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Hyperlipidemia-induced Atherosclerosis

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Abstract

Hyperlipidemic mice have become the predominant models in atherosclerosis research. Early studies using inbred mice were restricted by the need for severe dietary manipulations that generated diminutive lesions. The use of mice gained wider acceptability following the generation of genetically manipulated mice with enhanced susceptibilities for atherosclerotic lesion formation. The atherosclerotic lesions formed in these mice mimic several facets of the human disease. Mice with deficiencies of either apolipoprotein E or LDL receptors have been the most extensively researched to determine the effects of pharmacological and genetic interventions on lesion size and composition. This chapter will overview issues in the design of experimental studies using these mice. These include discussion of comparisons of the most common mouse models of atherosclerosis, the composition of the dietary stimulus to induce hyperlipidemia, the strain background of mice, the mode of atherosclerotic lesion analysis, methods for characterizing cellular compositions of lesions, and application of appropriate statistical tests. Mouse models have provided considerable insight into mechanisms of atherosclerotic lesion development.

Keywords

mice, cholesterol, atherosclerosis, saturated fat

Introduction

Atherosclerosis is the underlying cause of a majority of cardiovascular diseases, such as acute coronary syndrome and stroke. It is a complex process that develops in specific regions of arteries over protracted intervals. A commonly proposed sequence of atherosclerotic lesion development is as follows:

1. Lipoproteins are deposited in the subendothelial space and become modified to a form that promotes a monocyte chemoattractant response.
2. Monocytes attach to atherosclerosis-prone sites where they subsequently undergo diapedesis through an intact endothelial barrier.
3. Macrophages in the subendothelial space accumulate intracellular lipid. Also T lymphocytes are found in abundance at the site of macrophage recruitment.
4. Smooth muscle cells migrate and proliferate in the subendothelial space.
5. A hypocellular, lipid-rich core evolves as smooth muscle cells form a fibrous cap on the lumen surface of lesions.
6. At later stages, the fibrous cap thins and subsequently fractures to precipitate the development of thrombi. Alternatively, an erosive process can lead to the development of thrombi on the surface of lesions.

While this, and similar schemes, are commonly presented, there are many questions regarding the uniformity of this sequence in humans.¹ This uncertainty is attributable to several issues, including the chronicity and complexity of lesion development, the difficulty of acquiring tissues, and the shortcomings of currently available modalities for noninvasive characterization of arteries.

Given the prevalence of this disease, and its devastating consequences, there is a great need for animal models that recapitulate the human disease. Since the first description of experimental atherosclerosis in rabbits, many species have been models of the disease. Mice are a relatively recent species to be used as models of atherosclerosis. However, they are now the predominantly used species. This increased popularity is partly attributable to the practical and economic benefits of a small animal model, and also the relative ease of manipulating their genetics to define specific pathways in the atherogenic process.

As with most animal models of atherosclerosis, all mouse models require hyperlipidemia to generate lesions.² Hyperlipidemia in mice may be induced by diet, genetic manipulation, or a combination of both. The purpose of this chapter will be to discuss the relative merits of the most commonly used mouse models of atherosclerosis and provide an overview for the practical issues of lesion analysis.

Induction of Hyperlipidemia in mice

Diet

High density lipoproteins (HDL) are the only lipoproteins present in plasma of mice fed normal laboratory diets. Atherosclerosis is induced by the presence of cholesterol-delivering lipoprotein fractions such as chylomicron remnants, very low

density lipoproteins (VLDL), and low density lipoproteins (LDL). Early studies used severe dietary manipulations to increase plasma concentrations of cholesterol-delivering lipoproteins and induce atherosclerosis. These diets manipulated three components: saturated fat, cholesterol, and cholate.³ This approach was designed to increase plasma concentrations of VLDL and LDL by increasing cholesterol absorption and endogenous synthesis, while decreasing cholesterol secretion. Diets that are enriched in saturated fat and cholesterol are still commonly used in contemporary atherosclerosis studies. Most of the diets are enriched in saturated fat to a level of 21 per cent wt/wt (42 per cent of calories). The most common supplier of this diet is Harlan Teklad (Catalog #TD88137). Saturated fat is derived from several sources, the most common from milk, coconut, or lard. The consequences of these different sources of saturated fat have not been systematically evaluated. Finally, cholesterol has been enriched in diets to different extents that usually range from 0.15 to 1.25 per cent wt/wt. Again, systemic studies are lacking on the effects of cholesterol enrichment.

