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Development of experimental designs for atherosclerosis studies in mice

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Abstract

The mouse has become the de facto model for the majority of atherosclerosis studies. Studies involving the quantification of lesions in mouse models of the disease represent the basis of our evolving concepts on the biochemical and cellular mechanisms underlying the atherogenic process. Many issues of experimental design, including specific model, strain, gender, atherogenic stimulus, duration of study, group size, and statistical analysis may influence the outcome and interpretation of atherosclerosis studies. The selection of vascular bed in which to quantify atherosclerotic lesion size could also impact the interpretation of results. Early studies quantified atherosclerotic lesion size in either specific regions or all of the aortic sinus. Measurement of atherosclerosis throughout the aortic intimal surface has become a common mode for defining lesion size. It is likely that other vascular regions will be increasingly used. In addition to size, there is an increased emphasis on identifying and quantifying the cellular and chemical composition of atherosclerotic lesions.

Keywords: Atherosclerosis; Mouse; Image analysis

1. Introduction

Atherosclerotic lesions form in childhood although overt clinical diseases do not usually become apparent in most individuals for many decades [1]. Despite the widespread occurrence of atherosclerotic-related diseases, our understanding of the pathobiology of lesion formation is incomplete. The determination of the temporal changes in cellular and chemical components of lesions, and the mechanisms responsible for these changes, is hampered by several features. One of the principal impediments is the difficulty of obtaining atherosclerotic tissue in a sequential manner. Thus, the description of pathological changes in arteries is restricted to cross-sectional studies using tissues acquired in the cadaveric state or during surgery [2–4]. Advances in non-invasive imaging modalities should provide this information in the future, but the current technologies provide limited information on lesion composition. Other major factors that hinder elucidation of atherosclerotic mechanisms in humans include the chronicity of lesion evolution combined with the complex interactions of many chemical and cellular elements [5,6].

In view of these obstacles in defining atherogenesis in humans, animal models have provided valuable insight into the sequential events and the mechanisms responsible for initiation and maturation of lesion formation [7–10]. Many animal models have been used over the years, but historically there has been a preponderance of studies in monkeys, pigs, and rabbits [11]. The latter species were frequently used because of their sensitivity to diet-induced hypercholesterolemia and the availability of a widely used genetic variant, the Watanabe heritable hyperlipidemic rabbit, that is spontaneously

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hyperlipidemic due to functional deficiency of low density lipoprotein (LDL) receptors [12,13].

Contemporary atherosclerosis research is dominated by use of mouse models. The ability of mice to develop diet-induced atherosclerosis in a strain-dependent manner has been known for over two decades [14–18]. The use of mice in atherosclerosis research accelerated greatly with the development of genetically manipulated strains that exhibit extensive and mature lesions. The most extensively characterized of these strains are mice deficient in either apolipoprotein E (apoE) or LDL receptors [19–21]. There are now many mice available that develop atherosclerotic lesions, either spontaneously or during the feeding of modified diets, as a consequence of genetic engineering to overexpress, delete, or knockin many different genes [22,23]. All of these atherosclerosis-susceptible strains have a lipoprotein or lipid disorder as the precipitating factor for lesion development. However, a wide range of non-lipid and lipoprotein mechanisms modify the extent and characteristics of atherosclerosis [24,25].

There is considerable interest in defining factors that modify atherosclerosis, with a preponderance of these studies being performed in mouse models of the disease. To assist the laboratories that are entering this field, the purpose of this manuscript is to provide a discussion of the development of an experimental design for atherosclerosis studies in mice. We will also provide some discussion on the interpretive impact of decisions of experimental design.

2. Decisions in the development of an experimental design

The decision process in the development of an experimental design is likely to consider both scientific and practical issues. In Fig. 1, we list an example of a decision process in the determination of the experimental design, as will be discussed in the text.

