Using Mice to Dissect Genetic Factors in Atherosclerosis

Hooman Allayee, Anatole Ghazalpour, Aldons J. Lusis

Abstract—The genes that contribute to common, complex forms of atherosclerosis remain largely unknown. Genetic studies in humans have, for the most part, focused on identifying genes that predispose to the traditional risk factors, such as lipid levels and blood pressure, but apart from rare, single-gene disorders, there have been few successes to date. The use of mice to dissect the complex genetic etiology of atherosclerosis offers a viable alternative to human studies, because experimental parameters, such as environment, breeding scheme, and detailed phenotyping, can be controlled. Herein we review how mouse genetics can lead to the identification of genes, some of which would otherwise not have been considered as candidates for atherosclerosis, and provide an overview of the prospects for successful gene discovery in the future. (Arterioscler Thromb Vasc Biol. 2003;23:1501-1509.)

Key Words: atherosclerosis ■ genetic factors

Genetic factors contribute importantly to atherosclerosis in human populations, with a heritability of \( \approx 50\% \). Although there has been considerable success in identifying genes for rare disorders associated with atherosclerosis, our understanding of genes involved in the common forms is very incomplete. A large number of association studies with candidate genes have been performed, but few have been convincingly confirmed. Those that have been confirmed, including variations of hepatic lipase and HDL levels, apolipoprotein E (apoE) and LDL levels, apoCIII/apoAV and triglyceride levels, and paraoxonase 1 and myocardial infarction, as well as a handful of others, explain only a very small fraction of genetic susceptibility to atherosclerosis in humans. Clearly, a more complete understanding of the genetics of atherosclerosis would have important implications for diagnosis, treatment, and risk assessment (reviewed in Lusis et al\(^1\) and Lusis\(^2\)).

In principal, there are 2 ways to assign genes to physiologic processes. The first, called “forward genetics,” uses naturally occurring or induced mutations that affect the process. Usually, the underlying gene is identified by mapping the mutation with linkage analysis and then testing “positional candidates.” Alternatively, if a known gene is suspected of playing a role in a physiologic process, then variations of the gene can be examined by association in cases versus controls, the “candidate gene” approach. The second strategy, termed “reverse genetics,” involves the transgenic modification of a known gene, usually in a mouse or a rat, and then examining its effect on the physiologic process. Both forward and reverse genetics strategies have been crucially important for...
our field. In terms of forward genetics, the most important advances have come from human geneticists who have studied the rare mendelian disorders that perturb risk factors, such as lipid metabolism or blood pressure. For example, during the past few years, identification of genes for Tangier disease (ABCA1) and sitosterolemia (ABCG5 and ABCG8) has transformed our understanding of cholesterol transport. The forward genetics approach can also be applied to the dissection of complex genetic traits, which involve multiple genes and environmental influences. Here, progress has been relatively slow, and most of the successes relevant to atherosclerosis have involved previous biochemical knowledge of the underlying gene (a "candidate gene" strategy). One approach to the problem is to use naturally occurring variations in mice or rats, which simplifies the analysis. This is expected to provide information about a subset of the genetic variation that contributes to disease susceptibility in humans.

The Quantitative Trait Locus Approach

Like humans, inbred strains show genomic sequence variations every few thousand bases, which add up to hundreds of thousands across the genome. Most of these are inconsequential, but those that do influence gene expression or function are the basis for the variation in disease susceptibility (and all other genetically based traits) that is observed among strains. Similarly, such genetic variation in humans is also the basis for the differential disease susceptibility that individuals exhibit.

Using the quantitative trait locus (QTL) analysis approach has allowed the mapping of those specific variations that are responsible for differences in atherosclerosis susceptibility and other related traits. The overall strategy for this approach involves using either F2 or N2 mice generated from a cross (Figure 1) or recombinant inbred (RI) strains. Various sets of RI strains were the main source used for identifying loci that underlie atherosclerosis and related traits before the advent of the molecular and statistical tools required for analyzing a cross. Each RI strain is in fact inbred and has been developed from intercrossing 2 parental strains for 20 generations, during which time the genomes of the progenitors are broken into homozygous intervals of different length. By comparing...
the distribution of a trait (eg, atherosclerosis) in the strains from 1 RI set of animals with the distribution of the polymorphic genetic markers already typed for those strains, chromosomal loci that segregate with the trait of interest can be identified. In general, RI mapping has had low power and precision to detect QTLs, mainly owing to the small number of available strains in each set. Recently, Williams et al14 published a dense map for all RI sets that share C57BL/6J as a parental strain, which might provide a tool for RI mapping of some complex traits in the future.

