

Quantification of Atherosclerosis in Mice

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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^{-/-} 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
Targeted Deletions				
ApoE ^{-/-} (3,5,23)	Progress from predominantly foam cell lesion to lesions with necrotic cores and fibrous caps	Aortic root, aorta, carotid	No	Jackson
ApoE ^{-/-} (5,24)	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Taconic
LDL receptor ^{-/-} (25,26)	Lesions composed predominantly of lipid-laden foam cells	Aortic root, aorta	Yes	Jackson
Transgenics				
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
Compound				
ApoBec ^{-/-} × LDL receptor ^{-/-} (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^{-/-} and apoE^{-/-} mice. LDL receptor^{-/-} 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^{-/-} 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^{-/-} 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^{-/-} 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^{-/-} 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^{-/-} 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^{-/-} 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-gage 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-gage 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

held at the apex with forceps. Small spring scissors are used to cut toward the spine to sever the left and right carotid arteries and the left subclavian artery. The ease of visualizing these branches is largely dependent on the size of the thymus, which is related to factors such as the strain and age of the mouse. Once the arch region has been dissected free, the thoracic section is readily visible and can be removed by carefully cutting along the spine. The aorta and attached heart are dissected free by a final cut of the iliac arteries.

2. To separate the heart from the aorta, the anterior aspect of the heart is placed toward the operator, and the aorta is cut at the point where it emerges from the ventricular tissue.
3. The aorta is placed in a vial containing approx. 5 mL of freshly prepared 4% paraformaldehyde solution. We find that an overnight fixation facilitates the removal of the adventitial tissue. However, prolonged fixation can hinder this process. Therefore, the tissues are incubated overnight and then the fluid is exchanged for PBS.
4. For the heart, a scalpel is used to cut away approximately the lower 70% of the ventricular mass, as depicted in **Fig. 1A**. The upper cardiac portion is placed into a tissue mold and covered with OCT. The tissue is manipulated to ensure that the aortic root is filled with OCT. The aortic root needs to be oriented so that it is perpendicular to the bottom surface of the tissue mold. The mold is then placed on the Peltier stage of the cryostat, for rapid freezing.
5. Each mold should be individually wrapped in parafilm and stored in a freezer at -20°C in an air-tight container. OCT becomes rubbery and difficult to cut if it desiccates in a freezer.

3.3. Quantification of Lesions in the Aortic Root

1. The frozen tissue blocks are mounted in the cryostat with the ventricular tissue facing outward. Provided the tissue was placed correctly into the mold, placement of the block on the cryostat chuck will ensure that the aortic root is positioned perpendicular to the knife blade. The ventricular tissue is sectioned and discarded until the aortic sinus is reached. This is identified by the appearance of aortic valves or cusps. A diagrammatic scheme of the cutting is shown in **Fig. 1B** and an example of this region is shown in **Fig. 2A** (see Color Plate 2 following p. 144).
2. Slides are subsequently sectioned at 8- μm intervals. There is no generally accepted standard for the number of sections that are cut or the length of aorta that is sectioned. In our laboratory we usually retain every section for approx. 700 μm . From the start of section acquisition at the aortic sinus, we collect every section and sequentially place them on 10 slides. Therefore, each slide has approx. 9 sections that are 80 μm apart. (See **Note 2**.) The length of aorta that is sectioned is partially dependent on the severity of the disease. For investigations of aortic root atherosclerosis in mouse strains in which there is only a minor presence of disease, there may be no lesions present in distal portions of the sinus. Therefore, the length of aortic root that is sectioned could be reduced.