2.1. Selection of mouse model

The first choice in a mouse atherosclerosis study is to select one of the many available strains of wild-type or genetically engineered mice [22]. Early studies predominantly used C57BL/6 mice fed a diet that was supplemented with saturated fat, cholesterol, and cholate [17]. Use of wild-type C57BL/6 mice has largely been supplanted by genetically manipulated mice that are either endogenously hyperlipidemic or susceptible to diet-induced hyperlipidemia. The most commonly used genetically manipulated mice are those deficient in either apoE or LDL receptors. The former are hyperlipidemic and form atherosclerotic lesions on normal mouse diets [20,19]; while the latter require dietary supplementation of saturated fat and cholesterol to develop significant

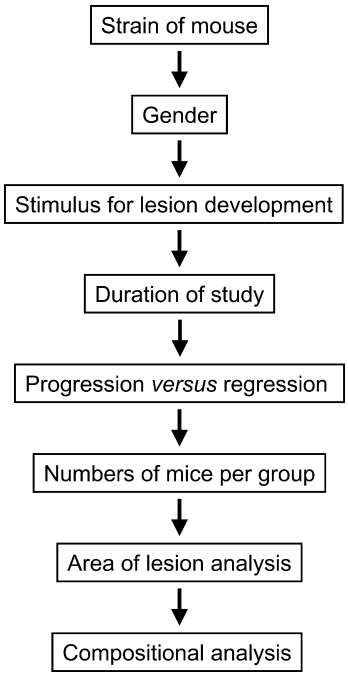

Fig. 1. The decision process in the development of an experimental design for atherosclerosis studies.

lesions [26,27]. Both apoE and LDL receptor-deficient mice are available from the Jackson Laboratory and other commercial companies. The available mice that have been backcrossed between 10 and 12 times into a C57BL/6 background.

Many genetically modified atherosclerosis-susceptible mice are now available that can provide mechanistic insight into the disease process [22,23,28]. Given the frequent use and relative ease of availability of apoE−/− and LDL receptor−/− mice, the decision process commonly involves a selection between these two strains. There are several factors to consider since in addition to the divergent lipoprotein characteristics, these mice also differ in immune function, susceptibility to obesity, and glucose homeostasis [29,30]. Therefore, the impact of these differences will be determinants in experimental designs.

The selection of a specific model will depend on the nature of the mechanistic insight that is required. For example, any intervention that lowers plasma cholesterol concentrations of apoB containing lipoproteins is likely to reduce the size of atherosclerotic lesions. Although there are some exceptions to this assumption [31,32], most lipid lowering strategies have reduced lesion size [33,34]. If direct vascular wall mechanisms of an intervention are being determined, any changes in plasma lipoprotein concentrations will complicate the interpretation. Judicious selection of an animal model can assist in the determination of the site of action of an intervention. For example, administration of estradiol reduces atherosclerotic lesion size in both apoE−/− and LDL receptor−/− mice. However, unlike apoE−/− mice, estradiol does not decrease plasma cholesterol

concentrations in LDL receptor-deficient mice which has been interpreted as a direct vessel wall effect [35,36].

Another issue in the decision of a specific mouse model is the cell type that is expected to be affected by the intervention. For example, atherosclerotic lesions of all mice contain macrophages and therefore may be appropriate for study of this cell type. However, smooth muscle cells are sparse in many mouse models of atherosclerosis, and investigators would need to be more selective of appropriate strains and conditions for studies on this cell type. ApoE−/− mice maintained on a normal laboratory diet are likely to have the largest content of smooth muscle cells in currently available models. All mouse models have a relatively sparse lesion infiltration of lymphocytes compared to the human disease [37,38]. Therefore, the extrapolation of any negative or modest phenotypic changes in acquired immune function from mice to humans should account for this difference [39–41].

2.2. Genetic manipulation of whole body versus restriction to bone marrow-derived stem cells

Early use of bone marrow transplantation demonstrated that repopulation of irradiated apoE−/−mice with cells from apoE+/+ donors normalized plasma cholesterol concentrations and ablated atherosclerotic lesions formation [42,43]. These initial reports spawned many atherosclerosis studies using this technique. One of the considerations of experimental design is the phenotype of the bone marrow-derived stem cells in relation to lipoprotein metabolism. These initial studies demonstrated the need to use donor cells from apoE−/− mice to repopulated irradiated apoE−/− recipients. Therefore, these studies require the use of compound-deficient mice. For application of this technique in LDL receptor−/− mice, there is not such a clear need for compound-deficient mice. All the current publications have demonstrated that LDL receptor phenotype of the donor cells does not significantly impact the size of atherosclerotic lesions when used to repopulate LDL receptor−/− mice [44–46]. This has the potential benefit that the complexity of developing compound-deficient mice is not needed for donors. Although the LDL receptor genotype of donor cells has no documented effects in LDL receptor-deficient recipients, it should be noted that the bone marrow cell LDL receptor genotype does affect the size of lesions in C57BL/6 mice [45,46].

