Aortic Arch Curvature and Atherosclerosis Have Overlapping Quantitative Trait Loci in a Cross Between 129S6/SvEvTac and C57BL/6J Apolipoprotein E–Null Mice

Hirofumi Tomita, Svetlana Zhilicheva, Shinja Kim, Nobuyo Maeda

Rationale: Apolipoprotein E-null mice with a 129S6/SvEvTac strain background (129-apoE) develop atherosclerotic plaques faster in the aortic arch but slower in the aortic root than those with a C57BL/6J background (B6-apoE). The shape of the aortic arch also differs in the 2 strains.

Objective: Because circulating plasma factors are the same at both locations, we tested the hypothesis that genetic factors affecting vascular geometry also affect the location and extent of atherosclerotic plaque development.

Methods and Results: Tests on the F2 progeny from a cross between 129-apoE-null and B6-apoE-null mice showed that the extent of atherosclerosis in the aortic arch is significantly correlated in males, but not in females, with the shape of arch curvature ($r=0.34$, $P<0.0001$) and weakly with the arch diameter ($r=0.20$, $P=0.02$). Quantitative trait locus (QTL) analysis identified 2 significant peaks for aortic arch lesion size on chromosome 1 (105 Mb, LOD=5.0, and 163 Mb, LOD=6.8), and a suggestive QTL on chromosome 15 (96 Mb, LOD=4.7). A significant QTL for aortic root lesion size was on chromosome 9 (61 Mb, LOD=6.9), but it was distinct from the QTLs for arch lesion size. Remarkably, the QTLs for susceptibility to atherosclerosis in the arch overlapped with a significant QTL that affects curvature of the arch on chromosome 1 (121 Mb, LOD=5.6) and a suggestive QTL on chromosome 15 (76 Mb, LOD=3.5).

Conclusions: The overlapping QTLs for curvature of the aortic arch and atherosclerosis support that the ontogeny of the aortic arch formation is a potential risk factor for atherosclerosis. (Circ Res. 2010;106:1052-1060.)

Key Words: apolipoprotein E-null mice  ■ quantitative trait locus  ■ atherosclerosis  ■ vascular geometry

Atherosclerosis is a complex trait resulting from interactions between multiple genetic and environmental factors. The spatial distribution of development of atherosclerotic plaques along the vasculature varies between individuals and by gender in humans, although lesions tend to develop close to arterial bifurcations and bends. This suggests that local hemodynamic forces, including shear stress, contribute to the regional development and progression of atherosclerosis. Because vessel geometry, which affects hemodynamic parameters, varies widely across human populations, the risk of developing atherosclerotic lesions might be higher in some individuals by virtue of their particular vascular geometry. However, the complexity of the relationships between vascular geometry, hemodynamics, and atherosclerosis combined with the genetic heterogeneity of human populations makes it extremely difficult to search for relevant genetic factors using human patients. Here, we reduce the genetic complexity by using inbred mice, and increase the incidence of lesions by using suitable mutants.

Apolipoprotein (apo)E-null mice spontaneously develop atherosclerotic plaques in the aortic root and aortic arch, but our recent report demonstrates that apoE-null mice on a 129S6/SvEvTac (129-apoE) and those on a C57BL/6J (B6-apoE) show several strain-specific differences in the patterns of plaque distributions in their aortas. For example, atherosclerotic lesions at the aortic arch develop more rapidly in 129-apoE mice than in B6-apoE mice. In contrast, lesions at the aortic root develop more slowly in 129-apoE mice than in B6-apoE mice. In addition, no significant male/female differences in the development of plaques are recognizable at either the aortic root or the aortic arch in 129-apoE mice, whereas there is a well-known gender effect in B6-apoE mice in which females develop more extensive plaques at the aortic root than males. The 2 strains also show distinct and easily recognizable differences in the geometry of the aortic arch. Additionally, computer simulations based on the differences in aortic arch geometry and hemodynamics of wild-type 129 and B6 mice support an interaction between these factors in determining the differences in plaque patterns in the aortic arch of apoE-null mice in these 2 strains. Because the aortic root and aortic arch are exposed to the same levels of plasma lipids and other circulating factors, it is possible to unravel genetic factors...
in these 2 strains that affect arterial geometry and atherosclerotic plaque development at the 2 locations in the aorta.

