

## Quantification of Atherosclerosis in Mice

Alan Daugherty and Stewart C. Whitman

### 1. Introduction

Traditionally, studies on the development of atherosclerosis have been performed in animals larger than mice, with a particular preponderance of studies in rabbits. Studies in the mid-1980s began to introduce the mouse as a model for the development of atherosclerosis. The extensive genomic information that was available on inbred mice proved to be attractive in identifying genetic links to atherosclerosis susceptibility. However, it was not until the availability of genetically engineered mice that this species became more widely accepted as a model that mimicked several aspects of the human disease.

This chapter briefly overviews the inbred and genetically engineered strains of mice that are available, discusses aspects of experimental design, and provides a detailed technical description of the most common methods of quantifying the extent of atherosclerosis.

#### 1.1. Strains of Mice Available for Atherosclerosis Research

One selection criterion for using a specific model should be the similarity between the atherosclerotic lesions that develop in mice compared with humans. A brief overview of the current thinking of the progression of human lesions is that the initial cellular event in the development of atherosclerosis is the adhesion of monocytes to an intact endothelial monolayer at specific regions in the arterial tree. A chemoattractant gradient induces diapedesis of monocytes, which enables them to migrate into the subendothelial space, where they become differentiated into macrophages. Macrophages become progressively engorged with lipid and form fatty streaks. At this stage of

progression, the lipid deposition in the subintimal space is predominantly in the intracellular space. The next major stage of progression is characterized by an acellular lipid-rich core covered by a fibrous cap consisting of smooth muscle cells and extracellular matrix (1). A final stage of progression involves a rupture or erosion of lesions and the formation of a thrombus. Although several strains of mice are used for atherosclerosis research, none of the presently available mouse models of atherosclerotic disease cover the complete sequence of human lesions.

Early atherosclerosis studies in mice were performed with inbred strains. These demonstrated that the most susceptible strain was C57BL/6, BALB/c mice were of intermediate susceptibility, and C3H mice were totally resistant (2). However, even in the C57BL/6 strain, lesions are restricted to developing in the aortic arch, are diminutive, and contain few cells.

Several genetically modified mouse models of atherosclerotic diseases are now becoming widely used. A list of the most frequently used models and some of their characteristics is given in **Table 1**. The most commonly used model is the apoE<sup>-/-</sup> mouse. This genotype was created in three independent laboratories, all of which seem to have the same general phenotypic characteristics (3-5).

A further consideration is the strain of the mouse that has been genetically engineered. As mentioned earlier, differences in atherosclerotic lesion susceptibility have been described in inbred strains. Recent evidence has also shown that strain has a marked effect on atherogenesis occurring under conditions of apoE deficiency. As with the inbred strains, apoE deficiency has the most striking effect on atherosclerosis in a C57BL/6 background. In contrast, apoE deficiency has a more modest effect on atherogenesis in FVB (6) and C3H (7) strains.

## **1.2. Protocol Considerations**

Many of the experimental design decisions have to be based on empirical information. When initiating a study on the development of atherosclerosis, the following questions need to be asked.

### **1.2.1. What Is the Most Appropriate Type of Mouse?**

As noted above, none of the currently available mouse models of atherosclerosis develop the full spectrum of lesions present in humans (1). Many mouse models develop lesions that resemble American Heart Association (AHA) type I and II; a more limited number have lesions that evolve into AHA type IV. No mouse models are currently available that develop the ruptured and eroded lesions that precipitate acute cardiovascular events in humans. Therefore, investigations that study lipid deposition and monocyte adhesions may be

**Table 1**  
**Selected Genetically Manipulated Mice That Have Been Used in Atherosclerosis Research**

Mouse	Characteristics of atherosclerosis	Area of lesion characterization	Modified diet required	Commercial availability
<b>Targeted Deletions</b>				
ApoE <sup>-/-</sup> (3,5,23)	Progress from predominantly foam cell lesion to lesions with necrotic cores and fibrous caps	Aortic root, aorta, carotid	No	Jackson
ApoE <sup>-/-</sup> (5,24)	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Taconic
LDL receptor <sup>-/-</sup> (25,26)	Lesions composed predominantly of lipid-laden foam cells	Aortic root, aorta	Yes	Jackson
<b>Transgenics</b>				
ApoB(20)	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Taconic
Apo (a) (27)	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Jackson
Cholesterol ester transfer	Lipid-rich lesions of undefined cellularity	Aortic root	Yes	Jackson
ApoE(arg112,Cys142) (29)	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Jackson
ApoE3Leiden (30)	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Jackson
apoC-III (31)	Not defined	Aortic root	Yes	Jackson
<b>Compound</b>				
ApoB <sup>-/-</sup> × LDL receptor <sup>-/-</sup> (32)	A range of lesions from lipid-laden foam cells to those containing fibrosis and smooth muscle hyperplasia	Aortic root, aorta	No	Jackson

performed in mice that develop lesions of simple morphology. However, the study of mechanisms of more complex tissue remodeling may require models that have mature aspects of the disease process (*see* details in **Table 1**).