In the era prior to genetic manipulation of mice, the inclusion of cholate was a critical component of hyperlipidemia-induced atherosclerosis. However, the inclusion of cholate has become a contentious issue. Although its presence enhances hypercholesterolemia, there is concern that cholate may directly promote inflammatory responses. Thus, the inclusion of cholate has invoked the criticism for its potential toxic role in inducing lesions by mechanisms that are not relevant to human disease. Clearly, the inclusion of cholate in diets does promote an inflammatory response. However, whether this is a direct effect of cholate or secondary to disturbances in cholesterol metabolism has not been defined. Furthermore, since inflammatory responses are now considered to be important mechanisms of atherosclerosis, the relevance of cholate induced responses cannot easily be dismissed. However, given the concern on the inclusion of cholate, it is preferable to omit it from diets in atherosclerosis studies.

Genetic manipulation

The most commonly used genetic manipulations in mouse atherosclerosis models are those that have increased plasma concentrations of VLDL and LDL. Deletion of apolipoprotein E (ApoE) was one of the initial approaches to induce atherogenic hyperlipidemia.^{4,5} ApoE, a ligand present on chylomicron remnants and VLDL, interacts with receptors responsible for removal of these particles from plasma. Thus, ApoE deficiency promotes hypercholesterolemia even when mice are fed a normal laboratory diet. VLDL and LDL account for the majority of particles in the plasma cholesterol distribution in ApoE^{-/-} mice. The composition of these lipoproteins is unusual in regard to both their apolipoprotein and lipid composition. For example, the VLDL fraction from these mice contains an unusual enrichment of ApoA1 and unesterified cholesterol. (Table 5.1)

Another most commonly used mouse model of atherosclerosis is deficiency of the LDL receptor.⁶ LDL receptor deficiency is an uncommon human genetic condition

that results in grossly elevated plasma concentrations of LDL cholesterol. In humans afflicted with the homozygous forms of this deficiency, fatal atherosclerosis will developed by the second decade of life. In contrast, mice with LDL receptor deficiency are only modestly hypercholesterolemic. This may be due to the lesser dependency of mice on LDL receptors to removal atherogenic lipoproteins. It should also be noted that the genetic deficiency of the LDL receptor-deficient mice differs from that of humans or Watanabe heritable hyperlipidemic rabbits. In humans or rabbits, there is no functional LDL receptors in patients afflicted with a homozygous form of this deficiency. In the case of mice, the gene was truncated at exon 4. Since the receptor binding region is presumably still synthesised in these mice, this may have a currently uncharacterised effect on plasma lipoproteins. In contrast to LDL receptor deficient humans, atherosclerosis has not been detected in mice lacking this gene when fed normal diet, except in later stages of life.

Mouse strain

Early studies demonstrated that different mouse strains have widely different susceptibilities to the development of atherosclerosis. In non genetically manipulated mice, the C57BL/6 strain is the most susceptible to lesion formation, and consequently, this background is universally used in atherosclerosis studies.

In genetically manipulated mice, it is clear that strain background of the mouse is also a powerful modifier of the extent of atherosclerotic lesion formation. The strain dependent effects are not attributable to changes in plasma lipoprotein concentrations, but instead are due to differences at the level of the aorta. For example, ApoE deficiency in C3H mice have much smaller lesions than in C57BL/6, despite equivalent hypercholesterolemia.⁷ Thus, strain background is a critical factor for interpretation of results and comparison between studies. Genetically targeted mice are commonly produced using stem cells from strains such as 129Sv/Ev. As a consequence, studies on recently developed genetically engineered mice have been frequently performed on hybrid mice of different strains. Currently, the ApoE^{-/-} and LDL receptor^{-/-} mice available from the Jackson Laboratory have been backcrossed at least 10 times into a C57BL/6 background.

