Determinants of Atherosclerosis Susceptibility in the C3H and C57BL/6 Mouse Model
Evidence for Involvement of Endothelial Cells but Not Blood Cells or Cholesterol Metabolism

Weibin Shi, Nicholas J. Wang, Diana M. Shih, Victor Z. Sun, Xuping Wang, Aldons J. Lusis

Abstract—Lipids, monocytes, and arterial wall cells are primary components involved in atherogenesis. Using the inbred mouse strains C57BL/6J (B6) and C3H/HeJ (C3H), which have been extensively studied as models of the genetic control of diet-induced atherosclerosis, we examined which of these components determine genetic susceptibility. To test whether dietary responsiveness is involved, a congenic strain of C3H carrying an apoE-null allele (apoE2/2) was constructed. Although C3H.apoE2/2 mice had higher plasma cholesterol levels, they developed much smaller lesions than their B6.apoE2/2 counterpart on either chow or Western diets. Reciprocal bone marrow transplantation between the strains, with congenics carrying the same H-2 haplotype, was performed to examine the role of monocytes. The atherosclerosis susceptibility was not altered in the recipient mice, indicating that variations in monocyte function were not involved. Endothelial cells isolated from the aorta of B6 mice exhibited a dramatic induction of monocyte chemotactic protein-1, macrophage colony–stimulating factor, vascular cell adhesion molecule-1, and heme oxygenase-1 in response to minimally modified LDL, whereas endothelial cells from C3H mice showed little or no induction. In a set of recombinant inbred strains derived from the B6 and C3H parental strains, endothelial responses to minimally modified LDL cosegregated with aortic lesion size. These data provide strong evidence that endothelial cells, but not monocytes or plasma lipid levels, account for the difference in susceptibility to atherosclerosis between the 2 mouse strains. (Circ Res. 2000;86:1078-1084.)

Key Words: hyperlipidemia ■ monocytes ■ endothelium ■ cells ■ atherosclerosis ■ inbred strains

Atherosclerosis is a complex disease of the large and medium-sized arteries that results from interactions among lipids, monocytes, and arterial wall cells. Increases in plasma LDL levels or decreases in HDL levels are major risk factors for the development of atherosclerosis. Endothelial cells (ECs) have been considered to play a crucial role in initiation of the disease. They oxidatively modify LDL, and in response to oxidized LDL or its components, ECs express monocyte chemotactic protein-1 (MCP-1), macrophage colony–stimulating factor (M-CSF), vascular cell adhesion molecule-1 (VCAM-1), and other adhesion molecules and growth factors that promote monocyte transmigration into the subendothelium and differentiation into macrophages. Subsequently, the macrophages ingest modified LDL to give rise to foam cells, the hallmark of early atherosclerosis. Monocytes are the primary source of foam cells in atherosclerotic lesions. There is convincing evidence that circulating monocytes significantly influence atherosclerosis progression in mouse models. The absence of M-CSF, a growth factor for monocyte-macrophages, markedly reduced atherosclerotic lesions in fat-fed wild-type mice, apoE2/2 mice, and LDL receptor–deficient mice. Also, the transplantation of bone marrow from apoE2/2 mice into wild-type mice greatly increased lesion development.

The variation in atherosclerosis susceptibility among inbred strains of mice provides a method of identifying the cellular and molecular interactions in atherogenesis. B6 and C3H mice are 2 commonly used inbred strains that differ strikingly in aortic fatty streak development when fed a high-fat, high-cholesterol diet with cholate. Because this atherogenic diet induces a marked reduction in plasma HDL levels of B6 mice but not in those of C3H mice, the alteration in HDL levels has been considered to be responsible for the difference in susceptibility. Genetic studies of the C3H and B6 mouse model have provided evidence for major gene effects, but the large nongenetic variance of lesion development has made genetic analysis difficult. The results of recent studies have suggested that inflammatory mechanisms may underlie the differences in susceptibility. We found that the atherogenic diet resulted in a dramatic induction in several inflammatory genes in the livers of B6 mice but not in those of C3H mice. Because of the small size of arteries

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in mice, these differences between the 2 strains have not been demonstrated at the level of arterial walls. The principal objective of the present study was to examine the contribution of lipids, monocytes, and ECs to atherosclerosis susceptibility and resistance in B6 and C3H mice.

