Genetic Locus in Mice That Blocks Development of Atherosclerosis Despite Extreme Hyperlipidemia

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Abstract—The genes contributing to the common forms of atherosclerosis are largely unknown. One approach to dissecting complex traits such as atherosclerosis is to use animal models, such as the mouse, to map and characterize the genetic loci involved. We now report the identification of a locus for aortic lesion formation on mouse chromosome 6 that exhibits a highly significant lod score of 6.7 in a genetic cross between the susceptible strain, C57BL/6J, and the resistant strain, CAST/Ei. The locus was confirmed by constructing a congenic strain in which the chromosome 6 segment from CAST/Ei was transferred to a C57BL/6J background in a series of backcrosses. The congenic strain was almost completely resistant to diet-induced atherosclerosis. The chromosome 6 segment was also transferred onto the background of an LDL receptor–null mutation and resulted again in almost complete resistance to aortic lesion formation. This locus also influenced insulin levels but did not affect plasma lipoprotein levels, blood pressure, or body fat. The chromosome 6 gene, which we call Arties (for arterial lesions), did not affect endothelial cell responses to oxidized LDL, but lesion formation was partially reduced through bone marrow transplantation. The locus contains the candidate gene peroxisome proliferator–activated receptor-γ, and the congenic mice exhibited significantly reduced expression of peroxisome proliferator–activated receptor-γ. (Circ Res. 2001;89:125-130.)

Key Words: congenic strains  insulin  peroxisome proliferator–activated receptor-γ
bone marrow transplantation  quantitative trait locus

Atherosclerosis is a disease of the large arteries that is the primary cause of heart disease and stroke. A large number of risk factors for the disease have been identified through epidemiological studies, including genetic traits, such as elevated cholesterol, elevated blood pressure, reduced HDL levels, and male sex, as well as environmental factors, such as smoking and a high-fat diet. Altogether, these risk factors do not appear to fully explain atherosclerosis. The search for genes contributing to the common multifactorial forms of atherosclerosis has been only modestly successful, with a major problem being the very complex etiology of the disease.1 One potentially powerful approach to understanding the genetic factors in the disease is the analysis of animal models such as mice. Inbred strains of mice differ greatly in susceptibility to the development of atherosclerotic lesions when maintained on high-fat diets; they also differ regarding the background of hyperlipidemia–inducing null mutations of apolipoprotein E or the LDL receptor (LDLR).2–7 We now report the mapping and partial characterization of a genetic locus on chromosome 6 that blocks the development of both early and advanced atherosclerotic lesions. A congenic strain carrying the locus exhibited little or no lesion formation even under conditions of extreme hyperlipidemia resulting from an LDLR mutation. The locus did not affect several risk factors normally associated with atherosclerosis, including LDL and VLDL levels, HDL levels, and blood pressure, suggesting the involvement of a novel pathway for disease susceptibility.

Materials and Methods

Animals
All mice, except the chromosome 6 congenic strain, were purchased from the Jackson Laboratories, Bar Harbor, Me, and were initially maintained on a regular chow diet. Mice were housed 3 or 4 per cage at 25°C on a 12-hour light-dark cycle. Animals were fasted overnight before blood was drawn. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the UCLA Animal Research Committee. The high-cholesterol high-fat diet (diet No. 90221, Harlan-Taklad) has been previously described.2,7

Construction of Chromosome 6 Congenic Mice
C57BL/6J×CAST/Ei (CAST×BL6)F1 mice were backcrossed to C57BL/6J (BL6) for 10 generations, with selection of each generation for the chromosome 6 locus from CAST/Ei (CAST) by using microsatellite markers. Brother-sister mating subsequently generated mice homozygous for the chromosome 6 locus from CAST.
Plasma Lipid and Insulin Measurements
Mice were fasted overnight and bled through retro-orbital veins under isoflurane anesthesia. Enzymatic assays for plasma total cholesterol, HDL cholesterol, and triglycerides were performed as described.9 Insulin levels were determined in duplicate by ELISA with a kit from Crystal Chemical (No. 1USKRO20).