One of the interpretative issues is the effect of the bone marrow transplantation process on lesion development. The one study that has directly compared the effects of irradiation in mice has noted a site-specific effect on lesion formation [47]. Mice that had undergone irradiation and repopulation had decreased size of lesions in the thoracic aorta, but increased size in the aortic sinus. Lesions in these mice also have differing composition, with increased lipid deposition and reduced collagen. Currently, there is no further insight into the mechanisms of this effect or how this should be considered in defining atherogenic mechanisms.

A major interpretative issue has arisen in defining the cells types within atherosclerotic lesions that are repopulated by the transplanted bone marrow cells. It is clear that bone marrow-derived cells have the potential to be differentiated in myeloid and lymphoid lines. The initial studies usually interpreted their results in terms of macrophage metabolism, largely based on this being the predominant cell type in mouse atherosclerotic lesions of a myeloid and lymphoid origin. However, this interpretation may need to be reassessed in light of evidence that bone marrow-derived stem cells have the potential to transdifferentiate. Presently, there are conflicting data that ranges from findings that only leukocytes in atherosclerotic lesions arise from bone marrow-derived cells, to studies demonstrating that a large proportion of endothelial and smooth muscle cells originate from this source [48–50]. While the discussion of the ability of bone marrow stem cells to transdifferentiate is beyond the scope of this paper, the potential for complex interpretive issues should be noted.

2.3. Background strain of mice

The early studies on inbred mice noted the profound effect of strain on the development of atherosclerosis. In fact, these differences were exploited in studies to identify genes responsible for the development of lesions [51,52,17].

The marked effects of strain background have also been shown in apoE-deficient mice. For example, lesion formation is dramatically less in C3H and FVB/N mice compared to C57BL/6 mice despite equivalent hyperlipidemia [53,54]. The effect of strain difference has potential importance in studies in which atherosclerosis in mice with a single gene deletion is compared to lesions in mice with compound gene deficiency. Therefore, one important issue in the development of compound-deficient mice is ensuring strain equivalency to the control mice. Thus, the unambiguous interpretation of data when comparing compound- and single-deficient mice is contingent on strain equivalency.

Two major strategies are employed in an effort to ensure any changes in lesion formation are attributable to a single gene when using compound-deficient mice. The most commonly employed approach is to backcross into an equivalent strain. The most common background strain in atherosclerosis studies is C57BL/6. Using random breeding approaches, it is usual to backcross 10 times. The number of backcrosses may be reduced by using the technique of speed congenics in which offspring from each mating are selected based on the greatest similarity to the target strain. While this will

decrease the time to development of strain equivalent mice, it is a significant technical barrier for many laboratories. However, such service is now offered on a contract basis at the Jackson Laboratory and Charles River Laboratories. The major disadvantage of the approach of backcrossing is the time and expense of generating the mice. An alternative approach is the breeding of atherosclerosis susceptible mice from parental strains that are heterozygous for a gene of interest. The littermates from this mating strategy may be compared for atherosclerotic lesion characteristics. Although there is likely to be genetic variance within these groups, this should be random. Therefore, any difference in lesion size of littermates should be the consequence of the single gene. The confidence in the interpretation of such studies is assisted by the use of large group numbers and a profound effect on atherosclerotic lesion formation.

2.4. Gender of mice

It is clear that gender could exert a major effect on the outcome of atherosclerosis studies [55,56]. Many studies have used female mice because of the assumption that they will develop a greater extent of atherosclerosis. While there have been studies that have demonstrated the development of larger lesions in female [57,58], this has not been a consistent finding [36,55]. Because of difference in the size of lesions and the potential for differing mechanisms, it is undesirable to perform studies with groups of mixed gender. It is not clear that there are distinct advantages to using one gender over the other. Given the number of gender-specific responses that have been observed, optimally, each study should include sufficient mice of both genders to permit gender-specific statistical analysis of atherosclerosis [59,56,55].