Materials and Methods

Construction of C3H apoE \(^{-/-}\) Mice

C3H apoE \(^{-/-}\) mice were created by crossing B6 apoE \(^{-/-}\) mice with C3H/HeJ mice. The resulting heterozygous apoE \(^{-/-}\) mice were then backcrossed to C3H mice for 4 generations. Mice homozygous for the apoE-null allele on a C3H background were subsequently generated by brother-sister mating.

Bone Marrow Transplantation

Two-month-old male C57BL6/J and C3H.SW mice, which share the same major histocompatible haplotype, H-2\(^{a}\), were used for bone marrow transplantation (BMT). Recipient mice were lethally irradiated. Bone marrow was harvested by flushing of the femurs and tibias of donor mice. Each recipient mouse was injected with \(10^7\) bone marrow cells through the tail veins.

Two weeks after BMT, overnight-fasted mice were bled, and DNA from the blood was analyzed with the use of polymorphic markers. The mice that expressed donor DNA were fed for 12 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.\(^19\)

The en face measurement of aortic lesions was made in apoE \(^{-/-}\) mice fed the Western diet. The aorta was stained with Sudan IV. The extent of the Sudan IV-positive area was quantified and expressed as a percent of the total aortic surface.

Plasma Lipid Measurements

Enzymatic assays for total and free cholesterol, HDL cholesterol, and triglycerides were performed as described by Hedrick et al.\(^20\)

Lipoprotein Isolation and Modification

LDL was isolated from the serum of healthy human donors as described by Havel et al.\(^21\) Minimally oxidized LDL was prepared through incubation of LDL with 7 \(\mu\)mol/L FeSO\(_4\) or 4 \(\mu\)mol/L CuSO\(_4\), as described previously.\(^22\)

Culture and Treatment of ECs

ECs from the thoracic aorta were isolated with an explantation technique. The thoracic aorta was gently cleansed of periadventitial fat and connective tissue and cut into rings \(\sim 3\) 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 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 DiI-Ac-LDL.

Confluent cells at passages 4 to 6 were treated for 4 hours with 200 \(\mu\)g/mL native LDL, 200 \(\mu\)g/mL minimally modified (MM)-LDL, 2 \(\mu\)g/mL LPS, or medium only.

RNA Extraction and Northern Blot Analysis

Total RNA of the ECs was isolated with TRIzol Reagents (GIBCO) according to the protocol from the manufacturer. The RNA was precipitated on agarose-formaldehyde gel, and transferred onto nylon membranes. The blots were hybridized with \(^{32}\)P-labeled mouse cDNA probes. The blots were exposed to Hyperfilm-ECL. The density of the bands was quantified with a densitometer and standardized with GAPDH.

Statistical Analysis

Data were presented as mean \(\pm\) SEM. ANOVA was used to determine differences between groups in lesions or lipid levels. When only 2 mean values were compared, the Student’s \(t\) test was used. Differences were considered statistically significant at \(P<0.05\).

Results

Plasma Lipid Levels and Aortic Lesions of C3H apoE-Deficient Mice

Both B6 apoE \(^{-/-}\) mice and C3H apoE \(^{-/-}\) mice exhibited hypercholesterolemia when on a normal chow diet, and it was aggravated by feeding the Western diet (Figure 1). The total cholesterol, free cholesterol, and triglyceride levels were slightly higher in C3H apoE \(^{-/-}\) mice than in B6 apoE \(^{-/-}\) mice on either chow (\(P=0.04\)) or Western diet (\(P<0.0001\)). On the chow, there were no significant differences in HDL cholesterol levels between C3H apoE \(^{-/-}\) (20.2 \(\pm\) 7.3 mg/L) and B6 apoE \(^{-/-}\) mice (21.0 \(\pm\) 2.8 mg/L). On the Western diet, HDL cholesterol levels were significantly elevated in C3H apoE \(^{-/-}\) mice (118.8 \(\pm\) 11.1 mg/L) (\(P=0.008\)) but not in B6 apoE \(^{-/-}\) mice (22.6 \(\pm\) 6.0 mg/L).