Quantitative trait locus (QTL) analysis is one of the most powerful methods for mapping genomic regions and allelic variations that are responsible for differences in complex traits such as atherosclerosis, and we have chosen to use this method for our study. There have been previous reports on the use of QTL analyses for atherosclerosis susceptibility, but these have focused on aortic root lesion size and plasma lipid levels with little attention being paid to the aortic arch lesion size and vascular geometry. Accordingly, we here describe the outcome of a QTL analysis of the F2 progeny of a cross between 129-apoE-null and B6-apoE-null parents. The results reveal several discrete chromosomal regions that affect vascular geometry, aortic root and aortic arch plaque developments, and provide evidence that differences in the genetic factors determining vascular geometry are potential risk factors for the development of atherosclerotic plaques.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Male 129-apoE mice were crossed with female B6-apoE mice to generate F1 mice, which were intercrossed to generate F2 progeny (138 males and 128 females). The F2 mice (24 weeks old) were perfused with 4% paraformaldehyde. The aortic arch was dissected free of surrounding tissue, placed in a flat transparent chamber, and its images were captured (Figure 1A). Plaque areas in the aortic arch and the diameters of the ascending aorta (DA1), transverse aorta (DA2), and descending aorta (DA3) were measured using Image J 1.40. The inner curvature of the aortic arch (angle) was defined as shown in Figure 1B. Briefly, a line 1 mm in length originating at the top of the inner curvature of the aortic arch (point A) was drawn parallel to the descending aorta. Next, a line was drawn perpendicular to the first line. The points where the second line intersected the ascending and descending aortas were points B and C, respectively. The angle BAC was defined as the angle of the aortic arch. Genomic DNA was isolated from the livers and single-nucleotide polymorphism (SNP) analysis was performed using the Illumina SNP panels. In our cross, 235 SNP markers were informative. QTL analyses were performed using R/qtl software, and the peak logarithm of odds (LOD) score was determined.

Results
Inheritance of Phenotypes in F2 Mice
After images of the dissected aortic arch were captured and the areas covered by lesions were measured, cross-sectional histological preparations of the ascending aortic arch at just proximal to the innominate artery were determined in some of the mice. Representative arch plaques from 129-apoE and B6-apoE mice are shown in Figure 1C. The plaque area in the aortic arch measured using the captured image was strongly correlated with the cross-sectional plaque size in the individual F2 male mice ($r=0.78$, $P<0.0001$, Figure 1D). This indicates that the plaque area measured in the captured image is a reasonable representation of atherosclerosis in the aortic arch, and we used this value in the analyses below.

Non-standard Abbreviations and Acronyms

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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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Figure 1. Atherosclerosis and geometric parameters in the aortic arch. A, Representative images of excised aortas from 9-month-old 129-apoE and B6-apoE male mice. B, Schema representing angle of the aortic arch (angle BAC), and diameters of ascending aorta (DA1), transverse aorta (DA2), and descending aorta (DA3). C, Representative cross-sectional histological preparations of the aortic arch in 9-month-old 129-apoE and B6-apoE male mice stained with Sudan IV and counterstained with hematoxylin. D, Relationship between arch lesion sizes measured by captured image and by cross-sectional preparations in F2 males ($n=111$).
Atherosclerotic lesion sizes, geometric parameters, body weight, and plasma lipids of parental, F1, and F2 mice are summarized in Table 1 and Online Figure I. As previously reported, the aortic root lesion sizes in 129-apoE mice were significantly smaller than those in B6-apoE mice in both sexes. In contrast, and again as previously reported, the aortic arch lesion sizes in 129-apoE mice were significantly bigger than those in B6-apoE mice. We found no significant male versus female differences in 129-apoE mice in the development of plaques in either the aortic root or the aortic arch, but female B6-apoE mice had significantly larger plaques than male B6-apoE mice at both locations. The root lesion sizes in the F1 and F2 mice were intermediate between those in the parental B6-apoE and 129-apoE mice, compatible with a codominant inheritance of this root lesion plaque size phenotype. The arch lesion sizes in the F1 and F2 males were also intermediate, but the F1 and F2 females had almost the same lesion size of the aortic arch as parental B6-apoE female mice. This is compatible with the presence of B6 alleles that are dominantly protective of the arch lesion in females.

There are striking differences in the geometry of the aortic arch between 129 and B6-apoE mice. The angle of the aortic arch in 129-apoE mice was significantly greater than in B6-apoE mice, reflecting a broader curvature. The angles in the F1 and F2 mice were intermediate between those in the 2 parental mice. The average diameter of the aorta at locations DA1, DA2, and DA3 (mean DA1–3) was significantly greater in B6-apoE than in 129-apoE mice. The mean DA1–3 in both F1 and F2 mice were similar to those in the parental B6-apoE mice, compatible with B6 alleles that are dominant in both sexes. Details of the aortic diameters of parental, F1, and F2 mice are given in Online Table I.

Plasma levels of total cholesterol in 129-apoE mice were significantly higher than in B6-apoE mice. There was no sex difference in 129-apoE mice, but cholesterol levels were significantly higher in B6-apoE males than in females. Plasma cholesterol levels in the F1 and F2 males were similar to those in the parental 129-apoE males, but higher than in the F1 and F2 females, which had intermediate cholesterol levels between the 2 parental strains. Plasma triglyceride levels were significantly higher in B6-apoE males than in 129-apoE males. The plasma triglyceride levels in the F1 and F2 mice were significantly higher than in either parental strains in both males and females. High-density lipoprotein cholesterol (HDL-C) levels were not different among groups.