Although the background strain of atherosclerosis-susceptible mice is now commonly defined, this can be a major issue when compound genetically deficient mice are developed to study a lesion modifier gene. In these studies, the experimental purpose is to define the effects of a single gene difference on atherosclerotic lesion development. Thus, the interpretation of studies will be compromised if the strain background of the single and compound deficient mice are not identical. Knowledge of the strain background of the mice is particularly important when the single and compound deficient mice are maintained as separate colonies. Currently, it is common to accept the equivalence of strains if both the genetically engineered mice used to generate the compound deficient mice have been backcrossed 10 times in a C57BL/6 background. The Jackson Laboratory and other commercial suppliers have genetic screening services that provide an indication of the percentage of genes that are of C57BL/6 origin. However, for practical reasons these services provide a

relatively small number of chromosomal markers. Consequently, these services will not fully validate all chromosomal regions, particularly those in the proximity of the genetically manipulated region. Also, genetic drift may occur in inbred colonies, and thus separate breeding colonies of single and compound deficient mice have the potential to acquire properties that may influence atherosclerosis development, independent of the genetically engineered effect.

The use of littermates circumvents the potential problem of different strain backgrounds imparting effects that may mask or exacerbate a specific genetic manipulation. For this approach to breeding, the common strategy is to maintain parental lines that are homozygous for the atherosclerosis-susceptible genotype (for example, ApoE^{-/-} or LDL receptor^{-/-}) and heterozygous for the gene of interest. This approach offers theoretical advantages of identifying the specific effects of a gene manipulation, rather than an effect attributable to variances in strain background. It also provides mice that are wild type, hetero-, and homozygous for the gene of interest to enable determination of gene dosage effects. However, there are some practical compromises. These include large breeding colonies to obtain the required number of wild type and homozygous mice. Also, there is a greater technical burden, since all offspring from this breeding strategy must be genotyped.

Environmental factors

Many animal facilities now house mice under barrier conditions. These sterile environments are obviously important from an institutional perspective in applying infection control to colonies. There are few formal studies that define the effects of sterile environments versus those that are usually termed as 'conventional.' However, there is anecdotal evidence that the extent of atherosclerosis and the mode of responding to an intervention may be altered by the housing environment.

Gender

There is some evidence that, contrary to humans, female mice may develop larger atherosclerotic lesions than males. Despite this premise, an overview of the literature, in which genders have been compared, indicates that there are no consistent gender difference in lesion size in either ApoE^{-/-} or LDL receptor^{-/-} mice. However, there have been many examples of gender-specific responses to interventions. These include administration of PPAR gamma agonists and interferon-gamma deficiency.^{8,9} Therefore, we always analyse data on a gender-specific basis. Also, if possible, we include both genders when determining the effects of a specific intervention.

Analysis of Atherosclerotic lesions

There are two common methods of quantifying atherosclerosis in mouse models. Probably the most frequently used method has been to quantify lesion size in the aortic root. This mode of analysis was initially described for mouse atherosclerosis in

the much cited publication from Paigen and colleagues.¹⁰ Another common method is the quantification of lesions on the intimal surface of the aorta, by a process that is frequently referred to as *en face*. A similar form of analysis was commonly used in larger animal models, prior to its introduction into the mouse atherosclerosis field by Palinski and colleagues.¹¹ There are also less commonly used forms of analysis such as quantification of the innominate artery.¹²

Aortic root lesion quantification

This form of analysis was described initially by Paigen *et al.*¹⁰ to quantify lesion size formed in C57BL/6 mice fed a diet enriched in saturated fat, cholesterol, and cholate. A novel approach to quantifying lesions was needed, since despite these relatively severe dietary manipulations, small lesions form only in the aortic root of these mice. A detailed technical description of this analysis has been published previously.¹³ The analysis of this region can be performed on either paraffin-embedded tissues or frozen tissues. The majority of studies that quantify atherosclerotic lesion size in the aortic root are performed on sections that have been cut using a cryostat. This method is preferred because of the greater ease with which the tissue can be orientated, as discussed later. Also, sections that will be stained for neutral lipids need to be frozen sections, since lipids are removed in the process of embedding tissues in paraffin.

Although similar approaches are used in most studies of the aortic root, there are many specific protocol issues for which there is no general agreement. These include:

1. The number of sections in which lesions are quantified.
2. The specific region of the root in which lesions are analysed (for example, sinus versus ascending aorta, or both).
3. The mode of analysis. For example, the analysis of neutral lipid stained regions *versus* tracing of the discernable intimal and medial boundaries of lesions.

