Genetic Backgrounds but Not Sizes of Atherosclerotic Lesions Determine Medial Destruction in the Aortic Root of Apolipoprotein E–Deficient Mice

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Objective—Destruction of the elastic media is the most striking histologic feature of atherosclerotic aortic aneurysms. Apolipoprotein E–deficient (apoE−/−) mice fed a Western diet develop advanced atherosclerotic lesions in the aorta. We sought to assess the integrity of atherosclerotic aortic walls in 2 apoE−/− strains, C57BL/6 (B6) and C3H/HeJ (C3H) that differ markedly in atherosclerosis susceptibility.

Methods and Results—C3H.apoE−/− mice developed much smaller atherosclerotic lesions than did B6.apoE−/− mice after being fed a Western diet for 16 weeks, but the C3H.apoE−/− mice exhibited destruction of the elastic media, including erosion, fragmentation, and focal dilatation beneath plaques. Gelatin and casein zymography showed proteolytic activity of matrix metalloproteinases (MMPs) -9, -2, and -12 in aortic tissues and of MMP-9 and -12 in macrophages from both strains. However, C3H.apoE−/− mice showed significantly increased MMP-2 and -12 activity in aortas and macrophages compared with those from B6.apoE−/− mice. MMP-9 activity was comparable in aortic tissues of the 2 strains, but it was significantly higher in macrophages from C3H.apoE−/− than from B6.apoE−/− mice.

Conclusions—Data indicate that genetic backgrounds but not sizes of atherosclerotic lesions determine medial destruction in the aortic root of apoE−/− mice and that an increase in MMP proteolytic activity might contribute to the medial destruction of aortic walls in C3H.apoE−/− mice. (Arterioscler Thromb Vasc Biol. 2003;23:1901-1906.)

Key Words: atherosclerosis ■ genetic predisposition ■ aortic aneurysms ■ matrix metalloproteinases

Atherosclerosis is a chronic inflammatory disease of large and medium arteries characterized by lipid deposition in the arterial wall.1,2 This disease progresses from foam cell lesions to advanced lesions with fibrous caps and necrotic lipid cores. Rupture of unstable plaques results in the formation of occluding thrombi, which trigger complications such as heart attack and stroke. On the other hand, inflammatory cells such as macrophages and lymphocytes in atherosclerotic lesions might infiltrate into the media and adventitia and impair the connective tissue, especially the elastic lamellae.3,4 The vessel wall is then unable to withstand the expansive force of blood pressure, and aneurysms ensue.

Aortic aneurysms are a common complication consequent to advanced atherosclerotic lesions in elderly individuals.3 The most characteristic histologic change of atherosclerotic aortic aneurysms is the destruction of medial elastic lamellae.3,4 In addition, medial and adventitial infiltration by macrophages and lymphocytes and loss of smooth muscle cells are striking in atherosclerotic aortic aneurysms in humans. Degeneration of the elastic media has been attributed to proteolytic degradation of structural proteins by proteases released by inflammatory cells. The best-studied group of such enzymes involved is the matrix metalloproteinases (MMPs), a family of enzymes that share the ability to degrade many molecules of the extracellular matrix. MMPs are released into tissues as inactive zymogens that can be activated by plasmin and other activators. The activity of MMPs is inhibited by the endogenous tissue inhibitors of metalloproteinases-1 through -4. Increased expression of MMP-1, -2, -3, -9, and -12 has been observed in aneurysm walls.5-8 Targeted gene disruption of MMP-2, -3, and -9 suppresses the development of experimental abdominal aortic aneurysms.9-11 The absence of tissue inhibitor of metalloproteinase-1 enhances MMP activity and promotes aneurysm formation.12

Apolipoprotein E–deficient (apoE−/−) mice have hyperlipidemia and develop all phases of atherosclerotic lesions seen in humans.13,14 Carmeliet et al15 reported that at the advanced
lesion stage, apoE<sup>−/−</sup> mice develop atherosclerosis-associated aneurysms in the aorta. However, subsequent studies failed to find evidence of aortic aneurysm formation in the mice. The reasons for the conflicting results are unknown. In the present study, we have provided experimental evidence that genetic backgrounds influence medial destruction beneath atherosclerotic lesions in apoE<sup>−/−</sup> mouse strains. Our results also suggest that elevation in MMP activity in aortas and macrophages might contribute to the medial destruction in C3H<sub>−/−</sub> mice.

