Atherosclerosis and Lipoproteins

Mapping, Genetic Isolation, and Characterization of Genetic Loci That Determine Resistance to Atherosclerosis in C3H Mice

Susanna S. Wang, Weibin Shi, Xuping Wang, Leandra Velky, Sarah Greenlee, Min T. Wang, Thomas A. Drake, Aldons J. Lusis

Objective—C3H/HeJ (C3H) mice are extremely resistant to atherosclerosis. To identify the genetic factors involved in lesion initiation, we studied a cross between C3H and the susceptible strain C57BL/6J (B6) on a hyperlipidemic (apolipoprotein E–null) background.

Methods and Results—Whereas a previous cross in mice fed a Western diet for 16 weeks revealed a very complex inheritance pattern with many significant lesion QTLs, the present cross, on a chow diet, revealed a single major locus on chromosome 9 (lod = 5.0, Ath29*), and a suggestive locus on chromosome 4 (lod = 2.6, Ath8). QTLs for plasma HDL, total cholesterol, and triglyceride levels were found on chromosome 1 over the ApoA2 gene. Neither of the lesion QTLs were associated with differences in plasma lipid levels or other systemic risk factors, consistent with the concept that genetic factors affecting cellular functions of the vessel wall are important determinants of atherosclerosis susceptibility. We generated a congenic strain for Ath29 and confirmed its contribution to lesion development. Toll-like receptor 4 (Tlr4), the lipopolysaccharide (LPS) receptor, is located in the Ath8 region and is known to be defective in C3H/HeJ mice. We constructed a congenic strain carrying a normal Tlr4 gene on the C3H ApoE–null background and found that the defective Tlr4 does not contribute significantly to lesion resistance during early lesion development.

Conclusions—We identified one major QTL on chromosome 9, Ath29, for early lesion development in the BXH ApoE+/– cross fed on a chow diet and confirmed its contribution in congenic mice. We have also determined that Tlr4 on the C3H ApoE–/– background does not contribute to early lesion development. *Ath29 is referred to as Ath22 in Su et al 2006. (Arterioscler Thromb Vasc Biol. 2007;27:2671-2676.)

Key Words: atherosclerosis ■ quantitative trait locus ■ C3H/HeJ

The genes underlying a number of Mendelian forms of atherosclerosis, such as familial hypercholesterolemia, have been identified, but the genetic factors involved in the common forms, explaining about 50% of all deaths in Western populations, remain largely unknown.1 One approach to the problem has been to study inbred strains of mice differing in atherosclerosis susceptibility. During the past 20 years, a number of strains of mice with varying susceptibility to the disease have been studied.2 C3H/HeJ (C3H) mice have proven to be particularly resistant to the disease.3 Whereas C57BL/6J (B6) and most other strains develop large, human-like atherosclerotic lesions on the background of the hyperlipidemia-inducing apolipoprotein E–null (ApoE–/–) mutation,4 C3H mice develop almost no lesions unless placed on a high-fat, Western diet.5 The resistance of C3H mice appears to be mediated in part by the failure of endothelial cells to respond to oxidized lipids. Thus, whereas oxidized LDL induces the expression of a variety of inflammatory genes such as monocyte chemotactic protein 1 (Mcp-1) and vascular cell adhesion molecule (VCAM) (Vcam-1) in aortic endothelial cells (ECs) from B6, ECs from C3H mice are almost totally resistant to the induction.5–7 Thus, studies of C3H mice provide an opportunity to identify novel mechanisms underlying susceptibility to atherosclerosis.

We now report the mapping of chromosomal loci contributing to atherosclerosis resistance in C3H mice on an ApoE–/– background. We studied 12-week-old mice maintained on a chow diet to assess genetic contributions to early lesion development. As in a previous study of mice maintained on a high-fat, high-cholesterol diet, the major locus identified in our study was on chromosome 9.* C3H/HeJ mice carry a defective allele of Toll-like receptor 4 (Tlr4), a gene that has been implicated in resistance to atherosclerosis in human studies9 as well as mice.10 Using a congenic mouse strain, we

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show that the Tlr4 gene does not have a significant impact on early atherosclerosis on the C3H background. We have also isolated the major locus contributing to atherosclerosis susceptibility on chromosome 9 as a congenic strain and characterized its effect on atherosclerosis. This strain should make fine mapping of the gene feasible.

Materials and Methods

Mice and Diets
C57BL/6J Apoe−/− (B6 Apoe−/−) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine and C3H/HeJ Apoe−/− (C3H Apoe−/−) mice were bred by backcrossing B6 Apoe−/− to C3H/HeJ for 10 generations as previously described. All mice were fed ad libidum and maintained on a 12-hour light/dark cycle. F2 mice were generated by crossing B6 Apoe−/− with C3H Apoe−/− and subsequently intercrossing the F1s. F2 mice were fed Purina Chow (Ralston-Purina Co) containing 4% fat and euthanized at 12 weeks of age. Before being euthanized, they were fasted overnight, anesthetized with Isoflurane, and bled through the retro-orbital sinus. Plasma was stored at −80°C.