**Relationships Between the Traits**

To investigate whether aortic geometry can contribute to plaque development, we analyzed the correlations between atherosclerotic plaque sizes and anatomic parameters in F2
mice (Online Figure II). There was a highly significant positive correlation between arch angle and aortic arch lesion size in males ($r=0.34$, $P<0.0001$). However, this correlation was not present in females ($r=0.10$, $P=0.28$). The mean DA1–3 was also positively correlated with arch lesion size in males ($r=0.20$, $P=0.02$), but not in females ($r=0.02$, $P=0.79$). Thus, plaque development in the aortic arch in males is more influenced by geometric factors than in females. In contrast, neither vessel angle nor diameter was correlated with aortic root lesion size. A positive correlation between arch lesion size and root lesion size was detected in males ($r=0.25$, $P=0.003$), but not in females ($r=0.15$, $P=0.09$), suggesting that some of the factors that influence plaque development at the 2 locations differ between the sexes.

We also analyzed the relationships between atherosclerotic plaque size and plasma lipid levels in our F2 mice (Online Figure III). Total cholesterol levels were weakly correlated with arch lesion size ($r=0.18$, $P=0.03$ in males and $r=0.15$, $P=0.09$ in females), but not with root lesion size ($r=-0.002$, $P=0.98$ in males and $r=-0.05$, $P=0.59$ in females). Plasma triglyceride and HDL-C levels were both negatively correlated with root lesion size in both sexes ($r=-0.25$ to $-0.18$, $P=0.003$ to 0.04). In contrast, there were no correlations between arch lesion size and plasma triglyceride or HDL-C levels.

**QTLs for Atherosclerosis at the Aortic Root**

QTL mapping was performed using the F2 mice. We analyzed the data in each sex separately, or jointly with sex treated as an interactive covariate (Figure 2). Details of the QTLs detected are summarized in Table 2 and Online Tables II–IV, and the allelic effects of the SNP markers near the peak on the trait are shown in Figure 3.

A highly significant QTL contributing to root lesion size was detected on chromosome (Chr) 9 at 39 Mb in males (Figure 2A, LOD=5.3). This locus accounts for 16% of the variance in males with the 2 alleles having additive effects on the trait. The B6 allele of the SNP (rs13480208) nearest to this locus was associated with an increased root lesion size, and mice homozygous for the B6 allele had twice larger plaque sizes than mice homozygous for the 129 allele at this locus (Figure 3A). In females, 4 suggestive QTLs contributing to root lesion size were identified on Chr1, 6, 9, and 14 with each locus accounting for around 9% of the variance (Figure 2B). The highest peak in females was located on Chr9 (LOD=2.8), but its location at 105 Mb is distal to the peak found in males. The alleles at this locus also have additive effects on the trait. The B6 allele of the nearest SNP (rs3694903) is associated with an increased root lesion size in females, but not in males (Figure 3B). When the male and female data were combined, the locus on Chr9 at 61 Mb remained significant, accounting for 9% of the variance in root lesion size. Two suggestive loci, one on Chr2 at 154 Mb and the other on Chr15 at 68 Mb each accounted for 4% and 7% of the variance in root lesion size (Figure 2C).

**QTLs for Atherosclerosis at the Aortic Arch**

We detected 2 significant QTLs on Chr1 in males that affect arch lesion size (Figure 2D). The distal peak on Chr1 at 163 Mb had a LOD score 5.3 and accounted for 16% of the variance in arch lesion size in males. The proximal peak at 105 Mb had a LOD score 3.9. The 129 alleles of both of these QTLs contributed to the increased arch lesion size with the alleles having an additive effect on the trait, as illustrated by the effects of SNP rs13476024 nearest to the proximal peak (Figure 3C), and SNP rs3685643 nearest to the distal peak (Figure 3D). Neither of the loci on Chr1 for arch lesion size in males was detected in females (Figure 2E). Instead, a significant QTL responsible for arch lesion size in females was found on Chr15 at 92 Mb (LOD=4.0), and the 129 alleles of this locus contributed to an increased arch lesion size additively (Figure 3E). This locus accounts for 14% of the variance in arch lesion size in females. When the data from both sexes were combined, the QTLs on Chr1 at 105 Mb and at 163 Mb remained significant with LOD scores of 5.0 and 6.8 respectively. The suggestive QTL on Chr15 at 96 Mb is also still present with a LOD score of 4.7 (Figure 2F).

Analysis using cross-sectional plaque size in the ascending aorta just proximal to innominate artery of F2 male mice (n=111) gave essentially the same mapping results (Online Figure IV).