In recent years, investigators have initiated QTL studies with crosses between inbred strains that differ in their susceptibility to atherosclerosis. Even though the use of this approach to dissect complex traits is still a long and laborious undertaking, the development of large numbers of genetic markers in mice has allowed the identification of some important genes for atherosclerosis (described later). QTL studies begin with construction of a cross between 2 selected inbred mouse strains, typically differing in expression of the parental origins of each portion of the genome can be determined by using polymorphic markers that distinguish the parental strains. QTL analysis basically analyzes whether the measured trait varies significantly across the population on the basis of the parental genotype at any given location in the genome, in the same manner as described for RI mapping. Polymorphic markers are selected to cover the entire genome at regular intervals. Specific software is available for this analysis, and statistical standards have been established to determine the significance of the results. When positive, the data are frequently presented as a graph with a curve representing the statistical likelihood, represented by a log-of-the-odds (LOD) score, of a genetic effect across the length of the chromosome (Figure 1B). For an F2 intercross, peak LOD scores >2.8 and 4.3 are taken to represent suggestive and significant evidence for linkage, respectively, although empirical significance levels can be generated for each data set by using permutation analysis. There will be a particular location along the chromosome where the likelihood of a genetic effect is greatest, referred to as the peak. From the data, a region of the chromosome where the likelihood of a genetic effect is greatest can be isolated on a common genetic background as a “congenic strain,” which simplifies the genetics for further analysis. This is accomplished by repeated backcrossing of an F1 animal to 1 of the parental strains and selecting at each generation only those individual animals that carry the QTL region from the opposite parent for further study. After 10 or more backcross generations, a congenic strain is created in which the genome is composed entirely of the background strain, with the exception of the region that encompasses the QTL, which is derived from the donor strain (Figure 1C). By phenotyping these animals for the trait(s) that was initially identified in the QTL analysis, such congenic strains allow confirmation of the mapping studies (Figure 1D) and, in essence, “mendelize” the complex trait, making fine mapping to ~1 Mb feasible. Examination of genes located in the finely mapped region, either with transgenic/knockout animals or through functional experiments, can then identify the underlying gene (Figure 1E). Ultimately, the human orthologs of such genes can be examined in populations comprising either families or patient cases and matched controls (Figure 1F).

A key aspect of this approach is that it does not require any foreknowledge of the affected gene(s). QTL mapping therefore offers the possibility of identifying entirely novel genes that might otherwise not have been considered. Because a gene product is part of a biologic pathway, identifying 1 member might lead to elucidation of other, interacting genes.

Mapping Genes for Atherosclerosis in Mice

In the 1960s and 1970s, a number of investigators, including Wissler and colleagues (Vesselinovitch and Wissler,5 Vesselinovitch et al7) developed diets capable of inducing mild hyperlipidemia when fed to mice. When maintained on these diets for several months, certain strains, but not others, developed fatty streak lesions in the proximal aorta.8 In the early 1980s, several groups characterized lipoproteins in mice and demonstrated significant genetic variations in lipoprotein levels and structures among inbred strains.9,10 In the mid-1980s, Paigen and colleagues (Paigen et al,11,12 Nishima et al13) modified the original Thompson diet and refined the methods for evaluation of aortic lesions. In the early 1990s, transgenic technologies led to the development of mouse models that can develop large, advanced lesions, in contrast to the fatty streak lesions observed after feeding of the atherogenic diets to wild-type mice (reviewed in Breslow14). As yet, robust mouse models for lesion rupture and thrombosis have not been developed, but recent observations by several laboratories suggest that apoE-null or LDL receptor (LDLR)–null mice can develop lesions that share many of the features of advanced human lesions and that might be capable of rupture.15–18 Molecular studies have also suggested potential mechanisms that could contribute to unstable plaque formation in mice.19–21

The first linkage studies of atherosclerosis susceptibility in mice were carried out with RI strains derived from susceptible strains, such as C57BL/6J, and resistant strains, such as C3H/HeJ, BALB/cJ, and A/J. These studies suggested that lesions were controlled by a small number of major genes, designated Ath1, Ath2, and Ath3.12,22–24 Subsequent studies indicated a more complex picture.25–29 As discussed later, the
HDL component of the Ath1 phenotype proved to be caused by polymorphisms of apoA-II.