Culture and Treatment of ECs
Endothelial cells (ECs) from the thoracic aorta were isolated with an explantation technique.10 The thoracic aorta was gently cleansed of periadventitial fat and connective tissue and cut into rings ≈3 mm in length. The aortic segments were placed on Matrigel (Collaborative Research) and incubated in DMEM supplemented with FBS, penicillin-streptomycin, heparin, EC growth supplements, and amphotericin B (Fungizone). The vessel rings were removed once cell outgrowth was observed. The cells were passaged with Dispase (Collaborative Research) and plated onto gelatin-coated dishes. The subsequent passages were performed with trypsin-EDTA. At passages used for experiments, all cells expressed the von Willebrand factor antigen and took up acetylated LDL.10 Confluent cells at passages 4 to 6 were treated for 4 hours with 200 μg/mL native LDL, 200 μg/mL minimally modified LDL, or medium only. The minimally modified LDL was prepared by brief iron or copper oxidation as described.10

Bone Marrow Transplantation
Two-month-old female BL6 mice were used as recipients as described for bone marrow transplantation.7 Recipient mice were lethally irradiated. Bone marrow was harvested from male BL6 mice or chromosome 6 congenic mice by flushing their femurs and tibias. Each recipient mouse was lethally irradiated and then injected with 107 bone marrow cells through the tail vein. Four weeks after transplantation, blood was drawn from overnight-fasted mice, and DNA from the blood was analyzed for the presence of a 250-bp sequence of the Y chromosome as described.7 The mice that expressed donor DNA were fed for 11 weeks with the atherogenic diet.

Tissue Preparation and Lesion Analysis
The methods that were used for the quantification of atheromatous lesions at the aortic root were as previously reported.9 Briefly, at euthanasia, the upper portion of the heart and the proximal aorta were obtained, embedded in OCT compound (Miles Laboratories), and stored at −70°C. Serial 10-μm-thick cryosections from the middle portion of the left ventricle to the aortic arch were collected and mounted on poly-D-lysine–coated slides. These sections were stained with oil red O and hematoxylin. The lipid-staining areas were counted in a blinded fashion by light microscopy. Selected sections were examined by using immunohistochemical analyses with antibodies specific for macrophages or smooth muscle cells as previously described.11

Statistical Analysis
All values are expressed as mean±SEM, with the exception of peroxisome proliferator–activated receptor-γ (PPAR-γ) levels, which were pooled samples and are expressed as means. ANOVA was used to determine differences between groups in lesions or lipid levels. Differences were considered statistically significant at P<0.05. Interval mapping and linkage analysis of an F2 intercross was performed by using the program Mapmaker.8 Theheritabilities of insulin levels and atherosclerosis, ie, the fraction of total variance of the (CAST×BL6)F2 cross attributable to genetic factors, were calculated as follows: heritability=(F2 variance−pooled parental variance)/F2 variance.

In this equation, the pooled parental variance provides an estimate of environmental variance (because the parental strains are genetically identical), and the (B6×CAST)F2 variance is the total variance (genetic+environmental).

Results
A survey of inbred strains of mice for susceptibility to atherosclerotic lesions after the feeding of a high-fat high-cholesterol diet revealed that CAST mice were very resistant to atherosclerosis despite having much higher levels of atherogenic LDL/VLDL cholesterol and plasma triglycerides but lower levels of antiatherogenic HDL cholesterol (Figure 1A). On a low-fat chow diet, CAST mice had approximately half the levels of total cholesterol that were found in BL6 mice; this occurrence was primarily due to decreased HDL cholesterol (Figure 1B). The lipid profile of CAST mice on the atherogenic diet (Figure 1C) would be expected to result in lesion development comparable to extremely hyperlipidemic mouse models of atherosclerosis, such as LDLR knockout mice or apolipoprotein E knockout mice.3–5 CAST mice exhibited slightly lower insulin levels than did BL6 mice (Figure 1D). Therefore, we concluded that CAST mice must possess strongly antiatherogenic genetic variations independent of plasma lipoprotein profiles.