**QTLs for the Geometry of Aortic Arch**

For the aortic arch angle, we identified 3 suggestive QTLs in males and 7 in females (Figure 2G and 2H). The male QTL on Chr1 at 126 Mb has the highest LOD score, 3.8, and accounts for 16% of the variance in males. Analysis with the nearest SNP (rs13476098) showed that the 129 allele was dominant in males but recessive in females (Figure 3F). In females, a QTL on Chr15 at 74 Mb had the highest LOD score 3.2 for this trait, with the 129 allele of the nearest SNP (rs3699312) being dominant over the B6 allele (Figure 3G). A broad peak on Chr1 at 86 Mb overlapping with a QTL in males accounts for ≈9% of the variance in females. In the combined analysis, the QTL on Chr1 at 121 Mb was significant. The locus on Chr15 at 76 Mb remained suggestive (Figure 2I).

The mean DA1–3 was predominantly governed by a QTL on Chr9 at 33 Mb (LOD=8.5) in males, which accounts for 25% of their variance (Figure 2J). The B6 allele of the SNP (rs3665206) nearest to this locus showed a larger diameter of the aorta with an additive effect (Figure 3H). Although this peak on Chr9 was not detected in females (Figure 2K), it remained significant when both sexes were analyzed together (Figure 2L). A suggestive QTL with a small contribution to mean DA1–3 was identified on Chr16 in females.

Like the QTLs for atherosclerosis, QTLs for aortic arch geometry were detected more easily in males than in females. However, analyses combining the 2 sexes indicate that loci on Chr1 at 121 Mb and on Chr9 at 33 Mb are still revealed as important determinants of the strain difference in the curvature and diameter of aortic arch, respectively (Figure 2I and 2L). Additionally, although body weights/size of animals may influence on the aortic geometry traits, QTLs for body weight did not map in the same chromosomal regions as geometry traits (Table 2, Online Tables II–IV, and Online Figure V and VI). Mapping results for the geometric traits were not altered when body weight was used as a covariate.
QTLs for Plasma Lipids

A distal region of Chr1 (170 to 180 Mb) was identified as including significant QTLs affecting all 3 plasma lipid levels in females (Figure 2N, 2Q, and 2T). The same QTLs in males were suggestive as contributing to plasma cholesterol and triglyceride levels (Figure 2M and 2P). A middle region of Chr9 (30 to 70 Mb) contained several QTLs contributing to plasma cholesterol and triglyceride levels independent of sex (Figure 2M, 2N, 2P, and 2Q). The 129 alleles at both loci on Chr1 and Chr9 conferred increased levels of plasma lipids. In addition, significant female-specific QTLs contributing to plasma cholesterol levels were detected on Chr2 at 8 Mb (LOD=5.0) and on Chr7 at 123 Mb (LOD=3.7) (Figure 2N). Similarly, a significant male-specific QTL affecting plasma triglyceride levels was identified on Chr7 at 49 Mb (LOD=4.6) (Figure 2P). In contrast to the QTLs for atherosclerosis and geometric traits, the peak LOD scores for plasma lipids were generally higher in females than in males.

Overlapping QTLs Determining Atherosclerosis and Vascular Geometry

The foregoing analyses have identified regions of Chr1, 9, and 15 that affect several of the traits of interest in the present context. We therefore examined these 3 chromosomes in

Figure 2. Genome-wide QTL analyses on atherosclerosis, vascular geometry, and plasma lipids in F2 males (left), females (middle), and combined (right). Atherosclerotic lesion sizes and plasma lipids were logarithmically transformed. Horizontal dashed lines and alternating long and short dash lines represent significant (P<0.05) and suggestive (P=0.63) levels, respectively, as determined by 1000 permutation tests.
more detail using the combined data from males and females (Figure 4). Of the 2 major QTL peaks for arch lesion size on Chr1 (105 and 163 Mb), the proximal and slightly smaller peak at 105 Mb clearly overlaps the QTL for arch angle at 121 Mb (Figure 4A). However, it does not overlap either the peak for plasma total cholesterol or triglyceride level. In contrast, the QTL at 163 Mb overlaps the locus for plasma total cholesterol level. Because the 129 alleles at both of the loci confer larger arch lesion sizes, the data are consistent with the possibility that 129 alleles of a gene on Chr1 that confers wider aortic curvature and the 129 alleles of another gene on Chr1 that confers higher plasma cholesterol level independently contribute to the atherosclerosis susceptibility in the aortic arch. QTLs for triglycerides and HDL-C on Chr1 overlap but peak distal to the QTL for atherosclerosis susceptibility.

The suggestive QTL for arch lesion size on Chr15 at 96 Mb also overlaps the suggestive QTL for angle that peaks at 76 Mb and the plasma triglyceride QTL at 91 Mb (Figure 4B). The 129 alleles at these loci, respectively, confer increased arch lesion size, wider angle, and lower triglyceride levels. Although parental 129-apoE mice have smaller plaques at the aortic root lesion compared to B6-apoE mice, a suggestive QTL for root lesion size on Chr15 at 68 Mb has the 129 allele conferring susceptibility. The arch lesion size QTL on Chr15 at 96 Mb results mainly from the female QTL, whereas the root lesion size QTL on Chr15 at 68 Mb results mainly from the male QTL. The confidence intervals of these 2 QTLs partially overlap, but their peak positions are distinct.