The mode of analysis performed by the author's laboratory is illustrated in Figure 5.1. Hearts are dissected from mice that have been perfused with saline to remove blood. For quantitative analysis, it is preferable to perfuse with fixative at arterial blood pressure. The caveat to this approach is that it severely compromises the characterization of lesions, since many antibody epitopes are masked by fixation. The aorta is cut from the excised heart at the region where it emerges from ventricular tissue on the anterior aspect of the heart. The heart is cut to remove approximately two-thirds of the ventricles. The tissue is then placed in embedding media and frozen. The accuracy of this approach relies on sections being cut perpendicular to the aortic axis. Thus, a critical step in this process is to ensure the orientation of the aorta is perpendicular in the embedding mold.

Frozen tissue blocks are placed in the cryostat and cut until the aortic valves become visible. Since the valves are difficult to see without visual aid, it is

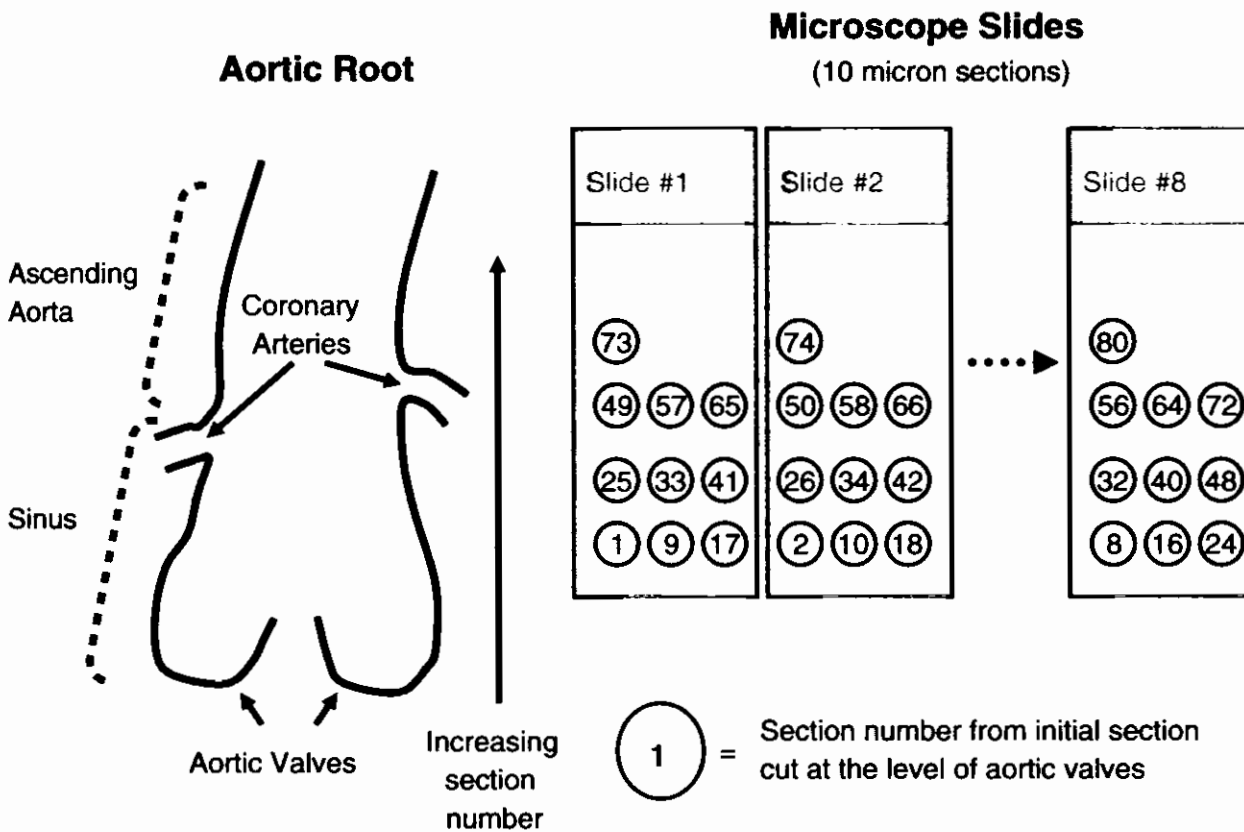


Figure 5.1 Diagrammatic representation of location of aortic root analysis of atherosclerosis and the mode of section layout on slides. For a color version of this figure, please see the images supplied on the accompanying CD

preferable to have a phase microscope in close proximity to permit frequent viewing of sections. Once the aortic valves are present, sections are collected in the manner illustrated in Figure 5.1. Ten micron sections are cut, with the first section being placed in the lower left corner of the first slide, and the second section in this same position on the second slide. This process continues for eight slides. The ninth section is then placed adjacent to the first section of the first slide. This process continues until approximately nine sections are placed on each slide. Upon completion of cutting, each slide has tissue sections at 80 μM intervals throughout the entire aortic root.