Methods

Mice and Protocols

B6<sup>−/−</sup> mice, which had been sequentially backcrossed with C57BL/6J mice for 10 generations, were purchased from the Jackson Laboratories (Bar Harbor, Me). C3H<sub>−/−</sub> mice were generated in our laboratory by initially crossing B6<sup>−/−</sup> mice with C3H/Hej mice. The resulting heterozygous apoE<sup>±</sup> mice were sequentially backcrossed to C3H mice for at least 4 generations, followed by brother-sister mating to generate homozygous apoE<sup>−/−</sup> mice. The mice were raised on a standard rodent chow containing 4% fat, 0.15% cholesterol, and 19.5% casein without sodium chloride (Ralston-Purina Co). At 8 weeks of age, mice (approximately half male and half female) were started on an adjusted, Western-type diet containing 42% fat, 0.15% cholesterol, and 19.5% casein without sodium chloride (TD 88137) and maintained on this diet for 16 weeks. All procedures were in accordance with current National Institutes of Health guidelines and approved by the University Animal Care and Use Committees.

Assessment of Atherosclerotic Lesions

The method for assessment of atheromatous lesions in the aorta was performed as previously reported by Qiao et al. In brief, animals were killed by cervical dislocation, and the heart and proximal aorta were excised and washed in phosphate-buffered saline. The basal portion of the heart and proximal aorta were embedded in mounting medium (OCT compound [Miles, Inc]), frozen on dry ice, and then stored at −70°C until being sectioned. Serial 10-μm-thick cryosections from the middle portion of the ventricles to the aortic arch were collected on poly-D-lysine–coated slides. In the region from the appearance to the disappearance of the aortic valves, every other section was collected. In all other regions, every fifth section was collected. The total number of sections examined for lesions ranged from 75 to 110 per mouse. Sections were stained with oil red O, hematoxylin, counterstained with fast green, and examined by light microscopy. For the en face assessment of aortic lesions, the aorta containing the ascending, arch, thoracic, and abdominal segments was dissected; gently cleaned of the adventitia; stained with Sudan IV; and imaged with commercially available software (Image-Pro Plus, Media Cybernetics).

To examine structural changes in detail, the aortas of mice were perfusion-fixed in situ by infusion at 80 mm Hg with 10% formalin. The basal portions of the heart and proximal aorta were then dissected, processed by standard histologic techniques, and embedded in paraffin. Serial 10-μm-thick sections were cut and stained for elastin with van Gieson’s stain (Sigma).

Gelatin and Casein Zymography

The activity of MMPs in aortic tissues and peritoneal macrophages was determined by gelatin and casein zymography. Six-week-old male mice were used for preparation of aortic proteins and macrophage cellular proteins. At this age, apoE<sup>−/−</sup> mice are known to have no detectable atherosclerotic lesions in the aorta. The aortas were washed thoroughly with phosphate-buffered saline containing 5 U/mL heparin through the left ventricle of the heart, cleaned of periadventitial fat and connective tissue, and snap-frozen in LN<sub>2</sub>. The frozen aortas were mechanically broken up, dispersed in a sample buffer (Invitrogen), and centrifuged at 500g for 10 minutes at 4°C; the supernatant was then collected and used for zymography. For isolation of macrophages, mice were injected intraperitoneally with 1 mL of 3% thioglycolate. Five days later, peritoneal macrophages were harvested by lavage of the peritoneal cavity with 40 mL cold phosphate-buffered saline. Red blood cells were removed by lysis with NH<sub>4</sub>Cl (150 mmol/L, pH 7.3). The remaining cells were suspended in the sample buffer and lysed by the freeze-thaw method. Ten micrometers of aortic or macrophage proteins was separated by electrophoresis on 10% gelatin or 12% casein zymogram gels (Invitrogen). The gels were subsequently incubated overnight at 37°C in a buffer provided by the manufacturer. Enzymatic activities were visualized as negative staining with Coomassie blue R-250 and quantified with a densitometer (Molecular Dynamics).

In Situ Zymography

In situ zymography was performed to localize gelatinolytic and caseinolytic activity in aortic tissues, as described by Faia et al. Cryosections (10 μm thick) of the proximal aorta from 12-month-old apoE<sup>−/−</sup> mice were overlaid on 0.5% low-melting-point agarose (Gibco) gels containing the assay solution (50 mmol/L Tris-HCl, 5 mmol/L CaCl<sub>2</sub>, 5 μmol/L ZnCl<sub>2</sub>, pH 7.5) and fluorescein-conjugated gelatin (50 μg/mL, G-1387; Molecular Probes) or casein (100 μg/mL, C-2990) and incubated overnight at 37°C. EDTA (10 mmol/L) was added to the assay solution as controls. Sections were then examined for fluorescence intensity with an epifluorescence microscope (Nikon TE300).