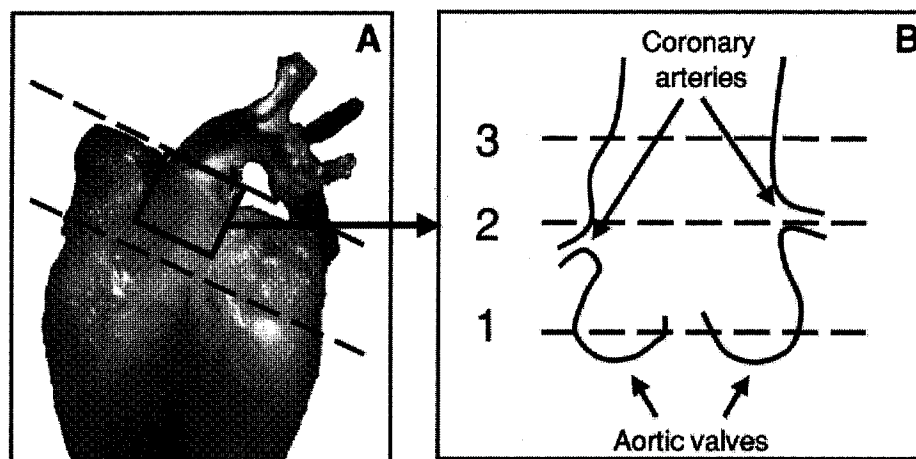


Fig. 1. A guide to the sectioning of the aortic root for quantification of atherosclerosis. (A) Tissue is cut in the regions represented by dotted lines to remove ventricular tissue and aorta. (B) Diagrammatic representation of the aortic root. The dotted lines indicate the regions used for examples in Fig. 2.

3. It should be noted that in the original description by Paigen et al. (19) of quantification of aortic atherosclerosis, the segment between the start of the aortic sinus and the orifices of the coronary artery was not used (see Fig. 2). However, most studies now include this region.
4. Sections are stained with hematoxylin to assist in tissue visualization. To quantify lesions, a stained slide is placed under a microscope, and images are captured to a computer at a magnification that permits clear definition of the lesion boundaries. Using the area quantification feature of the image analysis software, in each section, the area of lesion is defined by the internal elastic lamina and the luminal boundary. Data are commonly presented as the total lesion area in the number of sections quantified. Another useful presentation is to provide the total lesion area for sections at the specific regions of the aortic root, as described by Purcell-Huynh et al. (20).
5. Oil Red O staining is commonly used to assist in quantification of atherosclerotic lesions in mice. The color of the neutral lipid staining may enable the use of automated area determinations that are available in many image analysis packages. The usefulness of this approach is dependent on the composition of the lesions. It has utility in simple lesions that are uniformly stained for neutral lipid. However, in lesions that contain significant non-lipid-laden cells such as smooth muscle cells, extracellular matrix, and free cholesterol, then the extent of Oil Red O staining will not be representative of the entire area of the lesion.