There are practical issues relating to the housing of genders. Female mice can usually co-exist in groups, even when mixing mice from different litters. However, males of most strains can exhibit aggressive behavior and mice from different litters cannot be mixed unless this occurs at weaning age. Consequently, cages of male mice should be observed for any indication of injury related to adverse social interactions. This may necessitate the single housing of individual male mice.

2.5. Stimulus for the development of atherosclerosis

The genetic manipulation of some strains of mice can eliminate the need for additional stimuli for lesion formation. For example, apoE−/− mice are spontaneously hypercholesterolemic and do not need further manipulation for lesion development. However, different diets have been used to accelerate lesion formation, particularly those enriched in saturated fat and cholesterol. By gross pathology, the inclusion of saturated fat and cholesterol in the diet has been shown to promote the same atherogenic process as occurring during the feeding of normal laboratory diet [60]. This may also lead to a fundamental difference in the mechanisms responsible for generating lesions. For example, lack of mature lymphocytes in RAG1 and 2-deficient mice decreases the size of lesions in apoE−/− mice fed a normal diet, but not in those with augmented hypercholesterolemia by the feeding of diets enriched in saturated fat and cholesterol [39,40,61].

In many other strains, the diet has to be modified to develop detectable lesions. The most extreme diet was used in the original studies with C57BL/6 mice. This diet is enriched in saturated fat (21%wt/wt), cholesterol (1.25%), and cholate (0.5%). The cholate component has become the most controversial, because of its propensity to initiate inflammatory processes [62]. This component was probably needed in the wild-type C57BL/6 mice in order to develop atherosclerosis. Although the use of cholate is controversial, its inclusion is not needed in the era of genetically manipulated mice. Overall, there have not been extensive systematic studies of the role of different fats and cholesterol contents on the development of atherosclerosis in the most commonly used mouse models of atherosclerosis, although there is some limited information [27]. Therefore, dietary decisions are commonly based on empirical considerations. The most commonly used diet is enriched in saturated fat to 21%wt/wt with cholesterol in the range of 0.15 to 1.25%. This has become colloquially known as the "Western diet" given that it approximately mimics the average dietary composition consumed by humans in the Western hemisphere.

In addition to diet, the infusion of angiotensin II has recently emerged as a profound stimulus of atherogenesis, even in mice that already develop lesions such as LDL receptor−/− and apoE−/− [63–65]. This promotion of disease has a human correlate based on the results of the HOPE trial in which an ACE inhibitor markedly decreased the severity of atherosclerosis diseases despite a minimal change in blood pressure [66]. Also, angiotensin II has a major role in the development of hypercholesterolemia-induced lesions since either pharmacological or genetic attenuation of AT1 receptors reduced atherosclerosis in both LDL receptor−/− and apoE−/− mice [67–69]. It is not known whether the infusion of angiotensin II promotes the development of atherosclerosis by mechanisms that differ from hypercholesterolemia. Currently, there have been similar responses in atherosclerosis between hypercholesterolemia and angiotensin II infusion in mice with deficiencies of the chemokine receptor, CCR2 [70,71], estrogen [35,72], and osteopontin [73,74].

2.6. Duration of study

Atherosclerosis studies require prolonged housing of mice and are labor intensive to determine the size and

characteristics of lesions. As a consequence, the vast majority of studies are performed with lesions being defined at a single interval defined by the age of the mouse or the duration of the atherogenic stimulus. On the assumption that the extent of disease being studied by a manipulation is either unaltered or consistently changed, the single time point will provide an accurate evaluation [75]. However, one theoretical scenario is that the effect of the manipulation is only transient. Therefore, an early evaluation would determine a difference that would be missed if only late stages of the disease were defined. There are examples of transient effects on atherosclerosis. For example, endothelial-specific overexpression of 15-lipoxygenase [76] and deficiency of mature lymphocytes [41] led to transient increases and decreases in lesion size, respectively, that were not sustained. There is also the potential for a manipulation to only effect lesions at later stages of the disease process. Although no clear guidelines can be provided, for the determination of a drug or genetic effect compared to control groups, it would be optimal to determine atherosclerosis at more than one interval.

