных плаках (1.06±0.12 μm, Fisher exact test).

**Plaque Rupture**

После гистологического анализа, 51 из 98 мышей (52%) были найдены с интактным разрывом атеросклеротической плаки в брахимедиастинальной артерии. Примеры этих разрывов представлены на рисунке 2. Встречаемость внезапной смертности не различалась между животными, которые погибли внезапно (35 из 64) и животными, которые были терминальны (16 из 34, P=0.53, Fisher exact test).

Того не было различия в интактных плаках (P=0.69, Fisher exact test).

**Thrombus**

Все мыши, которые погибли внезапно, имели тромботический материал в просвете артерии, который не был получен из перфузии. Интенсивность тромбоза в перфузируемых животных с внезапными разрывами составила 73% (11 из 15), а интенсивность тромбоза не была видна в перфузируемых животных, которые не имели внезапных разрывов.

**Plaque Characteristics**

Сравнительные характеристики интактных и резорбтивных плак изучены в табл. 1. Средняя величина аденозного количество в интактном был значительно выше в разорванных плаках (2.66±0.16 μm) и интактных плаках (1.06±1.12 μm, P<0.0001). По предполагаемой высокой репрезентативности, внезапные разрывы были небольшим примером, где смертность составила 28/51 (52%), а внезапная смертность составила 35/64 (55%). Плаки интактных плак составили значительно тоньше в разорванных плаках (2.66±0.6 μm), чем в интактных плаках (4.7±0.6 μm, P=0.0004). Плаки были значимо больше в разорванных плаках (0.192±0.009 mm²) и интактных плаках (0.190±0.016 mm², P=0.0005). Основная поверхность образования в разорванных плаках значительно больше, чем в интактных плаках (57.7±1.9% против 35.3±3.3%, P<0.0001). Все плаки, исследованные, имели либо аминокислотный (или гликолипидный) жировой слой, но доля объема, занятого ядром, была значительно больше в интактных плаках с разрывами, чем в тех без. (50.7±2.2% против 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 ± 0.006 mm²; intact, 0.076 ± 0.007 mm²; $P = 0.73$).

**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

**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, μm</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, mm²</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, mm²</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.
The layered appearance of the brachiocephalic artery plaques has also been observed in human coronary arteries. Burke et al.11 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 in mice (\(r^2=0.252, P<0.0001\), Pearson’s parametric 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.11

**Lipid Core**

An increase in lipid core size has been linked to vulnerability of human plaques. Davies et al.12 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 \(\mu m\), is much less than the 23±19 \(\mu m\) reported for human ruptured coronary artery lesions.1 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 \(mm\),14 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 rupturing 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.11

**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.15 and Lominska et al.16 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.15,16 Furthermore, 129Sv mice absorb dietary cholesterol more effectively than C57BL/6 mice.17

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.18 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.19

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.

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References

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