Tissue sections can be visualised with standard histological staining. We frequently use just hematoxylin. Sections are occasionally stained with Oil Red O which helps in delineating small lesions in the aortic sinus. For quantification, images of each tissue section on a slide are captured using a standard digital camera. Lesion area measurements on each slide are performed with image analysis software (for example, Image-Pro, Media Cybernetics Inc.) We perform these measurements by manually outlining each lesion as defined by the lumen boundary and the internal elastic lamina. There have been attempts to automate this process, the most common being the determination of the area with Oil Red O staining. This mode can be appropriate if there is uniform neutral lipid staining throughout entire lesions. However, this condition is often not present, especially in more mature lesions that have areas of non-neutral lipid deposition containing unesterified cholesterol and extensive smooth muscle cells.

Following the acquisition of lesion areas on each individual section, we mean the data for sections in the same region. We have found the optimal landmark to normalise the location is the transition point of the aortic sinus and ascending aorta. This is defined as the last section in which aortic valve cusps are visible. This form of analysis permits a graph of the 'topology' of lesions throughout the aortic root. We have found this useful in previous studies since some effects have been limited to the extension of lesions into the ascending aorta. Other interventions may influence the thickness of lesions in the aortic sinus. Subsequent analysis can be performed in which there is a combination of sections to achieve a sum of lesion areas in a defined number of sections. Although there are no publications to date, determination of the lesion area of sequential aortic root sections would permit the determination of lesion volume in this region.

***En face* lesion quantification**

This form of analysis determines lesion area of all, or selected, regions of the aortic intima. For this mode of analysis, we dissect the aorta free from the point that it emerges from the anterior surface of cardiac ventricular tissue through to the ileal bifurcation, as noted above. Aortas are placed in a freshly prepared paraformaldehyde solution (4 per cent wt/wt in phosphate-buffered saline) overnight. Adventitial tissue is removed next day. Complete removal of adventitia can be difficult to achieve, but proficient operators can remove sufficient tissue within 15 min. Aortas are cut as described in Figure 5.2. The initial cut is performed in the lesser aortic curvature and is extended along the length of the vessel to the ileal bifurcation. A second cut is made on the midline of the greater curvature to the level of the subclavian artery. Atherosclerotic lesions in mice can be dislodged from the intimal surface, so care should be taken in the cutting process. The standardisation of these dissection lines is important to maintain a constant region area for lesion analysis.

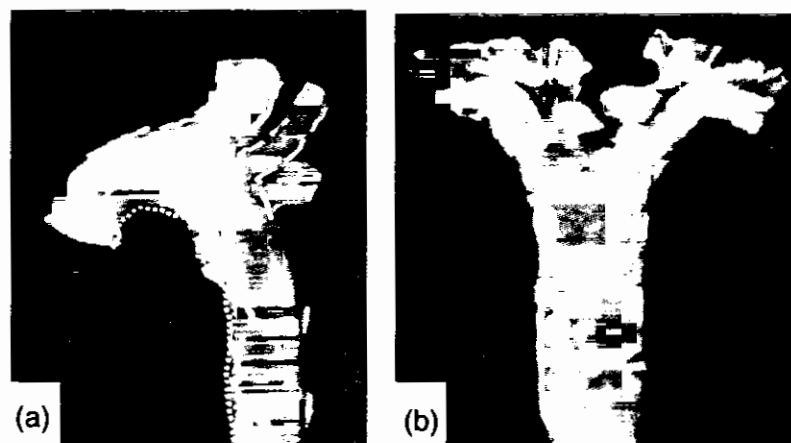


Figure 5.2 *En face* analysis of atherosclerosis lesions. (a) The dotted and dashed lines indicate the mode of cutting of the aortic arch region. (b) An example of an aorta pinned for image analysis of lesion area. For a color version of this figure, please see the images supplied on the accompanying CD