2.7. Studies of progression versus regression

The vast majority of mouse atherosclerosis studies define the effects of a genetic manipulation or pharmacological intervention that are initiated prior to the development of atherosclerotic lesions. This has provided valuable information on mechanisms of atherosclerosis initiation and propagation. However, it may not be so relevant to treatment in the clinical scenario that requires changes to existing lesions to deter subsequent growth, or even promote regression. Mechanisms of regression have been relatively understudied. One of the practical issues in performing studies on regression mechanisms is the protracted interval needed for the completion of these studies. Also, while pharmacological interventions are readily amenable to regression studies, the use of genetic manipulations is more limited. Fortunately, there are increasing options for influencing gene expression in a temporal manner for regression studies. One that has already been used is the adenoviral vector based expression [77,78]. The intravenous injection of these vectors leads to virtually exclusive expression in the liver and hence its use is most commonly restricted to changes in peripheral mechanisms rather than at the level of the arterial wall. However, the development of systems to control the temporal and spatial expression of genes should provide mechanistic insight into lesion regression [79].

2.8. Numbers of mice for each group

Determination of appropriate group sizes for a statistically reliable study will require knowledge of the variance of the control groups and the predicted change brought about by a manipulation. Literature values may be a poor gauge for the estimation of extent and variance of lesion size. In the authors' experience, there is considerable variance in the extent of atherosclerosis for the same model in different laboratories. While the source of this variance is unknown, it may be accounted for by a range of environmental factors such as type of bedding material, variation between different batches of a specific type of food, and housing conditions (i.e., barrier versus non-barrier facilities) [80].

Although most atherosclerosis studies are performed on inbred mice in controlled environments with standardized diets, there tends to be a high inherent variability in the extent of atherosclerosis formed. The size of lesions within groups of mice also frequently does not follow a Gaussian distribution. Therefore, the definition of relatively small changes in the size of atherosclerotic lesions generally requires reasonably large group sizes for robust statistical analysis. We commonly design our studies with 20 mice per group in total. In general, we pool numbers from two repeated studies to ensure reproducibility.

2.9. Vascular bed in which lesions will be quantified

The bulk of atherosclerosis quantification in mice has been performed on lesions that have formed in the aortic root. Historically, this was the only area in which atherosclerosis was consistently present in all pertinent models. With the advent of genetically manipulated mice, atherosclerotic lesion development occurs in other vascular areas. This has led to the use of en face analysis of lesions on the intimal surface of the aorta [58], as was routinely performed in larger animal models of atherosclerosis [81]. Since the technical details of quantifying lesions in these regions are described elsewhere [82], we will restrict comments to considerations of experimental design and interpretation.

2.9.1. Aortic root

Measurement of atherosclerotic lesion size was initially described in detail by Paigen et al. [83]. Briefly, this method entails the sequential tissue sectioning from the origin of the aortic valves to a region in the ascending aortic arch. This produces sections similar to those shown in Fig 2A. In our studies, we cut 10 μm frozen sections of the aortic root. For this analysis, up to nine tissue sections of aortic sinus at 80 μm intervals are placed on a single slide. This is accomplished by placing the initial sections in the lower left corner on each of eight slides. Sequential sections are then placed serially on these eight slides. By using this placement strategy for each slide, it is possible to create serial sections from the entire aortic root.