On Chr9, the major QTL for root lesion size overlaps the loci for plasma levels of cholesterol and triglyceride (Figure 4C). Although the 129 allele at this locus confers resistance to the root lesion size, the 129 allele for plasma lipids confers higher levels of cholesterol and triglyceride. A significant peak for aortic diameter also maps on Chr9, but its peak at 33 Mb is distinct from the other peaks.

Discussion
In the present study of the F2 progeny derived from a cross between apoE-null mice on 129 and B6 genetic backgrounds, we have identified QTLs affecting the development of

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Significant QTLs are in bold letters. Mean DA1–3 indicates average diameter of ascending, transverse, and descending aortas. Significance was determined by permutation testing: significant, P<0.05; suggestive, P<0.63. Confidence interval (CI) was defined as a 1 unit LOD dropoff.
plaques at 2 locations of the aortic tree, the root and the arch, and QTLs contributing to aortic geometry and plasma lipids. We find that major loci conferring atherosclerosis susceptibility at the root and arch are distinct from each other and are gender-specific. We also demonstrate that vessel diameter and shape of aortic arch curvature are genetically determined, and that the major QTLs on Chr1 and 15 that influence curvature of the aortic arch overlap the QTLs for atherosclerosis in the aortic arch.

Numerous QTLs for susceptibility to atherosclerosis (Ath) have been reported from genetic crosses between a variety of inbred strains of mice either maintained on an atherogenic diet or bred onto a sensitizing genetic background, such as apoE-null or low-density lipoprotein receptor–null.7–11 Most of these studies have focused on the development of plaques at the aortic root. Paigen et al described the first atherosclerosis susceptibility locus, Ath1, on the distal region of Chr1 in the crosses between the susceptible strain, B6, and resistant strains, C3H/He (C3H) or BALB/c. The B6 allele of Ath1 determines susceptibility to plaque formation at the aortic root in female mice fed an atherogenic diet.12 Subsequently, these investigators narrowed the Ath1 locus to a region between 161 to 164 Mb of Chr1, which contains ~11 genes, and they identified Tnfsf4, the gene coding for OX40 ligand, as most likely to be Ath1.13,14 However, Ath1 was not detected as a QTL in the B6-apoExC3H-apoE cross with the mice fed either normal chow or a western-type high fat diet.11,15 Our cross between 129-apoE and B6-apoE mice revealed a significant QTL on Chr9 with the 129 allele resistant to atherosclerosis in the root area. Although this QTL was detected only in males, its peak position at 61 Mb in the combined analysis with both sexes suggests that it could be Ath29, which was previously detected in chow-fed F2 females from a cross between B6-apoE and C3H-apoE mice. Ath29 also affects resistance to atherosclerosis in C3H mice.11

No other studies have addressed the atherosclerosis susceptibility in the aortic arch to date, although some factors, including fractalkine deficiency, have been reported to affect...
atherosclerosis at different locations differently in a single animal. Our analyses clearly show that the major genetic loci controlling arch lesion size are distinct from those controlling root lesion size in our cross, and neither of the 2 significant QTLs responsible for arch lesion size on Chr1 (105 and 163 Mb) appear to affect root lesion size. Although the higher peak at 163 Mb overlaps Ath1, Ath1 was found for root lesion size and its effects on aortic arch lesion size are not known. In addition, whereas the 129 alleles at the 2 loci controlling root lesion size and its effects on aortic arch lesion size are not known. In addition, whereas the 129 alleles at the 2 loci on Chr1 in our cross confer greater susceptibility in the arch lesion size than the B6 alleles, the B6 allele at Ath1 confers susceptibility over C3H or BALB/c allele. Further studies are necessary to determine whether or not the genes underlying these 2 seemingly different phenotypes are identical. We also note that there are strong gender effects for some of the phenotypes in our F2 progeny, suggesting potential maternal and/or lineage effects. Our F2 progeny was derived from parental crosses between female B6-apoE and male 129-apoE mice. Reciprocal crosses between female 129-apoE and male B6-apoE mice were not productive for unknown reasons (see the Online Data Supplement).

It is particularly noteworthy that only a few loci appear to determine the differences in vessel diameter and curvature of the aortic arch that we find in our 2 strains. Thus, we identified one major QTL on Chr9 affecting aortic diameter and another on Chr1 affecting arch angle. In this context, it is important to note that vessels affected by atherosclerotic plaques are known to undergo remodeling, including widening of curvatures and outward growth of vessels. However, in our evaluation we used 24-week-old F2 mice, an age at which the atherosclerosis is still not advanced sufficiently to cause significant remodeling. Although we cannot completely eliminate the possibility of vascular remodeling at this age, we stress that the strain differences in arch geometry are recognizable in wild-type mice. Additionally, the differently mapped QTLs for vessel diameter and arch angle indicate that vessel diameter does not affect arch angle.