Aortas are placed on black paper that overlays dental wax. Since there is an interaction between the paper and aorta, a relatively small number of pins (0.2 mm diameter minuten pins, Fine Science Tools, cat #26002-20) will be needed to keep tissues flat. Images are acquired of the intimal surface using a digital camera connected to a dissecting microscope. In our analysis, we routinely acquire photographs of unstained aortas. The staining of neutral lipids, for example using Sudan IV, has the potential to facilitate lesion identification. However, any residual adventitial adipose tissue will be strongly illuminated by neutral lipid staining and will be clearly visible through the translucent mouse aorta. This adventitial staining can confound the identification of lesions. Also, the neutral lipid stain will negate any subsequent analysis of tissue sterols that can be an additional mode of atherosclerotic lesion analysis.

As with the previous mode of analysis, we trace the lesions manually using the grossly discernable margins of lesions. Even with lipid-stained aortas, we have not been able to reliably set a threshold hue to automate lesion area quantification. Since there is some level of subjectivity to the decisions of lesion boundaries, the measurements should be verified by a second operator. In aortas with large atherosclerotic involvement, the distinction of lesion boundaries is clearly defined. Thus, the manual tracing can be completed rapidly and concordance between operators is usually high. In cases in which lesions are small and scattered, the ability to discern lesion boundaries becomes more subjective. In this case, we have two operators independently quantify the area of lesions.

Table 5.1 Comparison of some of the most commonly used mouse models of atherosclerosis when applied to gene manipulations in whole body and bone marrow-derived donor cells

| Mouse | Advantages | Disadvantages |
|---------------------|--|---|
| C57BL/6 | Availability Ease of breeding compared to generation of compound deficient mice | Requirement for cholate in diet Small lesions of simple morphology |
| ApoE ^{-/-} | Development of lesions on normal diet Complexity of lesions that can develop containing fibrous caps and necrotic cores | Unusual lipoprotein composition ApoE deficiency may have many confounding effects, for example on immune function Limited evidence that ApoE deficiency in humans promotes atherosclerosis Use in bone marrow transplantation studies requires donor cells to be ApoE ^{-/-} |
| LDL receptor | Rapid development of lesions Ease of use for bone marrow transplantation studies | Requirement for feeding a modified diet Lesions predominantly contain lipid laden macrophages with minimal other components |

Data are commonly represented as either absolute lesion area or as a percent of intimal area. Unless there is a change the intimal areas of aortas, in the comparative areas between groups should be the same with both these modes of data representation. During early stages of atherosclerotic development, lesions will be evident mostly in the arch. Thus, quantification of the thoracic and abdominal region may provide rather limited information, except with more advanced disease.

One of the potential shortcomings of this mode of analysis is that area measurements fail to account for any variances in lesion thickness between experimental groups. We have seen in previous studies that large changes in the extent of lesions (defined as thickness or volume) can occur in the absence of any change in coverage of intimal area.¹⁴ Therefore, during image analysis to quantify lesion area, notes should be made regarding lesion appearance, especially in relation to thickness. The evaluation of lesion thickness can be performed using standard histological approaches. However, a systematic evaluation of lesion thickness, even in a restricted area such as the aortic arch, would require the acquisition of many tissue sections and considerable effort. An alternative approach has been to determine the sterol content of the tissue. In unstained paraformaldehyde fixed tissues, sterols can be extracted and quantified using a gas chromatogram or enzymatic technique. In lesions that are predominantly lipid-laden, this provides an index of lesion volume.

Other considerations

Since there are different modes of analyzing atherosclerosis in mice, a question arises as to the relative benefits of measuring lesions in different regions, or if more than one arterial region should be quantified. Since neither the lesions that form in the aortic root or throughout the aortic wall have been implicated in atherosclerosis-induced diseases, it is difficult to rationalise that one area should be prioritised compared to another. Analysis of both areas of the same mouse is plausible, although this increases the workload of an already labor-intensive process. However, if both regions show the same result, this provides greater confidence in the effects of an intervention. In general, effects of interventions on lesions in aortic roots have paralleled those noted by *en face* analysis, although the effects of interventions to reduce atherosclerosis have usually been more profound by *en face* analysis than in the aortic root. There are also a limited number of studies that have noted different effects on atherosclerosis by these two approaches, although this is a minority of publications.