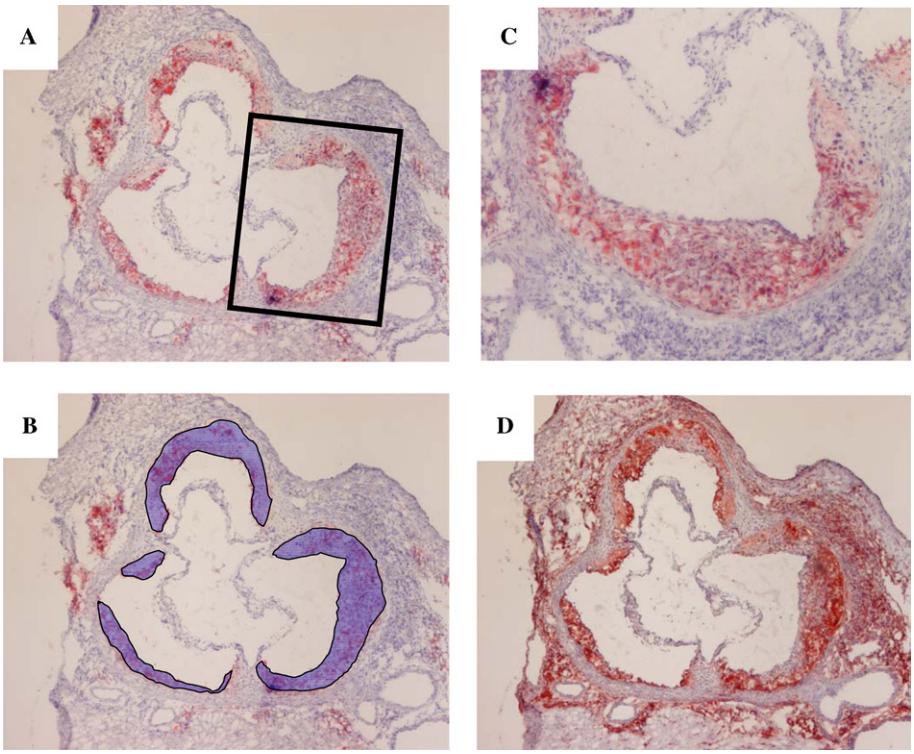

Fig. 2. Analysis of atherosclerosis in the aortic root. (A) An example of lesions formed in the aortic sinus of an LDL receptor-deficient mouse stained with oil red O. (B) The same tissue section as represented in A with manually traced overlays for the quantification of lesion size. (C) A higher magnification of a lesion, indicated by the box in Fig. 2A, staining with oil red O to demonstrate the inconsistent staining throughout the lesion. (D) An example of immunostaining of macrophages in mouse atherosclerotic lesions using a pan antibody available from Accurate Chemical Company.

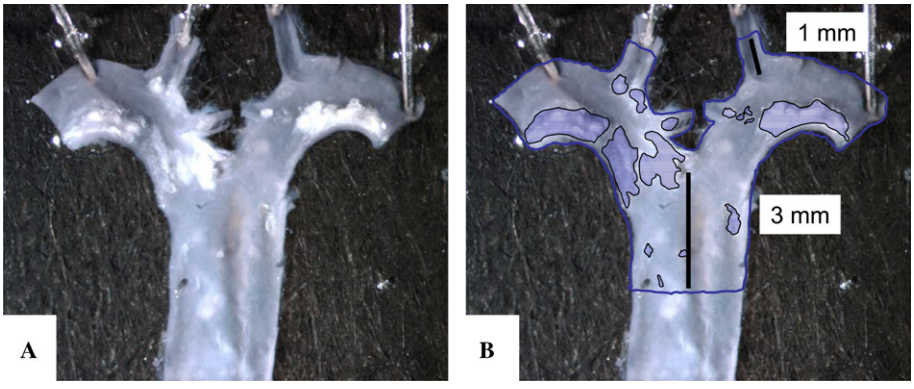

Fig. 3. Analysis of atherosclerosis on the aortic surface. (A) A photograph of an unstained aortic arch of an apoE−/− mouse. (B) An example of traced overlays on lesions to measure the area of disease that is commonly represented as a percentage of the intimal surface.

Lesions can be easily visualized with many stains, of which oil red O is one of the most common. The size of lesions can be quantified by image analysis software, preferably by an individual who is blinded to the experimental design. We perform this analysis by manually outlining the lesions from the internal elastic lamina to the luminal edge as shown in Fig. 2B. The development of automated quantification of lesion size is compromised by the inability of a stain to uniformly cover the entire area of the lesion. For example, as can be seen in Fig. 2C, oil red O provides an inconsistent stain within the lesion as may be expected in lesions that contain regions of extracellular matrix and unesterified cholesterol, in addition to the neutral lipid stained by oil red O. Another common mode of visualizing atherosclerotic lesions includes the immunocytochemical staining of macrophages as demonstrated in Fig. 2D. However, this form of staining is also unlikely to consistently define the complete margins of lesions.

There is no agreement on the number of sections that need to be measured for an authentic quantification of lesion size. There is clearly a variance of lesion size throughout the aortic root, and therefore any measurement needs to procure tissue from the equivalent region in

the root. The mode of analysis described above provides the area of cross-section of lesions throughout the aortic root [84]. In our studies, we orientate section relative to the disappearance of the aortic valve cusps and represent the lesion area throughout the root [85–87]. The definition of lesion size throughout the root is preferable to prevent data been inadvertently prejudiced by selection of sections that may come from different regions of the root.