Our most striking finding is that a highly significant QTL for arch lesion size overlaps completely with a locus for arch angle, independently of plasma lipid levels, and that there is a significant positive correlation between these 2 phenotypes in males. We recognize that there are hundreds of genes within the overlapping interval and that colocalization does not establish that a gene affecting atherosclerosis susceptibility in the aortic arch is identical to one leading to wider aortic curvature. Nevertheless, our observations open up the possibility that a gene influencing vessel geometry during embryo development can have alleles that increase the risk for atherosclerosis development later in life. There are many B6/129 SNPs in the coding sequences of genes in this interval. These include genes coding for the chemokine receptors CXCR4 and CXCR7, engrailed 1, dipeptidyl peptide 10, and GLI-Kruppel family member GLI2 (Online Figure VII). Variations in any of these may cause subtle differences in vessel formation during development and remodeling in disease. Narrowing the QTL intervals using strategies such as congenic mouse strains is necessary to identify the candidate genes.

Regardless of whether the colocalization of genetic factors influencing atherosclerosis and vascular geometry turns out to be valid or simply coincidental, our finding of QTLs that differentially influence the susceptibility for atherosclerosis at the root and the arch of the aorta is of great interest and is completely compatible with the ontogeny of the aorta. The vascular smooth muscle cells (VSMCs) of different region of the aorta arise from distinct progenitors. For example, neural crest–derived VSMCs contribute to the development of the ascending aorta, the arch of the aorta, and the 3 major vessels in the region. In contrast, VSMCs contributing to the base portion of the aorta, including the aortic root, are derived from a field of splanchic mesoderm beneath the floor of the foregut called the secondary heart field. The implications of these observations to our study are very persuasive: they suggest that the differences in the QTLs for atherosclerosis susceptibility in the aortic arch and root are a consequence of the different developmental origins of the 2 regions.

In conclusion, our study provides an entry to the study of genetic interactions between vascular geometry and atherosclerosis that are too complex and too subtle to decipher in heterogeneous human populations. Although much more work is required to identify the causative genes, our approach points the way to developing means of evaluating how genetic factors affecting vascular geometry influence the risk for atherosclerosis in humans.

Acknowledgments

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Sources of Funding

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Disclosures

None.

References

QTLs affecting the development of atherosclerosis at the aortic arch and root are distinct from each other and are gender-specific.

The geometric traits of the aortic arch are genetically determined by a small number of genetic loci.

QTLs affecting aortic arch lesion size overlap with those for shape of the aortic arch curvature.

Atherosclerotic plaque development at the aortic root is slower in apoE-null mice with a 129S6/SvEvTac strain background (129-apoE) than in those with a C57BL/6J background (B6-apoE). In contrast, 129-apoE mice develop more extensive atherosclerotic plaque lesions in the aortic arch than B6-apoE mice. The 2 strains also have easily recognizable differences in the aortic arch geometry. We identified quantitative trait loci (QTLs) affecting the development of atherosclerosis at the aortic arch and demonstrated that they are distinct from QTLs for atherosclerosis at the aortic root. Identification of QTLs determining vascular geometry of the aortic arch is novel. No QTL analyses on atherosclerosis at the aortic arch and vascular geometry have been performed previously. Most importantly, we found that QTLs affecting aortic arch lesion size overlap with those for curvature of the aortic arch. These findings suggest that the genes involved in the process of the aortic arch formation are a potential risk factor for atherosclerosis. Our study provides a novel concept of genetic interactions between vascular geometry and atherosclerosis and is a first step toward our understanding of genetic backgrounds affecting atherogenesis that are too complex and too subtle to decipher in heterogeneous human populations.
Aortic Arch Curvature and Atherosclerosis Have Overlapping Quantitative Trait Loci in a Cross Between 129S6/SvEvTac and C57BL/6J Apolipoprotein E−Null Mice

Hirofumi Tomita, Svetlana Zhilicheva, Shinja Kim and Nobuyo Maeda

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Supplement Material

Detailed Methods

Mice

129-apoE mice were generated by our laboratory as previously described (1). B6-apoE mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Male 129-apoE mice were crossed with female B6-apoE mice to generate F1 mice, which were intercrossed to generate F2 progeny (138 males and 128 females). Reciprocal crosses between 129-apoE females and B6-apoE males were not productive for unknown reasons. Crosses using eight 129-apoE females and four B6-apoE males resulted in two pregnancies producing one F1 female and two F1 males during the 10 months of breeding. F2 pups from this lineage were not included in the present study. Mice were maintained on a normal chow and bred in our mouse facility. All experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Plasma lipid measurements

The mice were fasted for 4 h, and blood samples were collected from the retro-orbital sinus. Blood samples were then centrifuged, and plasma levels of total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) were measured as previously described (2).