The loci contributing to atherosclerosis resistance in CAST mice were mapped by using an F2 intercross between CAST and BL6 mice. A total of ≈250 F2 mice were weaned onto rodent chow containing 6% of calories as fat, and then, at ≈4 months of age, they were switched to a high-fat high-cholesterol atherogenic diet for 15 weeks. The mice were typed for atherosclerotic lesions by examining cross sections in the proximal aorta as well as for levels of plasma lipoproteins, insulin, glucose, and leptin and for body fat composition. The distributions of aortic lesion sizes and plasma insulin levels in the parental, F1, and (CAST×BL6)F2 mice are presented in Figure 2. The estimated heritabilities of the traits approached 1 (0.99 for lesions and 0.92 for insulin levels). The distributions suggest a complex pattern of inheritance for both traits; eg, the aortic lesions of F2 animals exhibited a much wider range of sizes than the 2 parental strains, suggesting that CAST mice contribute proatherogenic as well as antiatherogenic genetic influences. The levels of LDL/VLDL cholesterol, HDL cholesterol, glucose, and leptin also exhibited complex inheritance patterns.12 A complete linkage map of the F2 animals was constructed by typing ≈110 microsatellite markers spanning all chromosomes except the Y chromosome, resulting in an average spacing of ≈10 cM between markers.12 A single major locus, mapping to the central region of mouse chromosome 6, was observed to contribute importantly to atherosclerotic lesion development (Figure 3). This locus exhibited a lod score of ≈6.7, a highly significant result given that the threshold for significance in an F2 intercross has been estimated to be 4.3.13 As previously observed,2 male F2 mice exhibited smaller atherosclerotic lesions than did female mice, but both sexes contributed to the lod score on chromosome 6. The chromosome 6 locus showed codominant inheritance, and the mice heterozygous for the region exhibited lesion development that was intermediate between the F2 mice homozygous for the CAST or BL6 parental alleles (Figure 3, inset). A suggestive quantitative trait locus (QTL) for insulin levels, with a lod score of 4.2, coincided with the QTL for aortic lesions (Figure 3). In contrast to aortic lesions, a number of other major loci also contributed to total levels of insulin in the F2 mice.12
To confirm the QTL for aortic lesions and to characterize the locus in more detail, a congenic strain containing the chromosomal region of the QTL from CAST mice on the background of BL6 mice was constructed. The congenic strain was derived by repeated backcrossing of progeny from a CAST x BL6 cross to the BL6 parental strain, with selection at each generation for genetic markers identifying that CAST chromosome 6 region. Thus, genetic markers D6mit102, D6mit105, D6mit256, D6mit111, D6mit14, and D6mit198 were typed in each generation of backcrossing to select for heterozygosity at the chromosome 6 locus (see Figure 3). After 10 generations of such backcrossing, mice heterozygous for the chromosome 6 locus (spanning markers D6mit102 and D6mit198) were intercrossed to produce a congenic strain (CON6) that contained the chromosome 6 CAST region on the background of strain BL6. Examination of the congenic mice maintained for 15 weeks on an atherogenic diet revealed that they were almost entirely resistant to the development of aortic lesions, with lesion size only a small percentage of the lesion size of strain BL6 mice and with significantly less lesion formation than exhibited by CAST mice (Figure 1A). The lesions in B6, CAST, and congenic mice had similar compositions, consisting primarily of foam cells and extracellular lipid and relatively little

Figure 1. Phenotypic differences between CAST, BL6, and CON6 mice. A, Fatty streak lesions in the proximal aorta of mice fed a high-fat high-cholesterol diet for 15 weeks were quantified in male (squares) and female (circles) mice of strains BL6, CAST, and CON6. Values are expressed as the mean±SEM. B, Plasma cholesterol levels in fasted mice fed a low-fat chow diet are shown. C, Plasma cholesterol levels in fasted mice fed an atherogenic diet for 15 weeks are shown. D, Plasma insulin levels in fasted mice fed a chow diet are shown.

Figure 2. Inheritance of plasma insulin levels and aortic lesion sizes in CAST x BL6 intercross. A, Distributions of plasma insulin levels (cholesterol diet) and lesion sizes in CAST, B6, (CAST x BL6)F1, and (CAST x BL6)F2 mice are shown. B, The lack of a significant correlation between plasma insulin levels and lesion sizes is illustrated by using regression analysis.