Overall, quantification of lesions in the aortic root is the most frequently used mode of quantifying atherosclerotic lesions in mice. One of the major drawbacks of this technique is the technical skill and time required to acquire serial sections throughout the entire aortic root.

2.9.2. En face analysis of the aortic intimal surface

Briefly, this technique involves the removal of the entire length of the aorta. The intimal surface is exposed, in its entirety, by a longitudinal cut through the inner curvature of the aortic arch and down the anterior aspect of the remaining aorta. A cut is also made through the greater curvature of the aortic arch to the subclavian branch. The aorta should be observed through a dissecting microscope during this cutting since mouse lesions may be displaced from the aorta during manipulations. The tissue is then pinned to a dark surface. If the lesions are sufficiently mature, they are clearly visible without staining, as can be seen in Fig. 3A. Atherosclerotic lesions can be traced for quantification of the area of lesion that is usually represented as a percent of the total intimal surface (Fig. 3B).

Lesions are also commonly visualized after a neutral lipid staining. This offers a limited advantage in large lesions, but can assist in the visualization of small lesions. The intense coloration of residual adipose on the adventitial surface following neutral lipid staining is clearly visible through the translucent mouse aorta and can lead to confusion. Staining of neutral lipids is needed to apply automated lesion size measurement techniques. This technique requires the setting of a threshold to define a color intensity that discriminates lesions from normal areas. The ease of setting a meaningful threshold can depend on the size and configuration of lesions. Alternatively, lesions can be traced manually. Since this requires some arbitrary decisions by the observer on lesion boundaries, it is preferable for at least 2 individuals quantify the lesions in every aorta. Even high quality image capture equipment can frequently produce images in which there may be some ambiguity in visualization of lesions. Therefore, it is preferable to observe the pinned aorta during the analysis of images to ensure that only lesions are being outlined.

Lesion size is normally represented as the percent of the intimal surface that is covered by atherosclerotic lesions. The meaningful interpretation of this requires that a standardized area of intima is quantified. Therefore, care should be taken to ensure consistency in the dissection, opening, and pinning of the aorta. We analyze lesions in the arch (ascending arch to 3 mm distal of the subclavian), thorax (arch to last intercostal branch), and abdomen (thorax to ileal bifurcation). Generally, lesions will initially develop in the arch and will only be present in measurable amounts in other aortic regions in animals with more advanced disease. Therefore, the sparse lesion presence in the thoracic and abdominal aorta may preclude the utility of lesion measurement in this region unless they are of significant size.

The measurement of lesions by this approach provides a two-dimensional size without taking into account the thickness of lesions. We have noted previously that lesion area was not a good indicator of lesion burden in which thickness and volume are also considerations [88]. Lesion thickness can be evaluated by histological sectioning, although this would be time consuming. An alternative approach is the quantification of the unesterified and esterified cholesterol content of aortic segments. Sterols can be extracted from tissues and quantified by enzymatic analysis [82] or gas chromatography [40]. Such analysis is not permissible if the tissue has been stained for neutral lipid.

2.9.3. Other vascular regions

The innominate artery has recently been proposed as a preferable region based on the relatively rapidity of lesion development and the more complex nature of the disease [89]. Although used in several studies on lesion characteristics, it has not been used extensively in quantitative studies. Its small size provides some technical challenges, but lesion formation in the innominate artery may provide valuable insight into the disease process.

2.10. Interpretation of lesion measurements

One of the decisions in study design is whether atherosclerotic lesions in one or more vascular beds should be quantified. Since there are examples of site selectivity in the effect of an intervention on atherosclerosis, there are advantages to obtain data in more than one region. If an intervention exhibits similar effects on lesion size in all regions quantified, this offers a straightforward interpretation on the effect on atherogenesis. Divergence of the effects of a manipulation on the size of lesions in different vascular beds provides a greater challenge to interpretation. There are several examples in which an intervention has had a lesser or no effect on lesion size in the aortic root while decreasing lesion size by the en face assay [90,91]. This may be partially attributed to the lesions being initiated in the aortic root with the options for discriminating differences diminishing over time. There is no obvious explanation for studies in which lesion size differences are noted in the aortic root but not by the en face approach.