Numerous QTL studies of atherosclerosis have now been reported from large genetic crosses between a variety of inbred strains of mice, maintained on either atherogenic diets or bred onto sensitized genetic backgrounds (such as apoE-null or LDLR-null; Table 1).22,24,30 A number of loci with highly significant evidence of linkage have been identified (Table 1 and Figure 2), and thus far, these studies have led to the identification of 2 genes involved in atherosclerosis (discussed subsequently).

Genetic studies in mice have also revealed information about pathways that contribute to genetic susceptibility to atherosclerosis, particularly in relation to lipid oxidation and inflammation.27 It is noteworthy that most of the loci for atherosclerosis identified in mice do not influence plasma lipid levels, blood pressure, or other systemic risk factors (see references in Table 1). This suggests the existence of important genetic factors that act at the cellular level, presumably by influencing vascular cells or leukocytes that infiltrate the vessel wall.

Most of the loci identified in the various crosses are unique (Table 1 and Figure 2). This is somewhat unexpected, if one assumes that genetic variations are common to different inbred strains of mice. One possible explanation for this observation is that experimental designs can influence the detection of loci; for example, the loci identified might depend on whether atherosclerosis is induced by feeding an atherogenic diet or by using a sensitized genetic background. Whether a particular locus attains statistical significance will also depend on the strength and number of loci that segregate in that cross. In any case, it appears that among the various inbred strains of mice, there exist many variations that also depend on whether atherosclerosis is induced by feeding an atherogenic diet or by using a sensitized genetic background. Whether a particular locus attains statistical significance will also depend on the strength and number of loci that segregate in that cross. In any case, it appears that among the various inbred strains of mice, there exist many variations that also depend on whether atherosclerosis is induced by feeding an atherogenic diet or by using a sensitized genetic background.

Pathologic studies in humans have revealed a great deal of variation in advanced atherosclerotic lesions and in the processes associated with myocardial infarction.40 Most genetic studies carried out in mice thus far have only examined lesion size, usually by quantifying lipid staining near the aortic root. Studies of lesion development at other sites (the distal aorta or the innominate artery, for example) might reveal distinct genetic influences.41 Curiously, few advanced lesions are observed in the coronary arteries in mouse models. In addition to lesion size, other parameters relevant to atherosclerosis, such as cellular composition, calcification, and lesion-related aneurysms, also vary among inbred strains. The fact that many common variations with dramatic effects on lesion development occur among inbred strains also has an important implication for reverse genetics strategies. Gene targeting is frequently performed on 1 genetic background (usually strain 129), and the mutant allele is then

<table>
<thead>
<tr>
<th>Locus (Genes)</th>
<th>Chromosome</th>
<th>Cross</th>
<th>LOD Score</th>
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<tr>
<td>Ath1 (apoA-II)</td>
<td>1</td>
<td>CXB and BXH RI strains</td>
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<td>14</td>
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<td>(B6 × DBA/2J) F2</td>
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</table>

Ath indicates atherosclerosis susceptibility locus; Artles, arterial lesion locus; Athsq, atherosclerosis susceptibility QTL. Not shown in the table are Ath4, Ath5, Ath12, Ath14, and Ath15. Ath4 is a locus affecting HDL levels and was identified in a subline of the Pera wild-type mouse. It is unknown if this locus has an effect on atherosclerosis. This subline has subsequently been reported as extinct.33 Athsq is the symbol reserved for the locus affecting non-HDL cholesterol levels but no gene or locus has been identified yet. Ath10, Ath12, Ath14, and Ath15 are names of atherosclerosis susceptibility loci that have not been associated with any identified chromosomal regions affecting lesion development in mice.

*No published name has been designated for these loci.
bred onto a separate background (frequently, strain C57BL/6J). During the breeding in this particular example, sequences flanking the targeted gene from strain 129 will be retained in the new congenic strain that is on a C57BL/6J background, and if only 4 or 5 generations of backcrossing are used, these flanking regions can be 10 or more centimorgans (cM) in size (20 million or more base pairs). If this flanking region contains a polymorphism that affects atherosclerosis, an incorrect conclusion regarding the function of the targeted gene might be drawn. This problem also applies to other traits relevant to atherosclerosis, such as plasma lipid metabolism and blood pressure, because many common variations also influence these traits.