At 12 weeks of age on the chow diet, the average area of aortic atherosclerotic lesions per section per animal in B6 apoE \(^{-/-}\) mice was 19 321 \(\pm\) 2992 \(\mu\)m\(^2\) (n = 15), but in C3H apoE \(^{-/-}\) mice, only 3 of 11 mice had lesions, with an...
average of 140±73 μm² (Figure 2) (P<0.0001). After 16 weeks on the Western diet, which started at 8 weeks of age, the mean area of aortic lesions was almost 10-fold greater in B6.apoE^2/2 mice (n=15) than in C3H.apoE^2/2 mice (n=10) (601 772±40 214 versus 63 000±5775 μm²) (P<0.0001).

The en face measurement of aortic lesions was made in mice maintained on the Western diet. Significantly reduced lesion development was detected in C3H.apoE^2/2 mice (5.3±0.9% versus 10.4±0.74% in B6.apoE^2/2 mice; P=0.0003).

Effect of BMT on Atherosclerosis
To examine the role of monocytes in atherogenesis, BMT was performed in 4 groups of mice: B6→C3H, C3H→B6, B6→B6, and C3H→C3H. In each group, 8 to 10 mice were used as recipients. An analysis of serum cholesterol and triglyceride levels 12 weeks after the atherogenic diet showed no significant differences between the mice receiving bone marrow from the other strain and those receiving bone marrow from the same strain (Figure 3). The mean area of aortic lesions was similar between groups C3H→B6 and B6→B6 (1477±496 versus 1503±472 μm²) (P=0.97; Figure 4). In addition, the mean lesion area between groups B6→C3H and C3H→C3H did not differ (43±33 versus 130±113 μm²) (P=0.48).

Responses of ECs to MM-LDL
As shown in Figure 5 and the Table, MM-LDL, prepared with Fe^{2+} or Cu^{2+} oxidation, induced marked production of MCP-1, M-CSF, VCAM-1, and heme oxygenase-1 (HO-1) mRNAs in ECs from susceptible B6 mice. In contrast, ECs from resistant C3H mice showed small or no induction of these mRNAs. Native LDL had no effect on gene induction in ECs from either strain. Interestingly, LPS induced prominent but similar expression of MCP-1, M-CSF, and VCAM-1 genes in both strains. Unlike MM-LDL, LPS had little effect on HO-1 expression. As a housekeeping gene, GAPDH mRNA was not induced. These results have been confirmed with multiple independent cultures of ECs from the 2 strains and with numerous separate preparations of oxidized LDL.

The AOP2 gene maps to a region of chromosome 1 that has been thought to harbor a gene that contributes to atherogen-
esis in a genetic cross between B6 and C3H mice. ECs of both B6 and C3H mice expressed similar baseline levels of AOP2 mRNA, and neither MM-LDL nor LPS influenced expression of the gene.

In a set of recombinant inbred (RI) strains (designated BXH) derived from the wild-type B6 and C3H strains, we examined the genetic segregation between endothelial responses to MM-LDL and atherosclerosis susceptibility. Because HO-1 mRNA was induced by MM-LDL but not by LPS, its induction was used to represent endothelial responses to MM-LDL. The size of aortic lesions after the atherogenic diet was fed for 15 weeks was used as the parameter for susceptibility to atherosclerosis. MM-LDL resulted in HO-1 mRNA induction in a strain-specific pattern, and strains with higher HO-1 induction had larger lesions (Fe-LDL r = 0.57, P = 0.0001; Cu-LDL r = 0.79, P = 0.0013) (Figure 6). Native LDL and LPS had little effect on HO-1 expression of ECs in these strains. These data indicate that EC responses to oxidized LDL cosegregate with atherosclerosis susceptibility.

Discussion

Inbred mouse strains B6 and C3H have been studied extensively as a model of the genetic control of atherogenesis. In response to an atherogenic diet, B6 mice develop fatty streak lesions in the proximal aorta, whereas C3H mice are totally resistant to fatty streak formation. The present study was undertaken to determine the levels (plasma lipids, monocytes, and arterial wall cells) at which genetic variations affect atherogenesis in these 2 mouse strains.

In wild-type mouse models, atherosclerosis is induced by feeding a high-fat, high-cholesterol diet that contains cholate.