The issue of relating mechanisms of atherosclerosis in mice to those occurring in humans is not clear. The regions in which atherosclerosis is quantified in mice are not those that are responsible for the overt cardiovascular disease in humans. In fact, the great vessels of the coronary circulation and the branches of the carotid arteries that are responsible for heart disease and stroke, respectively, in humans, are not sites that are prone to disease in mice. It is unclear whether these differences in location of lesions in mice is representative of the presumably differing hemodynamics between mice and humans.

2.11. Assessment of lesion composition

Although the quantification of lesion size has been the end-point of the majority of experimental atherosclerosis studies, there is general acceptance that the acute syndromes of atherosclerotic diseases are a consequence of abrupt changes that may be related to the cellular and chemical composition of lesions [92,93]. The determinants of lesions that portend the development of acute atherosclerotic-related syndromes, in the majority of cases, is thought to relate to a rupture of the fibrous cap in shoulder regions of lesions that exposes a thrombogenic strata containing macrophages [94]. The thrombogenic strata may also be the result of an erosive process [95]. In human lesions, there have been several schemes proposed to describe the pathological appearance of lesions that have a propensity to rupture [1,96]. We are still unsure of the extrapolation of these human lesion morphologies to mouse models. There have been some reports of plaque rupture and instability of mouse lesions [89,97–99], although these have not been consistent [100,101].

In the absence of consistently overt plaque rupture, lesion composition in mice is quantified as a surrogate to define a "vulnerable" phenotype. This is largely based on the relative composition of macrophages, smooth muscle cells, and extracellular matrix, as determined by either standard histological techniques or immunocytochemistry. These are straightforward and routine techniques.

However, application of quantitative approaches to these stains is a nascent field. Generally there are two modes of quantifying components of atherosclerotic lesions. One approach is the counting of a specific cell number. The ability to count a specific cell type can vary depending on the characteristics of the immunostaining. For cells such as T-lymphocytes, immunostaining results in discrete development of chromogen around a nucleus that ready permits discrimination of a single cell [38]. At the other end of the spectrum, immunostaining of macrophages results in more diffuse chromogen development due to the gross hypertrophy of this cell type during lipid engourgement combined with the difficulty in defining cell boundaries. The hypertrophy can also result in staining of a cell whose nucleus is not in the same plane as the tissue section. Once the cell number has been acquired, the data can be normalized to several variables including per lesion, lesion area, or percent of total cells. Unfortunately, the mode of normalization can influence the data. Finally, lesion composition will vary throughout the tissue, and therefore multiple sections should optimally be acquired per lesion.

The second general approach to quantification of lesion composition is to use image analysis to determine the area of histological staining or immunostaining within a region of interest. This approach commonly requires the setting of a threshold to determine the level of staining that is deemed to be specific. Since most staining procedures result in a range of hues, there is a level of subjectivity to setting the threshold. Therefore, the use of operators that are blinded to the experimental design is preferable. This type of analysis is most commonly normalized to lesion area and represented as a percent of area. As with cell number counting, the variation of composition throughout lesions necessitates quantification of multiple sections per lesion for meaningful interpretation. For immunostaining, the interpretation should also take into account the distribution of the antigen. For example, it is unlikely that a macrophage antigen, such as CD68, would be evenly spread throughout the cell on a tissue section. Therefore, data would be more accurately represented as "percent area of CD68 immunostaining" rather than the "percent area of macrophage immunostaining."

As noted at the onset, the quantification of lesion composition is becoming increasingly common. As briefly outline above, there are several potential issues that could impact the interpretation of these type of data and currently, there are no guidelines offered that would be generally accepted. However, this is clearly an important area of lesion analysis that would benefit from implementation of standardized approaches.

2.12. Statistical analysis

A basic tenet of experimental design is meaningful interpretation of data requires the application of appropriate statistical analysis. One specific issue that we frequently note is that the quantification of atherosclerosis provides data that frequently fails the test of equivalent variance and normality of data distribution that is a prerequisite for application of more sensitive parametric analysis. Many statistical software packages will alert the user on the appropriate application of parametric and non-parametric tests.

3. Conclusions

The quantification of the size of atherosclerotic lesions in mice has become a mainstay of research in the

mechanisms of the disease. There are many facets of experimental design that need to be considered to acquire data that is helpful in the advancement of the knowledge of the disease process. However, a thoughtful application for the development, execution, and interpretation of an experimental design is critical to provide insight into the atherogenic process.

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