Early studies used inbred mice, primarily of the C57BL/6 strain. However, as mentioned above, these mice develop small lesions, even when fed diets that have been modified to induce hyperlipidemia. Therefore, most contemporary studies have used a genetically modified variant, of which the most commonly used have been low-density lipoprotein (LDL) receptor<sup>-/-</sup> and apoE<sup>-/-</sup> mice. LDL receptor<sup>-/-</sup> mice require a modified diet to generate lesions. These lesions tend to have a simple morphology, in which lipid-laden macrophages are the predominant cell type throughout development. LDL receptor<sup>-/-</sup> mice are preferred by some investigators because the distribution of cholesterol within plasma lipoproteins bears more resemblance to humans in that most of the sterol is present in LDL. Lesions in apoE<sup>-/-</sup> mice start with a simple morphology of lipid-laden contained macrophages. As they mature, they will acquire a more complex morphology that includes acellular lipid cores containing cholesterol clefts and fibrous caps (8,9). Much of the cholesterol in apoE<sup>-/-</sup> mice is transported in the very low-density lipoprotein (VLDL) fraction. A characteristic of the LDL fraction is that it contains a more complex array of apolipoproteins other than apoB.

Therefore, deciding which mouse model to use will depend in part on the specific pathogenic process under investigation that is most accurately mimicked compared with humans.

#### 1.2.2. Should a Normal or Modified Diet Be Used?

Early studies using inbred mice required the use of a modified diet to generate a hyperlipidemic state, with subsequent development of atherosclerotic lesions. The most common modification was to have mice consume a diet highly enriched in saturated fat, cholesterol, and cholate (10). This diet is often referred to as the *Paigen diet*, after the investigator who popularized it. However, in the era in which genetically modified mice are available, such pronounced dietary manipulations are not needed. The apoE<sup>-/-</sup> mouse develops hypercholesterolemia and atherosclerosis when maintained on a normal diet. However, many studies have been performed on these mice during feeding of a diet that is primarily enriched with saturated fat and cholesterol to a level present in the diet of western industrial nations. This diet contains 40% of calories from fat (21% by weight) and is colloquially referred to as the *western diet*. It has been demonstrated that feeding this diet accelerates the development of atherosclerosis in apoE<sup>-/-</sup> mice without promoting gross changes in morphologic characteristics of lesions (8). However, feeding a saturated fat diet may influence the mechanism of the disease process. This is illustrated by

the study of total lymphocyte deficiency in apoE<sup>-/-</sup> mice. Total lymphocyte deficiency was produced by the deletion of either recombinant activator genes 1 or 2, which are required to produce mature B- and T-lymphocytes. Total lymphocyte deficiency had no effect on the extent of atherosclerosis development in mice that were fed a western diet. In contrast, there was a 42% decrease in lesion size in the aortic root when mice were fed a normal diet (11,12). Therefore, it is possible that the regulation of atherosclerosis by some mechanisms may be overridden by the production of severe hypercholesterolemia.

#### 1.2.3. What Duration of Study Is Needed?

There are evolving technologies in the noninvasive quantification of atherosclerosis in mice by modalities such as magnetic resonance imaging (13). However, such techniques are in a developmental phase, and lesion quantification presently requires termination of the mouse to acquire the vascular tissue. Therefore, decisions have to be made on the interval of tissue acquisitions. These decisions are facilitated by knowledge of the extent of atherosclerosis under specific circumstances. However, there appears to be substantial variation in the extent of atherosclerosis generated between investigators, even using the same strain of mice and the same diet. Therefore, each individual laboratory needs to define the extent of lesion formation in that environment.

The duration of the study is partially dictated by the underlying hypothesis being studied and the mode of analysis. If an intervention is being studied that inhibits the development of atherosclerosis, then a robust response needs to be generated in the control mice. Lesions form more rapidly in the aortic root, and therefore studies of shorter duration are permissible in this region compared with *en face* analysis of the entire aortic intima.

In the vast majority of studies, the extent of atherosclerosis is quantified at a single interval. There are many reasons for constraining studies to one interval, including the expense of the studies and the considerable work needed to complete the analysis. However, for some studies it may be important to define the effect of an intervention on the temporal characteristics of atherosclerosis.

#### 1.2.4. How Many Mice Should Be Used per Group?

The number of mice needed per group can be determined from power calculations. This requires prior knowledge of the variance within a control group. Given the differences in variance that are noted between investigators' laboratories, it is not possible to provide these estimates from literature values. Therefore, this information needs to be derived for each environment.