Plasma Lipid Measurements

Mice were fasted overnight before blood was collected from retroorbital veins under isoflurane anesthesia. Plasma total cholesterol, HDL cholesterol, and triglycerides were measured by enzymatic assays, as previously described by Hedrick et al.

Statistical Analysis

Data are presented as mean±SE, with n indicating the number of mice. Student’s t test was used to determine differences between strains B6 and C3H in lesion formation, MMP activities, and plasma lipid levels. Differences in phenotype frequencies between the 2 strains were tested by χ<sup>2</sup> analyses. Differences were considered statistically significant at P<0.05.

Results

Atherosclerotic Lesions and Aortic Wall Destruction

After being fed the Western diet for 16 weeks, B6<sup>−/−</sup> mice developed diffuse atherosclerotic lesions throughout the aorta. In contrast, atherosclerotic lesions of C3H<sub>−/−</sub> mice were primarily localized to the aortic root and arch (Figure 1). The size of the lesions in the aortic root was quantified after transverse cryosections were stained with oil red O. B6<sup>−/−</sup> mice (n=15) developed much larger atherosclerotic lesions than their C3H counterparts (n=10; 601 800±40 200 vs 63 000±5800 μm<sup>2</sup> per cross section per mouse; P<0.0001; Table 1). Although C3H<sub>−/−</sub> mice developed smaller atherosclerotic lesions, the media of their aortic walls showed erosion, disruption, or fragmentation by atherosclerotic lesions (Figure 2A and Table 1). Some plaques completely perforated the medial layer and protruded into the adventitia (Figure 2B). Focal dilatation of the media that was impaired by atherosclerotic lesions (Figure 2C) was observed in 5 of 10 mice (Figure 2C). Calcium deposits were also observed in the media that was impaired by atherosclerotic lesions (6 of 10 mice; Figure 2D). In contrast, in B6<sup>−/−</sup> mice (n=15), we did not observe erosion, disruption, or fragmentation of the media by adjacent athero-
sclerotic lesions, even though these mice had developed much larger lesions. In addition, in both strains, the aortic walls without atherosclerotic lesions did not show any signs of medial destruction.

To confirm the aforementioned findings, a separate set of experiments was performed in which the aortic roots of B6.apoE⁻/⁻ (n=5) and C3H.apoE⁻/⁻ mice (n=10) were processed by standard histologic techniques and stained with elastin–van Gieson’s stain. As shown in Figure 3, the elastic laminas of the media in B6 mice were continuous and did not show signs of impairment by atherosclerotic lesions. In contrast, atherosclerotic lesions in C3H mice infiltrated into the media of the aortic wall and degraded the elastic lamina in an internal-to-external gradient. The elastic lamina exhibited fragmentation or rupture of elastic layers. Autofluorescence analysis of endogenous elastin revealed that the elastic laminas were eroded or disrupted by atherosclerotic lesions (Figure 3C and 3D).

Activity of MMPs
Aortic proteins and macrophage cellular proteins prepared from 6-week-old male B6.apoE⁻/⁻ and C3H.apoE⁻/⁻ mice were analyzed by gelatin and casein zymography (Figure 4A). In aortic tissues, the predominant gelatinolytic bands occurred at 92, 72, and 62 kDa in both strains, corresponding to MMP-9 and pro–MMP-2 and the active form of MMP-2, respectively. In macrophages, the predominant gelatinolytic band occurred at 92 kDa in both strains, corresponding to MMP-9. In both aortic tissues and macrophages, the predominant caseinolytic band occurred at 22 kDa, corresponding to the active form of MMP-12. In both aortic tissues and macrophages, C3H.apoE⁻/⁻
mice showed significantly increased activity levels of pro-
MMP-2, MMP-2, and MMP-12 when compared with those of
B6.apoE⁺⁻ mice (P=0.00136 to 0.03; Figure 4B). In aortic
tissues, MMP-9 activity was comparable between the 2 strains
(P=0.95), but in macrophages, its activity was significantly
higher in C3H.apoE⁺⁻ mice than in B6.apoE⁺⁻ mice (P=0.030).