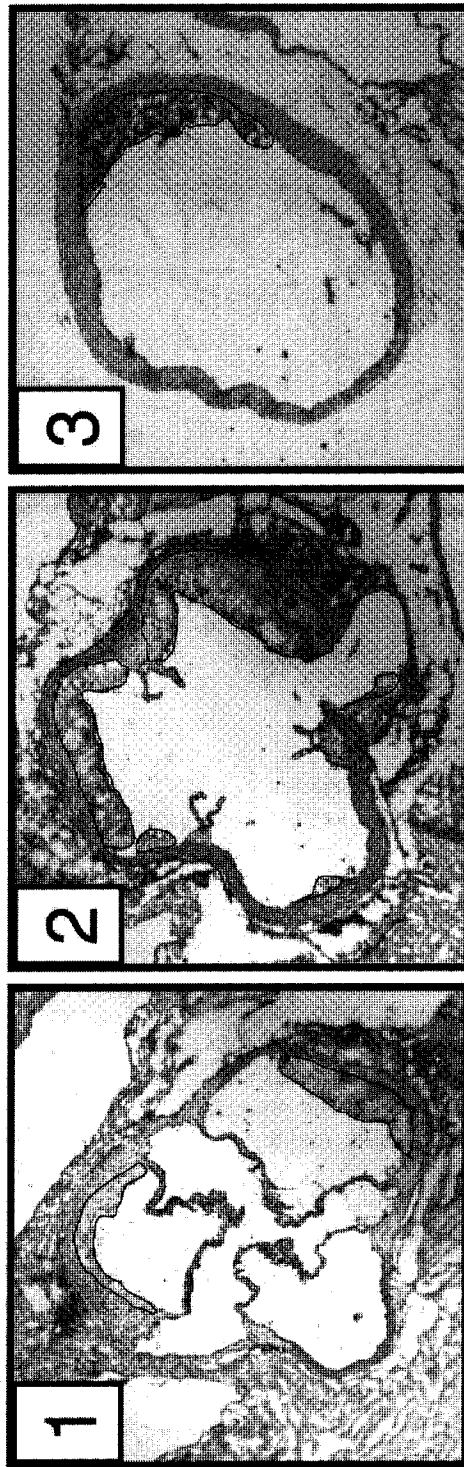


Fig. 2. Examples of sections in specific regions of aortic roots as indicated diagrammatically in Fig. 1. Sections are represented as follows: **1**, the start of the aortic sinus; **2**, the orifices of the coronary arteries marking the start of the ascending arch; **3**, the ascending aorta. The atherosclerotic lesions have been outlined for clarity. These examples are from aortic tissue of an apoE^{-/-} mouse.

3.4. Preparation of Tissue for Quantification of Intimal Lesions on Aortic Intima

1. Place the aorta on a dark background and use small forceps to gently pull adventitial tissue away from the aorta. This removal process needs to be completed without excessive manipulation of the aorta, and the tissue needs to be kept moist with PBS.
2. Following removal of the adventitial tissue, one of the arbitrary decisions that needs to be made is how much of the branches of the left and right carotids and left subclavian arteries should be retained. The branch points of these vessels from the aorta tend to be locations of predilection for lesion formation. Therefore, inconsistency in standardizing the tissue would adversely influence the results. Routinely we cut the carotid arteries 1 mm from the branch, whereas the left subclavian artery is removed at its point of origin.
3. Following preparation of the outside of the aorta, the tissue needs to be cut open to expose the intimal surface. This procedure involves introducing small spring scissors into the lumen of the vessel. One potential problem is that atherosclerotic lesions in the mouse can be relatively easily dislodged from the intimal surface (*see Note 3*). Fortunately, the mouse aorta is virtually transparent and lesions can be seen from the exterior of the vessel. Therefore, the cutting of the aorta should be performed under a dissecting microscope so that any dislodgement can be documented.
4. The first cut is through the lesser curvature of the aortic arch. The cut then continues down the entire length of the blood vessel.
5. Next, cut at the top of the aortic arch on the outer curvature from the ascending arch to the left subclavian artery.

3.5. Lipid Staining of Aortas

Lipid staining of aortas is not necessary for quantification of the intimal area covered by atherosclerotic lesions. However, it can be of assistance in contrasting the lesioned area against normal tissue. Also, if lesions are very small, lipid staining will be needed to visualize the disease.

1. Preheat a water bath to 37°C.
2. Prepare fixed aorta samples by cutting to expose the intima as described above (*see Subheading 3.4.*).
3. Place each tissue into a 0.5-mL Eppendorf tube and label appropriately.
4. Fill each tube with 400 μ L of Sudan IV (5 mg/mL in 70% isopropanol).
5. Place in water bath for 40 min at 37°C.
6. Remove sections of aorta and check extent of staining under a dissecting microscope. Periadventitial adipose tissue will stain very brightly with Sudan IV and should be removed before images of the aorta are collected. Keep tissues wet in PBS while cleaning.
7. Place the tissue in another 0.5-mL Eppendorf tube and fill with 400 μ L of the 70% isopropanol wash.