### Table 1: Induction of MCP-1, M-CSF, HO-1, VCAM-1, and AOP2 mRNA in Aortic Endothelial Cells From C57BL/6J (B6) and C3H/HeJ (C3H) Mice

<table>
<thead>
<tr>
<th></th>
<th>Fe-LDL</th>
<th>Cu-LDL</th>
<th>LDL</th>
<th>LPS</th>
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<tbody>
<tr>
<td>B6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>5.19±1.27*</td>
<td>2.17±0.68*</td>
<td>0.21±0.08</td>
<td>10.8±1.2*</td>
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<tr>
<td>M-CSF</td>
<td>1.12±0.28*</td>
<td>0.73±0.31*</td>
<td>0.30±0.08</td>
<td>1.85±0.3*</td>
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<tr>
<td>VCAM-1</td>
<td>1.27±0.40*</td>
<td>1.29±0.94*</td>
<td>0.33±0.19</td>
<td>3.6±0.40*</td>
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<tr>
<td>HO-1</td>
<td>7.46±1.41*</td>
<td>5.38±1.32*</td>
<td>0.41±0.04</td>
<td>0.49±0.07</td>
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<tr>
<td>AOP2</td>
<td>0.57±0.09</td>
<td>0.43±0.06</td>
<td>0.46±0.07</td>
<td>0.42±0.10</td>
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<tr>
<td>C3H</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.68±0.51*</td>
<td>0.10±0.03</td>
<td>0.11±0.03</td>
<td>10.26±1.84*</td>
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<tr>
<td>M-CSF</td>
<td>0.51±0.22</td>
<td>0.35±0.16</td>
<td>0.48±0.21</td>
<td>2.03±0.18*</td>
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<tr>
<td>VCAM-1</td>
<td>0.10±0.04</td>
<td>0.05±0.01</td>
<td>0.05±0.01</td>
<td>0.75±0.16*</td>
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<tr>
<td>HO-1</td>
<td>2.63±0.35*</td>
<td>1.69±0.21</td>
<td>1.44±0.09</td>
<td>1.35±0.21</td>
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<tr>
<td>AOP2</td>
<td>0.62±0.05</td>
<td>0.53±0.08</td>
<td>0.54±0.03</td>
<td>0.62±0.02</td>
</tr>
</tbody>
</table>

*P<0.05 vs LDL.

Endothelial cells were incubated for 4 hours with 200 μg/mL Fe2⁺ or Cu2⁺-modified LDL, native LDL, or 2 μg/mL LPS. The levels of mRNA were measured with Northern blot analysis and expressed as a ratio of densitometry units of particular mRNAs to GAPDH mRNA. Values are expressed as mean±SEM for 3 to 6 individual mice.
In response to this diet, strain B6 mice have reduced levels of HDL compared with C3H mice. Moreover, this diet results in a reduction in serum paraoxonase, an LDL-associated enzyme that protects against LDL oxidation, in B6 mice but not in C3H mice. The apoE/−/− mice represent a mouse model in which spontaneous hyperlipidemia and atherosclerosis occur with a low-fat, low-cholesterol diet. In the present study, we constructed congenic apoE/−/− mice on a C3H genetic background by repeatedly backcrossing B6.apoE/−/− mice to C3H mice, with selection for the apoE-null allele, followed by intercrossing to produce homozygous apoE-null mice on the C3H background. The resulting apoE/−/− mice had a background of ∼96% C3H. Consistent with the finding with the wild-type mice fed the atherogenic diet, C3H.apoE/−/− mice were highly resistant to atherosclerosis, developing much smaller lesions than B6.apoE/−/− mice on either chow or a Western diet. The resistance to atherosclerosis is unlikely to be associated with HDL levels because there were no significant differences in HDL levels between the 2 strains of mice on the chow diet. As seen in wild-type mice, the Western diet elevated the HDL level in C3H.apoE/−/− mice but not in B6.apoE/−/− mice. A recent study in which the apoE-null allele was transferred onto the genetic background of strain FVB/NT also revealed a strong effect of genetic background on atherosclerosis susceptibility and plasma lipid levels.

Macrophage-derived foam cells are the principal cellular elements in early atherosclerotic lesions. Moreover, foam cells produce cytokines and growth factors that play an important role in the development and progression of atherosclerosis. In the present study, the role of monocytes/macrophages in atherosclerosis susceptibility was examined through reciprocal transplantation of the bone marrow of B6 and C3H mice. B6 and C3H mice differ in monocytes (or other blood elements) do not play an important role in the genetic difference in atherosclerosis susceptibility between the 2 strains.