Although the mouse is clearly the most useful mammal for genetic studies, the rat, the classic mammal for physiologic studies, is also proving to be useful for examining certain traits relevant to atherosclerosis, such as plasma lipid metabolism and blood pressure, because many common variations also influence these traits.

From QTL to Gene to Humans

The first gene to be identified as contributing to atherosclerosis susceptibility from genetic studies in mice was Apoa2, which encodes apoA-II, an abundant component of HDL. Polymorphisms of the gene among different mouse strains determine apoA-II production or turnover, and a congenic strain that contains an allele for high levels of apoA-II exhibited increased atherosclerosis. Subsequent transgenic studies with both mouse apoA-II and human apoA-II indicated that apoA-II–rich HDL promotes atherogenesis. This might be due to the reduced antioxidant potential that apoA-II–enriched HDL exhibits. These observations have also been extended to humans, in whom increased apoA-II levels have been associated with metabolic disorders.
Ultimately, the goal of identifying genes for atherosclerosis in mice is to extrapolate those results to studies in humans and to develop novel diagnostic and therapeutic strategies. Studies of the Artles locus on chromosome 6 provide such an example (Figure 1). Initially, Artles was identified in an F$_2$ cross between inbred strains C57BL/6J and CAST/Ei and exhibited a highly significant LOD score of 6.7 (Figure 1B). Interestingly, there was coincident linkage of insulin levels with this locus, raising the possibility that the underlying gene has pleiotropic effects on atherosclerosis and other related metabolic traits (Figure 1B). Subsequently, a congenic strain was developed to confirm the functional importance of the QTL and to identify the underlying gene that causes resistance to lesion formation. Congenic animals carrying the middle region of chromosome 6 derived from CAST/Ei (Figure 1C) confirmed the phenotype of Artles because these mice exhibited dramatic resistance to aortic lesion formation and had lower insulin levels compared with control mice (Figure 1D). Among the candidate genes in the congenic interval was 5-lipoxygenase (5-LO), an enzyme involved in leukotriene production. 5-LO is expressed primarily in leukocytes and has been studied mainly in the context of acute, not chronic, inflammation. Based partly on the results of bone marrow transplantation experiments, which implied the involvement of monocytes/macrophages or other leukocytes, 5-LO was examined as a positional candidate. To evaluate 5-LO as a candidate gene, Mehrabian et al.$^{39}$ crossed 5-LO-knockout mice with LDLR-null mice and fed them a high-fat, high-cholesterol diet. Importantly, these mice exhibited a profound reduction in aortic lesion formation, despite having cholesterol levels exceeding 500 mg/dL (Figure 1E). Conservative amino acid substitutions at the 3' end of the protein were also identified between CAST/Ei and C57BL/6J, but it was not known whether these variations influenced 5-LO function.$^{39}$ By creating the same substitutions at the conserved positions in the human enzyme, Habenicht and colleagues (Kuhn et al.$^{32}$) have recently tested the functionality of these amino acid changes and found that the CAST/Ei form of 5-LO has a marked decrease in activity and expression levels. Taken together, these studies provide strong evidence that 5-LO is the gene that underlies Artles and demonstrate the power of the QTL approach for identifying genes associated with atherosclerosis.

In preliminary studies of a human population, there is evidence to suggest that genetic variation in the 5-LO gene also affects similar phenotypes in humans. For example, a promoter polymorphism consisting of a variable number of Sp1 transcription factor binding sites was associated with carotid intima-media thickness, an accepted surrogate marker for atherosclerosis. Specifically, intima-media thickness in those individuals homozygous for either the 3 or 4 allele (D alleles) of the polymorphism was significantly increased, by nearly 100 µm, compared with individuals with other genotypes.$^{53}$ Furthermore, the same individuals also exhibited significantly higher levels of insulin, C-reactive protein, and interleukin-6. These results are consistent with the observations in 5-LO-deficient mice and support the concept that 5-LO has pleiotropic effects on the development of atherosclerosis, as well as known risk factors associated with it. Pharmacologic inhibitors of 5-LO and other enzymes in the leukotriene synthesis pathway are presently available, raising the possibility of potentially novel therapeutic strategies.