Tlr4 Congenic Studies
To produce C3H.Apoε−/− Tlr4lps−/− (normal response to lipopolysaccharide [LPS]) congenic mice, C3H Apoe−/−, which are Tlr4lps−/− (defective LPS response) because of a spontaneous mutation, were intercrossed with C3H/DiSnA Apoe−/− Tlr4lps−/− mice and C3H Apoe−/− Tlr4−/− F2 offspring were selected. Genotyping for Apoe and Tlr4 was done by polymerase chain reaction (PCR). PCR for Apoe was performed as previously described. The Tlr4 primer sequences are as follows: F 5′-tcaagaagaggtgga3′ and R 5′-tcaagaatacacaagcgc3′. The Tlr4 PCR product was digested with NlaIII (CATG): the Tlr4lps−/− allele contains a unique restriction enzyme site yielding a 98-bp product. The progeny of the F1 heterozygotes gave rise to approximately a 1:2:1 ratio of Tlr4lps−/−, Tlr4lps−/−, and Tlr4lps−/− respectively. C3H/DiSnA mice were obtained from Dr. Peter DeMant, Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, NY, who had bred the strain in his laboratory. The Tlr4lps−/− allele arose on the C3H/HeJ strain after the separation of the two colonies.

Histological Analyses
The aorta was sectioned and lesions were quantified as previously described. After the mice were euthanized, the heart and proximal aorta were excised and washed in PBS. The apex and lower half of the ventricles were cut off. The remaining specimen was embedded in Tissue-Tek (Miles), frozen on dry ice, and stored at −80°C until the aortic valves appeared. From then on, every fifth 10-μm sectioning. Serial cryosections were prepared through the ventricle and on chromosome 10 at 58 cM (lod = 2.5; supplemental Figure IIa). The B6 allele conferred susceptibility in all cases.

Linkage and Data Analysis
Genotype and phenotype data were imported into Windows QTL Cartographer version 2.0. Linkage analysis was performed using microsatellite markers. One significant QTL for atherosclerosis was found on chromosome 9 at about 27 cM (Figure 1a). Two suggestive QTLs were found on chromosome 4 (lod = 2.6) at 28 cM (Figure 1b) and on chromosome 10 at 58 cM (lod = 2.5; supplemental Figure IIa). The B6 allele conferred susceptibility in all cases. Plasma lipid levels were measured to determine whether any correlation existed between lesion size and lipid levels. QTL analysis was also performed on lipid levels to identify loci that regulate lipids and to determine whether any lipid QTLs colocalized with the lesion QTLs. Using the Spearman
rank correlation, the correlation coefficient ($r$) was calculated between lesion size and LDL/very low-density lipoprotein, high-density lipoprotein, total cholesterol, triglycerides, and free fatty acids. No significant correlations were found between any of the lipid traits and lesions (supplemental Table I). Significant QTLs on chromosome 1 at 92 cM were found for HDL (lod=7.1) and triglycerides (lod=4.4; Figure 2). A suggestive QTL for total cholesterol (lod=3.0) was also identified at the same locus. A suggestive QTL for HDL (lod=3.8) and triglycerides (lod=2.5) was found on chromosome 10 at 14 cM. Hence, no QTL overlaps were found between lesions and lipids (supplemental Figure IIa and IIb).

**Confirmation and Characterization of the Chromosome 9 Locus**

We constructed mice congenic for the chromosome 9 locus by introgressing that region of C3H onto a B6 Apoe$^{−/−}$ background by backcrossing for 8 generations. As determined by genotyping, the congenic region extended from 15 to 61 cM between markers D9mit297 and D9mit16. Female congenics had a mean lesion size of 9094±1871 $\mu$m$^2$ per section and B6 Apoe$^{−/−}$ littermate controls had a mean size of 36319±4518 $\mu$m$^2$ per section. Thus, the lesions in the female congenics were about 25% the size of the controls ($P<0.0001$). Male congenics had a mean lesion size of 11375±1649 $\mu$m$^2$ per section and control B6 Apoe$^{−/−}$ had a size of 20896±3481 $\mu$m$^2$ per section. Thus, the congenic lesions of the males were about 54% the size of the controls ($P=0.013$). These results confirm the contribution of this locus to atherosclerosis (Figure 3). The majority of studies of
atherosclerosis in mice are consistent with our sex-biased results; female mice almost always develop larger lesions than male mice, and the reasons underlying this sex bias are not known. The congenic mice resemble C3H Apoe+/− mice in their lesion development. Male and female C3H Apoe+/− mice exhibit extremely small lesions that are statistically not different in size, likewise for our congenics. We speculate that the chromosome 9 region harbors an allele that may act more strongly in females compared with males.