We used 2 different models (dietary and apoE knockout) to determine the influence of monocytes and plasma lipids on atherosclerosis susceptibility of B6 and C3H mice. This raises the concern that the genetic control of atherosclerosis susceptibility may differ between the 2 models. However, the 2 models have yielded generally consistent findings in a number of studies. For example, the expression of a human apoA-I transgene dramatically reduced lesion formation in both models. Similarly, a null mutation of M-CSF nearly abolished lesion formation in both models. In addition, T or B lymphocyte deficiencies exhibited similar effects in the 2 models. Finally, recent studies have shown that a null mutation of paraoxonase increased lesion formation ∼2-fold in both models (D.M.S. and A.J.L., unpublished observations).

Accumulated evidence indicates that LDL oxidation plays an important role in atherogenesis. Minimally or mildly oxidized species of LDL are potent inducers of inflammatory genes. Among them, M-CSF, MCP-1, and VCAM-1 are highly elevated in atherosclerotic lesions compared with normal artery and highly induced in ECs by oxidized LDL. HO-1, an enzyme that catalyzes heme to biliverdin, carbon monoxide, and free iron, is a sensitive indicator of cellular oxidative stress and is highly elevated in atherosclerotic lesions. The present data clearly show that ECs from susceptible B6 mice exhibit induction of mRNA for MCP-1, M-CSF, VCAM-1, and HO-1 in response to MM-LDL, whereas ECs from C3H mice exhibit little or no induction (Figure 5). In contrast to MM-LDL, LPS induced similar expression of MCP-1, M-CSF, and VCAM-1 in ECs from the 2 strains and had no effect on HO-1 expression. These findings are consistent with the observation that in ECs, MM-LDL specifically induces adhesion molecules for monocytes, whereas LPS induces adhesion molecules for both neutrophils and monocytes. Our results are in agreement with previous in vivo findings that the injection of MM-LDL or the feeding of an atherogenic diet induced a greater production of MCP-1, M-CSF, and HO-1 in B6 mice than in C3H mice.

To determine whether endothelial responsiveness to MM-LDL is associated with genetic susceptibility to atherosclerosis, we examined endothelial responses to MM-LDL in a set of RI strains derived from the wild-type B6 and C3H mice. Each RI strain represents a unique mixture of genes derived from the parental strains, and depending on the combination of genes that was inherited, each strain exhibits a particular degree of susceptibility to atherosclerosis. In the present study, we examined the induction of HO-1 mRNA because it was highly induced by MM-LDL but was not by LPS. We found that endothelial responses to MM-LDL segregated with the susceptibility to aortic atherosclerotic lesions (Figure 6). This finding provides genetic evidence that variations in endothelial responses to MM-LDL are closely linked with aortic atherosclerotic lesion formation. Thus, it is likely that feeding of the atherogenic diet to mice results in lipid accumulation in the aorta and other arteries, where the lipids become oxidatively modified. The oxidized lipids then stimulate ECs to express MCP-1, M-CSF, VCAM-1, and other proinflammatory molecules, resulting in monocyte infiltration and foam cell formation. It appears that because the ECs of B6 mice are much more responsive to oxidized lipoproteins than are the ECs of C3H mice, the degree of inflammation and monocyte infiltration is greater in B6 than in C3H mice.

The AOP2 gene, which encodes an antioxidant protein, maps to distal mouse chromosome 1 near the putative location of the Ath-1 gene. A recent study suggested that the AOP2 gene may in fact correspond to Ath-1. Our present results do not support this conclusion, because no differences were observed in AOP2 expression between strains in response to MM-LDL (Figure 5).

In C3H mice, a naturally occurring mutation on chromosome 4 renders most cells, including lymphocytes and macrophages, insensitive to LPS-induced cytokine release. A
recent study showed that the LPS allele of C3H mice corresponds to a missense mutation in exon 3 of the Toll-like receptor-4 gene.\textsuperscript{43} The Toll-like receptor-4 has been suggested to transduce the LPS signal across the plasma membrane. However, our present results indicated that ECs from C3H mice were as responsive to LPS as those from B6 mice with respect to MCP-1, M-CSF, and VCAM-1 mRNA induction. These data suggest that receptor subtypes on ECs that mediate the effect of LPS may differ from those on lymphocytes and macrophages.

The demonstration that the differences in atherosclerosis susceptibility between inbred mouse strains B6 and C3H are due, at least in part, to genetic differences in endothelial responses provides the first clear evidence for genetic factors in atherosclerosis that act at the level of the vessel wall.

Acknowledgment

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References


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