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fibrous material (data not shown). With respect to plasma lipoprotein levels, the congenic mice resembled the background BL6 strain on a chow diet (Figure 1B) and on an atherogenic diet (Figure 1C). The levels of serum paraoxonase, an antioxidant protein that destroys certain oxidized phospholipids and protects against atherosclerosis, were similar in the BL6 and congenic mice (data not shown). In agreement with the QTL results, insulin levels in the congenic mice were significantly different from levels in the BL6 background strain but were rather similar to levels in CAST mice (Figure 1D). In all 3 strains, males tended to have higher insulin levels than did females (Figure 1D). Although the locus controls both insulin levels and lesion formation, there was no overall correlation between the 2 traits among F2 mice (Figure 2B). Most likely, this is attributable to the fact that multiple, independently segregating loci contribute to the traits.

BL6 mice fed an atherogenic diet develop fatty streak lesions, but they do not develop advanced fibrous lesions. In contrast, certain knockout models, such as the LDLR knockout mouse, develop advanced lesions throughout the aortic tree. To study the effects of the chromosome 6 locus on advanced atherosclerotic lesions, the congenic region was crossed onto the background of an LDLR knockout mouse and tested for lesion development. After the feeding of an atherogenic diet, lesions were assessed in the LDLR knockout mice in the presence or absence of the chromosome 6 region from CAST (Figure 4A). As expected, compared with the BL6 mice, the LDLR knockout mice exhibited greatly elevated LDL/VLDL cholesterol levels, and the lesions in the LDLR knockout mice were \( \approx 30 \) times larger than those in the BL6 mice maintained on the high-fat diet. However, the LDLR knockout/congenic mice had very small lesions (Figure 4A). In the experiment shown in Figure 4A, 4 of the 7 LDL receptor knockout mice containing the chromosome 6 congenic region contained no detectable lipid-staining lesions in the aorta (Figure 4A). The lesions in BL6 and congenic mice carrying the LDLR null allele were similar in composition, consisting primarily of foam cells and extracellular lipid, with a small amount of fibrous material and smooth muscle cells. The lipoprotein profiles of the LDLR knockout mice with and without the chromosome 6 congenic region were similar, although there was a small difference in HDL levels (Figure 4B).

To test the possible involvement of ECs in the genetic variation in atherosclerosis, cultures of aortic ECs were established from BL6 and the congenic mice and were tested for inflammatory responses to oxidized LDL. We have previously shown that ECs from the atherosclerosis-resistant strain C3H are unresponsive to oxidized LDL but that ECs from the susceptible BL6 strain are responsive and that in a genetic cross between the strains, responsiveness segregated with lesion formation. In contrast, the ECs of both the congenic strain mice and BL6 mice responded equally to copper- or iron-oxidized LDL induction of several inflammatory genes, including monocyte chemotactic protein-1 and heme oxygenase (data not shown). Thus, EC responsiveness to oxidized LDL does not explain the chromosome 6 QTL for resistance to atherogenesis.
Therefore, we sequenced the PPARγ gene from either BL6 mice (BL6/BL6) or the chromosome 6 congenic mice (CON6/BL6). After a period to allow full engraftment, the mice were placed on a high-fat high-cholesterol diet for 11 weeks, when aortas were removed and atherosclerosis was quantified.

To test whether bone marrow–derived cells are involved in the difference in atherogenesis, bone marrow transplantation experiments were performed with BL6 mice used as the recipients and either the congenic or BL6 mice used as the donors. The transplanted mice were then fed an atherogenic diet for 11 weeks, at which time aortic lesion development was assessed. The mice receiving marrow from congenic mice exhibited ≈3-fold smaller lesions than did mice receiving BL6 bone marrow (Figure 5). Thus, the chromosome 6 gene (or genes) appears to act in part by influencing functions of cells derived from bone marrow, presumably monocyte/macrophages or lymphocytes. However, because the difference in lesion size between the congenic strain and BL6 mouse was ≈20-fold, other factors or cell types appear to be involved as well.

Several candidate genes for insulin levels and atherosclerosis occur in the chromosome 6 interval (Figure 3). These include the genes encoding the apolipoprotein B mRNA editing enzyme (ApoBec)\(^1\) and the adhesion molecule (Pecam). Levels of expression of these were found to be similar in CAST and BL6 mice (data not shown). Nevertheless, it remains possible that structural variations of these candidates could affect their functions.