Anatomical and plaque measurements

At the age of 24 weeks, mice were anesthetized with 2.5% avertin and then perfused with 4% paraformaldehyde under physiological pressures. Segments of the proximal aorta and the portion of the heart containing the aortic root were embedded and sectioned, and area of the atherosclerotic lesions in the aortic root were measured as previously described (2,3). To evaluate anatomical parameters and plaque lesions of the aortic arch, the aortic tree was dissected free of surrounding tissue under a dissection microscope. The aortic samples were then placed in a flat transparent chamber of 1.2 mm depth, and their images were captured. Anatomical parameters and plaque areas in the aortic arch were measured using
Image J 1.40 software. Cross-sectional histological preparations of the ascending aortic arch just proximal to the innominate artery were made at 50 μm intervals and the mean cross sectional plaque size within the aortic arch were determined from three cross sections.

**Genotyping**

Genomic DNA was isolated from the livers using DNeasy Tissue Kit (Qiagen, Hilden, Germany), and double-stranded DNA was quantified using PocoGreen dsDNA Assay Kit (Molecular Probes, Eugene OR, USA). Single nucleotide polymorphism (SNP) analysis was performed using the Illumina BeadArray technology with Mouse Low Density Linkage SNP panels, which is an optimized set of 377 SNPs covering the 19 autosomes and the X chromosome and includes approximately four SNPs per each 27Mb interval across the entire mouse genome. In our cross, 235 SNP markers were informative. DNA from parental and F1 mice were used as controls for each marker.

**QTL mapping**

Using R/qtl software, QTL analyses were performed in each sex separately, or jointly with sex treated as an interactive covariate (4). Because values of lesion areas of the aortic root and arch, and plasma levels of total cholesterol, triglycerides, and HDL-C were not linearly distributed, these data were logarithmically transformed before QTL analysis. The peak logarithm of odds (LOD) score on the candidate chromosomal regions was determined and 1000 permutation tests were used for analysis of each trait to define the genome-wide LOD score threshold required to be significant or suggestive (5). Loci that exceeded the 95th percentile of the permutation distribution were defined as significant (p<0.05) and those exceeding the 37th percentile were suggestive (p<0.63). The allelic contributions of genotypes at a SNP marker closest to the peak LOD position were analyzed in the F2 mice.

**Statistical analysis**

Differences between two groups were compared with Mann-Whitney's U-test. One-way ANOVA was used for statistical analysis among multiple groups followed by Tukey-Kramer’s honestly significant
difference test. Linear Pearson analyses were performed for the correlation study. Differences were considered significant when p values were <0.05.
Supplemental References


**Online Table I.** Aortic diameters of parental, F1, and F2 mice.

<table>
<thead>
<tr>
<th></th>
<th>129-apoE (n)</th>
<th>B6-apoE (n)</th>
<th>F1 (n)</th>
<th>F2 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DA1</strong> M</td>
<td>1.17±0.13* † (19)</td>
<td>1.30±0.13 (22)</td>
<td>1.29±0.12* (15)</td>
<td>1.30±0.14* (138)</td>
</tr>
<tr>
<td>(mm) F</td>
<td>1.03±0.10† (16)</td>
<td>1.22±0.15 (18)</td>
<td>1.16±0.13 (17)</td>
<td>1.17±0.11 (127)</td>
</tr>
<tr>
<td><strong>DA2</strong> M</td>
<td>1.19±0.11* † (19)</td>
<td>1.34±0.12* (22)</td>
<td>1.29±0.13* (15)</td>
<td>1.29±0.12* (138)</td>
</tr>
<tr>
<td>(mm) F</td>
<td>1.03±0.07† (16)</td>
<td>1.24±0.11 (18)</td>
<td>1.19±0.08 (17)</td>
<td>1.18±0.09 (127)</td>
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<tr>
<td><strong>DA3</strong> M</td>
<td>1.14±0.12* (19)</td>
<td>1.13±0.08* (22)</td>
<td>1.18±0.07* (15)</td>
<td>1.18±0.08* (138)</td>
</tr>
<tr>
<td>(mm) F</td>
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<td>1.05±0.07 (18)</td>
<td>1.06±0.08 (17)</td>
<td>1.07±0.09 (127)</td>
</tr>
</tbody>
</table>