**Tlr4 on the C3H Background Does Not Significantly Influence Lesion Development**

*Tlr4*, located on chromosome 4 at 33 cM, was just distal to the suggestive chromosome 4 peak. Because *Tlr4* has been associated with atherosclerosis in humans and mice and is known to be defective in C3H/HeJ, we investigated the role of *Tlr4* in atherosclerosis in C3H/HeJ mice. We generated C3H Apoe+/− mice that carry the functional *Tlr4* gene from C3H/DisNA by intercrossing the strains. The mean lesion size for C3H Apoe+/− *Tlr4*+/− was 384±127 μm²/section, that of C3H Apoe+/− *Tlr4*+/− was 221±105 μm²/section, and that of B6 Apoe+/− *Tlr4*+/− was 15580±1220 μm²/section. Thus, the congenic C3H Apoe+/− *Tlr4*+/− showed no significant difference in lesion size compared with C3H Apoe+/− *Tlr4*+/−, though both of these groups were significantly different from B6 Apoe+/− *Tlr4*+/− (P<0.0001). These data suggest that *Tlr4* does not contribute significantly to the extreme resistance of C3H mice to early lesion development, and that *Tlr4* is not a cause of the difference in lesion initiation between C3H and B6.

**Discussion**

C3H mice are unusually resistant to atherosclerosis, and we now report a genetic analysis of a cross between strain C3H and the susceptible strain B6 on the background of hyperlipidemia because of an *Apoe*+/− mutation. Several significant conclusions have emerged. First, the cross does not replicate the *Ath1* locus identified using C57BL/6J (B6) × C3H/HeJ (C3H) recombinant inbred (RI) strains fed a cholic acid diet.

Second, a major locus on chromosome 9 was identified and confirmed using a congenic strategy. Third, the *Tlr4* gene was shown to not be a significant factor in the resistance of C3H mice to atherosclerosis. Finally, a chromosome 1 locus for plasma lipids identified in a BXH cross on a chow diet was replicated. Below, we discuss these points in turn.

*Ath1* was the first locus identified for atherosclerosis susceptibility in mice in BXH and B6XBALB/c recombinant inbred strains. It was determined to be the major locus located on chromosome 1 at 90 cM contributing to differences in diet-induced atherosclerosis in female mice. Recent studies suggest that variations in *Tnfsf4* underlie *Ath1*. The reason for our failure to replicate the *Ath1* locus is unclear. It is possible that QTLs found on the high fat cholic acid diet are different from those found in *Apoe*+/− mice fed chow diet, or we may not have been sufficiently powered to detect it. Studies of BXH recombinant inbred strains maintained on a cholic acid diet performed in our laboratory were not consistent with a chromosome 1 location for a major atherosclerosis susceptibility gene. It is noteworthy that the early study treated atherosclerosis as a nominal rather than quantitative trait. Another BXH *Apoe*+/− F2 intercross fed on a 12-week Western diet also failed to replicate the locus. The major locus contributing to atherosclerosis in this hyperlipidemic cross was on chromosome 9, which is designated *Ath29*. More recently, we performed a second cross with those strains maintained on a Western diet for 16 weeks and observed a number of additional loci contributing to lesion development. The chromosome 9 locus in the present study likely replicates the *Ath29* QTL previously found in both BXH *Apoe*+/− F2 crosses fed the Western diet.

There are many notable genes within the 95% confidence interval for *Ath29* (17 to 33 cM). Of the 529 genes in the interval, more than 100 of them are olfactory receptors. A short list of candidate genes can be found in the Table.
for atherosclerosis in this cross, we note that the Apoa1, Apoa4, Apoc3, Apoa5 cluster, which has been associated with familial combined hypercholesterolemia,\(^9\) falls in the region. Another gene, Sortilin-related Receptor, Sort1, has structural homology with the Ldrl family,\(^20\) and has been shown to bind both apolipoprotein E and lipoprotein lipase (Lpl) and is expressed in the lesions of Apoe null mice.\(^21\) Nicotinamide n-methyltransferase (Nnm) is involved in the metabolism of homocysteine, whose levels in the plasma are an independent risk factor for CAD. Plasma homocysteine levels were linked to atherosclerosis in this cross, perhaps because of the hyperlipidemic Apo\(^e^-\) background. Sterol O-acyltransferase (Sout1) is another candidate for the lipid QTLs on chromosome 1. Also known as acyl coenzyme A (CoA): cholesterol acyltransferase, it is an endoplasmic reticulum protein that forms cholesterol esters from cholesterol. It is also involved in adrenocortical lipid depletion in AKR/J mice.\(^5\)

In conclusion, the mapping and genetic isolation of the chromosome 9 locus conferring atherosclerosis resistance of C3H mice should allow fine mapping of the region and the identification of the underlying gene. This task should be aided by the recent development of an expression quantitative trait locus database for C3H X B6 mice.\(^6\)

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**Disclosures**

None.

**References**


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Table I – Spearman Correlation between lesion size and lipid traits.

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Supplementary Figure Legend

Figure I. Distributions of (a) atherosclerotic lesion size and (b) log transformed atherosclerotic lesion size.

Figure II. Genomewide lod scores for atherosclerosis (a) and various plasma lipids (b) including HDL, triglycerides and total cholesterol.
Figure I

a

Distribution of Atherosclerosis

b

Distribution of log Atherosclerosis
Figure IIa

log Atherosclerosis

Lipids

Genomewide Position (cM)

Lod Score

Genomewide Position (cM)