Studies that quantify atherosclerotic lesions are frequently characterized by wide variances in the data. These wide variances occur even in mouse

studies performed with inbred strains, which adds genetic equivalence to a standardized environment of such variables as feeding and housing. The wide variance, combined with a common lack of normal distribution for the data, frequently leads to an inability to perform the most commonly used parametric statistical tests. Although parametric tests have a considerably enhanced sensitivity over nonparametric tests, their inappropriate use can lead to a type I statistical error. In our studies, we use the SigmaStat (SPSS) statistical package, which provides information on the appropriate use of a specific statistical test. (See Note 1.)

#### 1.2.5. Which Arteries Should Be Used for Quantifying Atherosclerosis?

The majority of atherosclerosis determinations in mice are performed in the aortic root or in the entire aortic tree. This emphasis is due to the early experience with wild-type C57BL/6 mice fed modified diets in which lesions only formed in these regions. Subsequent experiments used *en face* quantification of the entire aorta (14), in a process that had been used in several other species, particularly rabbits (15,16). Use of this technique requires genetically modified mice since these are the only strains in which significant disease occurs throughout the aorta. In our hands, the quantification of atherosclerosis by the *en face* technique is considerably more rapid than using the aortic root.

The carotid artery has been used extensively in vascular damage and transplant studies (17,18), but has had scant use in atherosclerosis studies. The coronary bed is of obvious interest, although there is no publication demonstrating a formal quantification process in this region.

## 2. Materials

1. Paraformaldehyde solution (4% w/v) for tissue fixation. Dissolve paraformaldehyde in phosphate-buffered saline (PBS). This will require boiling the solution in a loosely covered flask, which must be performed in a fume hood. The solution should be made on the day of the experiment.
2. Tissue molds (Fisher Scientific, cat. no. 22-038217).
3. OCT for embedding tissue (Fisher Scientific, cat. no. NC9509852).
4. Dissection equipment. In addition to general dissection equipment, small equipment is needed including small spring scissors (Fine Science Tools, Foster City, CA, cat. no. 1500-02); small forceps (Fine Science Tools, cat. no. 11065-07); and 0.2-mm minuten pins (Fine Science Tools; cat. no. 26002-20).
5. 1-mL syringes with 23-gage needles.
6. Hardware needed includes a dissection microscope such as a Nikon SMZ, an upright microscope, and a camera for acquiring images on a computer. For image analysis, we use ImagePro (Media Cybernetics).
7. Wako cholesterol kit (Wako, Richmond VA, cat. no. 276-64909).

### 3. Methods

#### 3.1. Preparation of the Mouse

1. Anesthetize mouse with either a parental injection (ketamine/xylazine; 100 mg/kg and 1 mg/kg, respectively) or by inhalation with metaflurane. Place mouse on the dorsal side and cut the skin on the ventral side from the base of the abdomen to the underside of the chin. Cut the abdominal skin until the xyphoid process is in view. Lift the xyphoid process with hemostats, make cuts on either side of the rib cage, and cut the diaphragm carefully. Then make two cuts down either side of the rib cage to reveal the heart. Now displace the ribs to provide clear access to the heart.
2. Exsanguinate the mouse by placing a 23-gauge needle through the apex of the ventricle. We find that it is generally easiest to use the right ventricle since the position of the needle tip within the chamber is more readily apparent than in the left ventricle. It is best to enter the ventricle at an angle parallel to the septum of the heart. During drawing of the blood, periodically rotate the needle 360°: this will prevent blocking of the needle opening by the walls of the ventricle. For a 25-g mice, it should be possible to acquire approx. 0.8–1 mL of blood.
3. Perfusion of the heart and aorta is performed via a cannula placed in the left ventricle. Remove the right atrium to allow fluid to escape from the body. Again, a 23-gauge needle works well for introduction of the perfusate. As with blood collection, try to enter the left ventricle at an angle parallel to the septum of the heart. First perfuse the mouse with approx. 20 mL of PBS to remove blood. Removal of the blood is a great assistance to the dissection of the aorta, especially in the abdominal region.
4. There are some circumstances in which it will be preferable to fix the tissue. This should be performed at arterial pressure of 80–100 mmHg. We use 4% paraformaldehyde dissolved in PBS. The perfusion is performed in a fume hood with an appropriate method of recapturing the fluid. Perfusion of the mouse for 30 min is sufficient to achieve reasonable fixation.

#### 3.2. Dissection of the Heart and Aorta

1. For quantification of atherosclerosis in the aortic tree, it is imperative that the entire tissue from heart to ileal bifurcation be removed without damage. We initially dissect the aorta free in the abdominal region. The intestinal tissue is displaced, and the most accessible region of the aorta, distal to the renal branches, is dissected free. It is easy to continue this dissection down to the ileal bifurcation. Probably the most difficult part of the dissection is between the renal branches and the diaphragm. The ease with which this region of the aorta can be seen varies considerably between strains and is also dependent on variables such as diet. Generally, until sufficient experience has been obtained, it is best to perform conservative cuts at some distance from the aorta.

Once the abdominal region has been dissected free, we subsequently move to the proximal regions. The lungs and esophagus are removed, and the heart is