To determine the cell types that express MMPs in the aortic
wall, in situ zymography with fluorescent gelatin and casein
was performed on the aortas of 12-month-old apoE⁻⁻ mice
that had been fed a chow diet (Figure 4C). As shown in A
through D, strong gelatinolytic activity was detected in
atherosclerotic lesions, especially at the shoulder of the
lesions. Mild gelatinolytic activity was detected in the media
of the arterial wall without atherosclerotic lesions. Enzymatic
activity was suppressed by the addition of 10 mmol/L EDTA,
indicating that the gelatinolytic activity was derived from
MMPs. In situ zymography with casein indicated that casein-
olytic activity was pronounced in the media of the aortic
wall and at the caps of atherosclerotic lesions (E and G),
whereas it was less pronounced at the core of athero-
 sclerotic lesions. The enzymatic activity was mildly inhibited by
EDTA (F and H).

**Plasma Lipid Levels**

After being fed the Western diet for 16 weeks, both B6.apoE⁻⁻
and C3H.apoE⁺⁻ mice developed extreme hypercholesterol-
emia (Figure 5). The total cholesterol level was 1187±59
mg/dL in B6.apoE⁺⁻ mice and 1088±40 mg/dL in C3H.apoE⁺⁻
mice, although the difference was not statistically significant
(P=0.10). C3H.apoE⁺⁻ mice had significantly increased lev-
els of HDL cholesterol (119±11 vs 27±4 mg/dL;
P=0.00001) and triglycerides (105±13 vs 29±7 mg/dL;
P=0.00014) compared with B6.apoE⁺⁻ mice.

**Discussion**

In the present study, we examined phenotypic differences in
the integrity of atherosclerotic aortic walls in 2 apoE⁻⁻ mouse
strains, B6 and C3H, which are known to differ markedly in
atherosclerosis susceptibility. The major finding was that
C3H.apoE⁺⁻ mice exhibited atherosclerosis-associated medial
destruction, whereas B6.apoE⁺⁻ mice were resistant to medial
destruction, despite the fact that they developed much larger
atherosclerotic lesions. Carmeliet and colleagues¹⁵ first re-
ported that apoE⁻⁻ mice, which were bred on a mixed genetic
background of 75% B6 and 25% 129SvJ, developed ath-
 erosclerotic aneurysms in the aortas when they were fed an
atherogenic diet with cholate for 10 weeks. However, 2
subsequent studies reported that on the B6 genetic back-
ground, apoE⁻⁻ mice developed severe aortic dissection
and aortic aneurysms. The current study supports the notion
that B6 and C3H strains differ in their susceptibility to
atherosclerosis.

**Figure 4 (continued).** Chow diet. Cryosections were overlaid on low-
melting-point agarose (0.5%) gels containing the assay solution
(50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, 5 mmol/L ZnCl₂, pH 7.5) and
fluorescein-conjugated gelatin (50 g/mL) or casein (100 g/mL)
and incubated overnight at 37 °C. Gelatinolytic activity was obvious
in atherosclerotic lesions and was mild in the medial arterial wall (A
and C) but inhibited after addition of 10 mmol/L EDTA (B and D).
Caseinolytic activity was pronounced in the medial arterial wall and
at the cap of atherosclerotic lesions (E and G), whereas it was less
pronounced at the core of atherosclerotic lesions. Enzymatic activ-
ity was mildly inhibited by EDTA (F and H).
...the development and progression of aortic aneurysms. In those studies, the apoE−/− mice were at least 7 months old or were fed a Western-type diet for 16 weeks. Values are mean ± SEM for 5 to 10 mice. *P < 0.05 vs B6.apoE−/− mice.