A particularly attractive candidate was the gene for PPAR\(_{\gamma}\).\(^2\) This ligand-activated transcription factor influences both insulin metabolism and macrophage functions involved in foam cell formation.\(^3\) The expression of the protein was examined in CAST, BL6, and the chromosome 6 congenic mice in various tissues, including peritoneal macrophages, adipose tissue, and intestine. The expression of PPAR\(_{\gamma}\) in peritoneal macrophages, both at the level of mRNA (Figure 6A) and protein (Figure 6B), was reduced by ≈60% in CAST mice and the congenic mice compared with BL6 or C3H mice. A similar reduction in PPAR\(_{\gamma}\) expression in CAST mice was observed in all 3 tissues examined. The fact that the congenic mice showed reduced expression suggested that the genetic difference was due to a cis-acting element. Therefore, we sequenced the PPAR\(_{\gamma}\) cDNA and ≈1 kb of the promoter region in the 2 strains. With the exception of 1 silent variation, the cDNA coding sequence was identical. However, the promoter from CAST mice exhibited a 6-bp deletion, relative to BL6 mice, located 416 bp upstream from the PPAR\(_{\gamma}\) translation start site (Figure 6C). This sequence (TTAGGA) may be part of a transcription factor binding site, although further studies are required to demonstrate this. This variation may explain the QTL for insulin levels, inasmuch as heterozygous PPAR\(_{\gamma}\) knockout mice have improved insulin sensitivity.\(^4\) However, the PPAR\(_{\gamma}\) variation may not explain the dramatic reduction in atherogenesis, inasmuch as recent studies have indicated that PPAR\(_{\gamma}\) has a protective role in atherogenesis.\(^5\) In particular, recent studies using 2 different PPAR\(_{\gamma}\) agonists revealed antiatherogenic effects in LDLR-null mice.\(^6\) Nevertheless, PPAR\(_{\gamma}\) should not be dismissed as a candidate, and further studies are required to more definitively determine its role.

**Discussion**

The above data indicate that a gene located in the central portion of chromosome 6 is capable of dramatically reducing atherosclerosis even on the background of an extreme hyperlipidemia model. We designate this gene Artles (for arterial lesions). The protective effect of the gene is greater than that of any knockout or transgenic model studied thus far, including adhesion molecules, chemotactic molecules, and growth factors. The gene does not appear to act by influencing the levels of LDL/VLDL, HDL, or serum paraoxonase, although...
the locus does have an effect on insulin levels. It seems unlikely that the difference in insulin levels is the primary cause of the resistance to atherosclerosis, because other loci in this same cross, including a locus on chromosome 2,12 had effects on insulin levels greater than those of the chromosome 6 locus yet did not affect the development of atherosclerotic lesions. Moreover, insulin levels did not correlate with lesions in the F2 cross. It is noteworthy that a locus for type 1 diabetes in the nonobese diabetic mouse was mapped to a similar region of chromosome 6.21 The syntenic human locus, on chromosome 10, also contains a type 1 diabetes gene.21 We have not been able to find any other risk factors in these animals (including blood pressure) that might contribute to lesion formation. Thus, the chromosome 6 locus appears to act by influencing an as-yet-unknown pathway for the development of atherosclerosis.

Given the dramatic effect of the chromosome 6 gene on atherosclerosis, it should be possible to perform fine mapping and positional cloning of the chromosome 6 gene. It is possible that the locus contains >1 gene contributing to lesion formation, although the fact that the locus on chromosome 6 was the only locus yielding even a suggestive lod score (>2.5) makes this possibility unlikely.

Some genetic epidemiological studies have suggested that known risk factors explain only ∼50% of the genetic component in atherosclerosis (review in Reference 1), although such estimates are likely to exhibit considerable error. Indeed, because atherosclerosis is a disease of the vessel wall, it would not be unexpected that certain genetic variations might influence cellular functions independent of systemic factors, such as lipoprotein levels or blood pressure. The identification of such novel pathways contributing to atherosclerosis could have great clinical and diagnostic significance. Although very effective drugs for lowering cholesterol and blood pressure have been developed, atherosclerosis remains by far the leading cause of death in westernized countries. Knowledge of cellular pathways contributing to the disease might provide new diagnostic approaches for risk assessment as well as new targets for drug development.

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