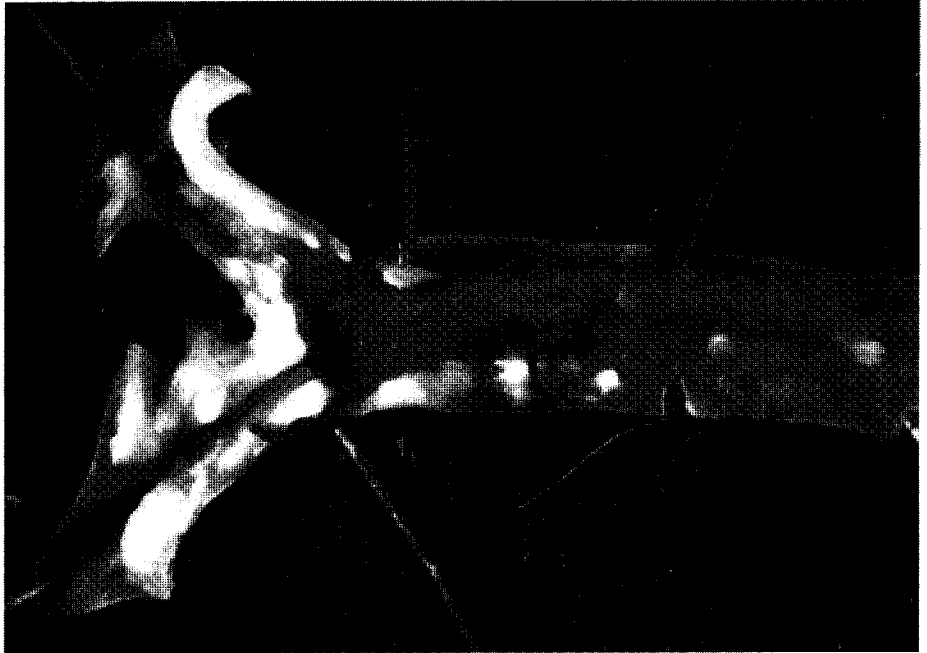


Fig. 3. An example of the arch region of an aorta pinned out for acquisition of an image that is used to quantify atherosclerosis by the *en face* technique described in **Subheading 4.5.3**. The bright white material is unstained atherosclerotic lesions.

8. Agitate by hand for 15 s and return to water bath for 5 min.
9. Check the extent of lipid staining under the dissecting microscope. If additional destaining is required, incubation with 70% isopropanol should be repeated until there is optimal contrast between lesion and the normal segments of aorta.

3.6. En Face Determination of Percent of the Intimal Area Covered by Atherosclerotic Lesions

1. The aorta is pinned on black paper placed over dental wax using minuten pins, as shown in **Fig. 3**. We find that the use of paper provides some modest adhesion of the aorta and minimizes the number of pins required to obtain flat aortic tissues. A ruler is placed next to each image to assist in calibration of the image. Tissues need to be maintained under moist conditions.
2. An image is captured to the computer. We collect this image using a digital camera connected to a dissecting microscope that has a reducing and polarizing lens to obtain an appropriate field and reduce glare, respectively.
3. In the case of unstained aortas, sometimes glare will provide the appearance of a lesion on the image. For lipid-stained lesions, any residual adipose tissue on

the adventitial surface will be clearly seen, since the mouse aorta is translucent. Therefore, we verify the location of the lesions by comparing the image to the pinned tissue.

4. Image analysis software is used to determine the area of the intima and lesions. We manually trace the outline of the intima and lesions. We have not been able to obtain images with sufficient contrast to perform these measurements reliably in an automated mode with image analysis software. Even with stained lesions, it is our experience that the staining is so variable that it is difficult to set color thresholds to provide data that are reliably the same as a manual measurement.
5. A second observer should verify the data generated by the primary operator. (*See Note 4.*)
6. We represent the data for the three major regions of the aorta: arch, thoracic, and abdominal. We define these regions as follows: Arch, root to 3 mm beyond the left subclavian; thoracic, end of arch segment to the last intercostal branch; and abdominal, end of thoracic segment to the ileal bifurcation.

3.7. Tissue Sterol Analysis

There are several reasons to determine tissue sterol content. One is that it acts as an independent parameter of atherosclerosis. Second, we have observed examples in which the lesion area is not changed, but visual inspection shows that the lesions are thicker (21,22; *see Note 5*). In these cases, assuming the lesions are primarily lipid-containing, this measurement provides an index of lesion volume. Finally, the determination of the ratio of esterified to unesterified cholesterol can provide mechanistic insight into the mode of lipid deposition. It is important to note that tissue sterol analysis cannot be performed on aortas that have been stained with Sudan IV.