The major finding of this study is that genetic backgrounds but not sizes of atherosclerotic lesions determine medial destruction in apoE−/− mice. This finding provides an explanation for the puzzling association between atherosclerosis and aortic aneurysm formation. Indeed, although the great majority of aortic aneurysms are associated with atherosclerosis, many patients suffering from atherosclerotic disease never develop aortic aneurysms. The factors that contribute to the progression from an intimal lesion (atherosclerosis) to major medial damage (aneurysms) are not well known. However, genetic factors appear to be a major determinant for the progression leading to aortic aneurysm formation. Indeed, prospective family studies indicate that male siblings of individuals affected with aortic aneurysms have an increased risk of 11% to 32% for developing the disorder compared with the general population risk of 2% to 5%. Hypercholesterolemia has been shown to increase monocyte infiltration in injured aortic walls and promote aortic aneurysm formation in rabbits. However, in our experiments, we found that the 2 apoE−/− strains had comparable plasma levels of total cholesterol. Thus, hypercholesterolemia is unlikely to explain the difference between them in medial destruction. C3H.apoE−/− mice had a dramatically increased HDL cholesterol level in comparison with B6.apoE−/− mice. HDL is known to inhibit monocyte infiltration and alleviate inflammation; thus, the increased HDL cholesterol level could not explain the medial destruction of C3H mice. Studies of the mechanisms responsible for aneurysm formation and progression have been hampered by the lack of availability of animal models. Carrell et al maintained that an ideal model of aortic aneurysms should include all of the pathologic features observed in the human condition, such as atherosclerosis, disruption of elastic lamellae in the tunica media, and adventitial inflammation. However, few animal models reproduce all of these features. In rabbits, hyperlipidemia results in extensive aortic atherosclerosis but does not induce aortic aneurysms. To induce aneurysms, CaCl2 and thioglycolate have to be applied to the adventitia to enhance aortic wall inflammation. Aortic aneurysms can be induced in B6.apoE−/− mice by angiotensin infusion, but in this model, aneurysms are independent of atherosclerotic lesions. In contrast, C3H.apoE−/− mice develop spontaneous atherosclerotic aneurysm formation in C3H/apoE−/− mice (Figures 2 and 3). Thus, the increase in activity of MMP-9 and MMP-12 in macrophages observed in this study could contribute significantly to the destruction of the media in C3H mice. In macrophages, we found that MMP-2 activity was not detectable in B6 mice and was very limited in C3H mice. This finding is consistent with the immunohistochemical result of Davis et al, who reported that MMP-2 is expressed by smooth muscle cells and fibroblasts but rarely expressed by macrophages. These observations suggest that MMP-2 produced by macrophages contributed less significantly to the medial destruction in C3H mice.

MMP-2, MMP-9, and MMP-12 are able to directly degrade elastin or fibrillar collagen; we thus investigated their activities in aortic tissues and macrophages from the 2 apoE−/− strains. A notable finding of this study is the obvious activity of all 3 MMPs detected in aortas, even though they had no atherosclerotic lesions. In a recent study, Galis et al also detected apparent MMP-9 and MMP-2 activity in the carotid arteries of mice. MMP-12 is known to be produced by monocytes/macrophages. Aortic intimal smooth muscle cells, and cultured medial smooth muscle cells that lose contractility. In this study, we found that MMP-12 was also produced by normal aortas of mice. Interestingly, although the activity of MMP-9 in the aorta was comparable between the 2 strains, C3H mice exhibited significantly increased proteolytic activity of MMP-2 and MMP-12 when compared with B6 mice. Given that MMP-2 and MMP-12 have potent elastolytic ability, it is plausible to speculate that these 2 MMPs produced by arterial wall cells played a role in the destruction of the elastic media in C3H mice.

In this study, we found that medial destruction was associated with infiltration of the arterial walls by atherosclerotic lesions in C3H.apoE−/− mice (Figures 2 and 3). Thus, the increase in activity of MMP-9 and MMP-12 in macrophages observed in this study could contribute significantly to the destruction of the media in C3H mice. In macrophages, we found that MMP-2 activity was not detectable in B6 mice and was very limited in C3H mice. This finding is consistent with the immunohistochemical result of Davis et al, who reported that MMP-2 is expressed by smooth muscle cells and fibroblasts but rarely expressed by macrophages. These observations suggest that MMP-2 produced by macrophages contributed less significantly to the medial destruction in C3H mice.

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rosis and display disruption of the elastic lamellae and infiltration by macrophages, thus providing an experimental tool to study atherosclerotic aortic aneurysms. However, it is worth noting that although C3H/apoE−/− mice exhibited obvious medial destruction, there was no gross aneurysmal dilatation of the aortic walls. The dissociation between medial destruction and aneurysmal dilatation might be explained by the finding that advanced atherosclerotic lesions of apoE−/− mice contain a large fraction of fibrotic tissues as well as calcification,13,19 which are known to limit dilatation of the vessel wall.

In summary, we have observed the experimental evidence that genetic factors influence atherosclerotic medial wall destruction, at least partially, by modulating the activity of MMPs. Also, these genetic factors seem to be independent of factors that influence the size of atherosclerotic lesions.

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