Values are means±SD. All mice are 24 weeks of age. DA1, DA2, and DA3 indicate diameter of ascending, transverse, and descending aortas, respectively; M, male; F, female. *p<0.05 against females within each group. †p<0.05 between 129-apoE and B6-apoE mice within each sex. DA1 and DA2 were significantly bigger in B6-apoE mice than in 129-apoE mice. DA1 and DA2 in the F1 and F2 mice were similar to those in the parental B6-apoE mice. While there was no difference in DA3 between 129-apoE and B6-apoE males, there was a small but significant difference in DA3 between 129 and B6-apoE females. DA3 in the F1 and F2 females were similar to those in the parental B6-apoE mice.
**Online Table II.** Identified QTLs affecting atherosclerotic lesion sizes, anatomical phenotypes, body weight, and plasma lipids in F2 males.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chr</th>
<th>Peak (Mb)</th>
<th>CI (Mb)</th>
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<th>Nearest marker</th>
<th>Variance (%)</th>
<th>Significance</th>
<th>High allele</th>
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Significant QTLs are in bold letters. DA1, DA2, and DA3 indicate diameter of ascending, transverse, and descending aortas, respectively; Mean DA1-3, average diameter of DA1, DA2, and DA3; Chr,
chromosome; Mb, megabase; LOD, logarithm of odds. Root and arch lesion sizes, cholesterol, triglyceride, and HDL-C were logarithmically transformed. Significance was determined by permutation testing: significant $p<0.05$, suggestive $p<0.63$. Confidence interval (CI) was defined as a one unit LOD drop-off.
Online Table III. Identified QTLs affecting atherosclerotic lesion sizes, anatomical phenotypes, body weight, and plasma lipids in F2 females.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chr (Mb)</th>
<th>Peak CI (Mb)</th>
<th>LOD</th>
<th>Nearest marker</th>
<th>Variance (%)</th>
<th>Significance</th>
<th>High allele</th>
</tr>
</thead>
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<tr>
<td>Root lesion</td>
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Significant QTLs are in bold letters. Abbreviations are shown in Online Table II.
Online Table IV. Identified QTLs affecting atherosclerotic lesion sizes, anatomical phenotypes, body weight, and plasma lipids using the combined data from F2 males and females with sex treated as an interactive covariate.

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Significant QTLs are in bold letters. Abbreviations are shown in Online Table II.
Online Figure I

A. Male

B. Female

C. Male

D. Female

E. Male

F. Female

G. Male

H. Female

Online Figure I
Online Figure I. Distributions of root lesion size (A and B), arch lesion size (C and D), angle (E and F), mean diameter of aortic arch (mean DA1-3) (G and H), body weight (I and J), cholesterol (K and L), triglyceride (M and N), and HDL-C (O and P) in the parental, F1, and F2 mice. Atherosclerotic lesion sizes and plasma levels of cholesterol, triglyceride, and HDL-C were logarithmically transformed. Horizontal lines represent the mean of each group.
Online Figure II. Relationships between atherosclerotic lesion sizes and geometric parameters in F2 mice. Atherosclerotic lesion sizes were logarithmically transformed. A-D. Relationships between arch lesion sizes and angle (A and B) or mean diameter of aortic arch (mean DA1-3) (C and D). E and F. Relationships between arch lesion size and root lesion size. G-J. Relationships between root lesion sizes and angle (G and H) or mean DA1-3 (I and J).
Online Figure III. Relationships between atherosclerotic lesion sizes and plasma lipids in F2 mice. Atherosclerotic lesion sizes and plasma lipids were logarithmically transformed. A-F. Relationships between arch lesion sizes and cholesterol (A and B), triglyceride (C and D), or HDL-C (E and F). G-L. Relationships between root lesion sizes and cholesterol (G and H), triglyceride (I and J), or HDL-C (K and L).
Online Figure IV. Genome-wide QTL analysis on aortic arch lesion sizes measured by cross-sectional histological preparations in male F2 population (n=111). Lesion sizes were logarithmically transformed. Horizontal dashed line and alternate long and short dash line represent significant (p=0.05) and suggestive (p=0.63) levels, respectively, as determined by 1000 permutation tests. Two significant peaks were detected on chromosome 1 at 121 and 159Mb.
Online Figure V. Genome-wide QTL analyses on body weight in F2 males (A) and in F2 females (B). The combined data from F2 males and females were analyzed with sex treated as an interactive covariate (C). Horizontal dashed lines and alternate long and short dash lines represent significant (p=0.05) and suggestive (p=0.63) levels, respectively, as determined by 1000 permutation tests.
Online Figure VI. Relationships between body weight and angle (A and B) or mean diameter of aortic arch (mean DA1-3) (C and D) in F2 males and females. There are no significant correlations between body weight and geometric traits, except that body weight has a small but positive correlation with aortic diameter in individual F2 females.
Online Figure VII. Haplotype block pattern in the interval of chromosome 1 where QTLs for arch lesion size and arch angle overlap. SNP data of C57BL/6J and 129S1/SvlmJ strain backgrounds from the Perlegen Mouse SNP Browser are used, because 129S6/SvEvTac strain data are not available. Identical regions between the two strains are in yellow, and different regions are in red. Arrows show the map position of the representative candidate genes. **Cxcr4** and **Cxcr7** indicate chemokine (C-X-C motif) receptors 4 and 7, respectively; **Cntnap5b**, contactin associated protein-like 5B; **Serpnb8**, serine (or cysteine) peptidase inhibitor, clade B, member 8; **Gli2**, GLI-Kruppel family member GLI2; **En1**, engrailed 1; **Dpp10**, dipeptidylpeptidase 10.