Determination of lesion composition

Atherosclerotic lesions have complex cellular compositions that change at different stages of development. Therefore, in addition to quantifying the size, it is useful to determine the cellular composition of lesions. Table 5.2 provides examples of

Table 5.2 Examples of antibodies used in immunocytochemical studies to characterize cellular composition of atherosclerotic lesions

| Cell type | Antigen | Antibody | Source | Reference |
|--------------------|------------------------------|--------------|----------------------|-----------|
| Macrophage | Mouse peritoneal macrophages | AIA31240 | Accurate Chemical Co | 19,20 |
| | CD11b | MAC-1 | Serotec | 21 |
| | Unknown | MOMA-2 | Serotec | 17 |
| | CD68 | FA-11 | Serotec | 22 |
| | F4/80 | CI:A3-1 | Serotec | |
| T lymphocyte - pan | CD90 (Thy1.2) | F7D5 | Serotec | 24 |
| CD4 | CD4 | GK1.5 (L3T4) | Chemicon | 24,25 |
| CD8 | CD8 | YTS105.18 | Serotec | 24 |
| B lymphocyte | Mouse CD19 | 1D3 | Pharmingen | 26 |
| Endothelial | CD31 - PECAM | MEC13.3 | BD Pharmingen | 26 |
| Smooth muscle | Alpha actin | 1A4 | Sigma/Dako | 17 |

antibodies used to identify resident cells of the arterial wall (endothelial and smooth muscle) and the most prominent cell types that infiltrate the evolving atherosclerotic lesion (macrophages, T lymphocytes and subclasses, and B lymphocytes). Of the many cell types present in atherosclerosis, perhaps the most difficult to immunodetect in a uniform manner are macrophages, since this cell type is so heterogeneous in lesions. Many of the monoclonal antibodies that react against macrophage antigens are differentiation dependent and therefore react with macrophage subsets. To overcome this, we have used antisera developed in rabbits that have been injected with mouse peritoneal macrophages. The use of a cell as the immunising agent leads to the development of antibodies against many macrophage antigens. This should provide an increased likelihood of detecting all macrophages.

Conventional immunocytochemical procedures are used to detect cells in lesions. However, one issue that should be addressed is the high non-specific background of chromogen development that often occurs within atherosclerotic tissue. This issue amplifies the need for appropriate control experiments. At a minimum, controls should include performing the procedure in the absence of the primary antibody to determine non specific chromogen development through the secondary antibody and detection complex. It is preferable to include a control for the primary antibody that will include non-immune sera for antisera, non-immune antibodies for purified IgG or IgM, and isotype-matched antibodies against irrelevant antigens for monoclonal antibodies.

Statistical analysis

The development of experimental atherosclerosis is usually characterised by inherent variability of lesion size within groups. This may provide some challenges in terms of the number of animals needed to demonstrate an effect. It is also common to acquire

data in which numbers do not conform to equivalence of variance and normality of distribution. Therefore, the basic tenets of statistical analysis apply in that data should be determined for suitability for analysis by parametric or non-parametric tests. Application of the correct statistical test is facilitated by many software packages. In our laboratory, we use SigmaStat which has a user friendly interface and an interactive mode that suggests the optimal test for a specific dataset.

Conclusions

Since the generation of ApoE^{-/-} and LDL receptor^{-/-} mice in the early 1990s, there has been a dramatic increase in the use of the mouse as an atherosclerosis model for studying potential mechanisms of human disease. Despite these many studies, there is a lack of consensus on many facets of atherosclerotic lesion quantification and characterization. An establishment of rigorous standards may reduce the number of studies in which data conflicts. For example, there is conflicting data for the effects of scavenger receptor deficiency on atherosclerotic lesion formation.¹⁵⁻¹⁷

There is continuing debate on the extent to which lesion development in the mouse mimics human disease. There are specific facets of the early stages of the human disease that are faithfully recapitulated, especially the development of lipid-laden macrophages. Also, some models experience extensive fibrotic components and development of acellular cores containing crystals of unesterified cholesterol. However, lesions in mice infrequently progress to late stages of human disease as defined by American Heart Association class IV and V.¹⁸ As a consequence, mice seldom manifest the diseases that occur so rampantly in humans as a consequence of atherosclerosis. It is likely that future efforts will be directed to devise models in which acute coronary syndromes and strokes will be generated routinely.

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