1. Determine the intimal area of the aortic segment from the captured image, as described in **Subheading 3.4**.
2. Transfer each sample into a Kimax tube.
3. Add 3 mL of 95% methanol and allow to sit for 1 h at room temperature in capped tubes. For short incubations (<2–4 h), we use marbles in place of tube caps because the marbles are much faster to place and remove.
4. Add 6 mL of chloroform and mix, ensuring that all tissues are floating and solvents have mixed in the capped tubes.
5. Allow to sit overnight at room temperature in capped tubes.
6. Add 2 mL of saline. Vortex well (approx. 1 min) and allow phases to separate for 1 h. The separation of phases can be enhanced by low-speed centrifugation (200g, 10 min).
7. Transfer bottom chloroform phase into a 16 × 100-mm disposable glass tube using a glass pipet.
8. Using a stock cholesterol solution (200 mg/dL; generally provided in the Wako assay kit), a standard curve is generated by pipeting duplicate aliquots into 16 × 100-mm disposable glass tubes, as shown below:

Final cholesterol concentration (μg)	μL to pipet
blank	0
1	5
2	10
5	25
10	50
20	100

9. Evaporate standards and samples to dryness under a gentle flow of N_2 with mild heating (at 37°C). For standards, add 1 mL of chloroform plus 1 mL of 1% Triton X-100 in chloroform, and mix by vortexing. For samples, add 450 μL of chloroform plus 450 μL of 1% Triton X-100 in chloroform, and mix by vortexing.
10. Evaporate the standards and samples again to dryness under N_2 with heating (37°C). For standards, add 500 μL of deionized water and for samples add 225 μL . Roll or vortex until the Triton X-100 goes into solution and incubate at 37°C for 15 min in a water bath.
11. Pipette duplicate 50- μL aliquots of each standard and each sample into individual wells of two microtiter plates. One of the microtiter plates will be used to determine the total cholesterol content of each sample, and the second microtiter plate will be used to determine the unesterified cholesterol content of each sample. Note the number of plates you will need before you prepare the standards; 500 μL of each standard should be enough for two total cholesterol and two unesterified cholesterol plates.
4. Add 150 μL of either total cholesterol or unesterified cholesterol reagent per well and mix. Incubate for 10 min at 37°C in a dry oven and then for 50 min at room temperature prior to reading absorbance on a plate reader using the correct wavelength as specified by the manufacturer of the cholesterol assay. The dilution correction factor will be 4.5 (225 μL of sample/50 μL of sample/well).

3.8. Summary

At present there are no standards for the assessment of atherosclerosis in mice. Therefore, the methods detailed here should be taken as examples. In addition to quantitating the size of lesions, the composition of the lesion may also be an important parameter. Therefore, although a detailed description is beyond the scope of this chapter, investigators are encouraged to perform standard histologic and immunocytochemical analysis of lesions.

4. Notes

1. The quantification of atherosclerosis is inherently variable, and data are frequently not normally distributed. Data must be tested to define whether the application of either parametric or nonparametric analysis is appropriate.
2. For aortic root analysis, care needs to be taken in defining landmarks so that lesion sizes are compared in sections from the same region. Furthermore, lesions

in the sinus may not be reflective of changes in the ascending aorta, and it is preferable to acquire sections from all of the root region.

3. Atherosclerotic lesions in mice are relatively easily displaced from the luminal surface. To minimize lesion loss, tissues should be handled with care, and cutting to expose the intimal surface should be performed under conditions in which visualization of the lesions is possible.
4. The determination of the extent of atherosclerosis in mice frequently requires some subjective judgments to define the boundaries of lesions. Therefore, lesions should be quantified by more than one observer. Blinding of studies is preferable.
5. For *en face* analysis, a similar area of lesion coverage of the intima does not necessarily mean that lesion size is the same. Frequently, careful visual inspection of the tissue illustrates that lesion thickness may be different in the absence of changes in area. For lipid-rich lesions, this difference can be defined using the measurement of tissue sterols.

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