### The Road Ahead

A major impact of the mouse on our understanding of atherosclerosis has involved its genetic manipulation (ie, transgenic and knockout models) to examine mechanistic questions. However, studies of naturally occurring variations in the mouse, such as those described earlier, have tremendous potential to reveal new genes and pathways. Thus far, many loci for atherosclerosis-related traits have been mapped, but few genes have been identified. Recently, there has been important progress in the development of genomics and bioinformatics tools that will accelerate this process (Table 2). It seems likely that, as we enter the postgenome era of elucidating gene function, combined genetic/genomic approaches will prove increasingly useful for the identification of novel pathways relevant to atherosclerosis.$^{44-58}$

First and foremost, the currently available sequence of 5 mouse strains will allow rapid screening of candidate genes within QTLs and congenic regions. Once the QTL has been narrowed to several hundred kilobases, the entire sequence can be downloaded and examined to search for both known and unknown genes as candidates.

Recent studies have also identified numerous polymorphisms that differ among 8 strains, adding further to the tools...
that geneticists have at their disposal for gene identification.99,100 If the fine-mapping example described earlier were used in a cross between C57BL/6J and DBA/2J, for example, the available genomics resources would allow all of the nucleotide substitutions between C57BL/6J and DBA/2J in that region, as well as the entire sequence, to be determined. By prioritizing those genetic variations that occur in or near genes, researchers will be able to focus their effort on the most likely genetic causes first.

Among the important new developments is the availability of genome-wide congenic strains.11,12 As described earlier, once a QTL has been identified, congenic strains are created through repeated backcrossing, which can take 2 to 4 years, even with marker-assisted breeding. However, 3 panels of congenic strains that span the genome continuously have already been constructed between C57BL/6J and A/J, C57BL/6J and CAST/Ei, and C57BL/6J B6 and DBA/2J. These resources will be of immense use for atherosclerosis-related QTLs, because rather than creating a congenic strain for each identified locus, the desired strain(s) can be obtained from the corresponding panel, and one can directly proceed to confirming the QTL and fine mapping.

The feasibility of performing large-scale arrays for RNA expression in affected tissues is also highly complementary to QTL mapping. As demonstrated recently, traditional QTL analysis when combined with expression array experiments is an extremely powerful approach and yields tremendous insight into the genetic complexity that underlies common diseases.63 In essence, measuring transcript abundances in specific tissues can be thought of as measuring any other quantitative trait (eg, cholesterol), except that microarray technology allows the simultaneous quantification of thousands of genes (ie, traits). Analogous to how QTLs for lipid levels or atherosclerosis have been identified in mouse crosses, QTLs have also now been identified for mRNA expression (eQTLs).64 In the future, therefore, the criteria for prioritizing positional candidates will also include those genes that yield eQTLs over their own physical location, the linkage of which is coincident with the “clinical” QTL that was originally identified. On another level, by performing concurrent measurement and analysis of multiple traits, gene-gene interactions can be identified, including a possible common genetic basis for apparently diverse traits, such as bone density and lipid metabolism.64 This can be performed with combinations of clinical traits and/or expression array traits, adding further to the wealth of knowledge that this approach is likely to provide.

Recently, the in vivo use of small, interfering RNA molecules (siRNA) to attenuate gene expression has come to the forefront and has the potential to allow rapid screening of positional candidate genes. This novel technique involves the injection of short, double-stranded RNA oligonucleotides into mice through the tail vein under high hydrostatic pressure.65,66 The siRNA oligonucleotides, which are homologous to a target gene, are taken up by different tissues (the liver is a particularly good tissue) and cause the selective degradation of that gene’s mRNA. Compared with the time and expense it takes to create transgenic or knockout animal strains, siRNA strategies hold tremendous promise for functional testing of candidate genes. However, a major drawback is the short and transient inhibition of gene expression, on the order of 24 to 48 hours. Thus, for traits such as atherosclerosis or obesity, which can take months to develop, the use of siRNA might not be entirely feasible.

Conclusions

Common forms of atherosclerosis have a very complex etiology, and because of this complexity, it has proven difficult to apply the mapping strategies that have revolutionized our understanding of mendelian disorders. QTL approaches in mice offer a viable alternative to the problem. The main difficulty encountered in this approach has been the challenge of fine mapping to reduce the number of positional candidate genes. Several new tools in mouse genetics promise to accelerate the identification of genes that underlie QTLs. Ultimately, the identification of atherosclerosis genes (in both mice and humans) will lead to a better understanding of the pathophysiologic processes that are perturbed in and are manifested as disease and to the focused discovery of novel therapeutic agents and strategies.

References


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