Subphysiologic Apolipoprotein E (ApoE) Plasma Levels Inhibit Neointimal Formation After Arterial Injury in ApoE-Deficient Mice


Objective.—Apolipoprotein E (apoE) reduces mouse atherosclerosis progression independent of plasma cholesterol level effects. A mouse artery injury model was used to examine whether apoE exhibits beneficial lipid-independent effects on neointimal formation.

Methods and Results.—ApoE-deficient (apoE−/−), wild-type (WT), and transgenic apoE−/− mice (secreting apoE at different levels from adrenal glands) underwent femoral artery injury. Mice with low expression of plasma apoE (0.1% of WT) had cholesterol levels approximately half those of apoE−/− littermates (but still ∼6× >WT). Mice with higher expression (HE; 2% to 3% of WT) of plasma apoE had cholesterol levels approximately twice those of WT. Injured WT mouse (versus apoE−/−) arteries had a smaller mean intima-to-media (I/M) ratio (0.87 versus 1.96; P<0.05). HE mice tended to have lower mean I/M ratios (1.3; P>0.05 versus apoE−/− mice). Multiple regression analysis indicated that apoE levels were significantly associated with reduced I/M ratios, but plasma cholesterol levels were not, before or after adjusting for apoE. In addition, foam cell content of the neointima and media of injured arteries, a negative prognostic indicator in postangioplasty human lesions, was inversely related to plasma apoE levels.

Conclusions.—Similar to its effects on atherosclerosis progression, in a mouse model of restenosis, a subphysiological level of apoE was associated with beneficial effects on lesion size/composition. (Arterioscler Thromb Vasc Biol. 2004; 24:1460-1465.)

Key Words: apolipoprotein E ■ arterial injury ■ neointimal formation ■ restenosis ■ mouse

Atherosclerosis complications are the most common causes of death in Western societies. In the United States, heart disease and stroke account for almost half of all deaths. Atherosclerosis is a chronic process resulting from interaction between normal and modified lipoproteins, monocyte-derived macrophages, lymphocytes, and normal cellular elements of the arterial wall. Endothelial dysfunction and the retention and oxidation of cholesteryl ester-rich lipoproteins in the subendothelial space initiate complex inflammatory reactions, which can ultimately lead to plaque formation and rupture.

Restenosis after angioplasty is a common clinical problem, even with the widespread use of stents. In certain aspects, restenosis represents an accelerated form of arteriosclerosis, in which neointima formation is a prominent feature. After endothelial denudation, there is proliferation and migration of vascular smooth muscle cells (SMCs) from the tunica media to the intima. In addition, abnormal vascular remodeling associated with inefficient compensatory enlargement or even constriction of the arterial wall contributes to luminal stenosis. Despite the use of stents, restenosis occurs in 20% to 30% of patients in the first 6 months after coronary angioplasty procedures.

Apolipoprotein E (apoE) plays a critical role in cholesterol homeostasis. It serves as the ligand for receptor-mediated clearance of several classes of lipoproteins, including chylomicrons, very low-density lipoprotein (LDL), high-density lipoprotein subclasses, and lipoprotein remnants. In apoE-deficient (apoE−/−) mice (in which the apoE gene is inactivated), the hyperlipidemia is marked and results in the spontaneous development of atherosclerotic lesions throughout the arterial tree. Because of similarities in histopathologic characteristics between lesions of apoE−/− mice and patients, these mice are widely used as a model of human atherosclerosis and restenosis.

Previous studies have suggested that apoE has antiatherogenic properties independent from its lipid-lowering effect.
### Results

#### Plasma Cholesterol and ApoE Levels

Plasma cholesterol and apoE levels at the time of death are summarized in Table 1. The data show that only very small apoE levels were apparently necessary to reduce plasma cholesterol levels. For example, at 0.1% of WT apoE, plasma cholesterol in low expressors was half that in apoE−/− littermates (but still ∼6-fold more than the WT level). In higher expressors, at 2% to 3% of WT apoE, plasma cholesterol levels were lower by 80%, to 2.3-fold higher than the WT level.

#### Cholesterol and ApoE Effects on Neointimal Formation and Arterial Remodeling

Four weeks after injury, neointimal formation was apparent in femoral arteries from all 4 groups (Figure 1A). For comparative purposes, the preinjury histological appearances of apoE−/− mice were also examined, and as shown in Figure 1B, it was entirely normal. A representative section from a mouse in each injury group is shown in Figure 1A, and by inspection, the amount of neointima appears to be in the order WT<high expression(HE)<low expression (LE) <apoE−/− (ie, inversely associated with the apoE level). The combined data confirmed the visual impression and are summarized in Table 2. ApoE−/− mice had significantly higher I/M ratios than WT mice (1.96±0.2 versus 0.87±0.2; P<0.04). Although not statistically significant, but clearly visible as a trend, LE mice (1.59±0.2) and HE mice (1.3±0.2) had smaller I/M ratios in comparison with their apoE−/− counterparts (Table 2).

Because of positive vessel remodeling after injury, arteries have the potential to conserve luminal area. Thus, we were interested in determining whether the increase in neointimal area resulted in any loss of lumen. The assessment of histopathologic stenosis revealed that greater intimal formation was associated with increasing luminal stenosis (Table 2). Nonetheless, there may still have been some outward (ie, positive) remodeling of the injured artery. Indeed, a regression analysis showed that the area encompassed by the external elastic lamina increased with increasing intimal area (data not shown), indicating a positive remodeling of the artery in response to lesion formation. However, given the histopathologic stenosis data, the degree of arterial enlargement was not sufficient to prevent luminal narrowing.

### Methods

The Methods section is available online at http://atvb.ahajournals.org.
in the intima of the LE artery and appeared to be in deeper regions internal elastic lamina (IEL). Foam cells were less prominent in region of the intima and in deeper regions on both sides of the accumulations of foam cells in the immediate subendothelial immunostaining, the arteries from apoE mice. As seen in Figure 2, as judged by morphology or morphological differences between the lesions of the 4 groups of Besides the differences in neointimal size, we found striking morphological differences between the lesions of the 4 groups of mice. As seen in Figure 2, as judged by morphology or immunostaining, the arteries from apoE mice showed large accumulations of foam cells in the immediate subendothelial region of the intima and in deeper regions on both sides of the internal elastic lamina (IEL). Foam cells were less prominent in the intima of the LE artery and appeared to be in deeper regions of the intima and in the media near the IEL. In contrast, no foam cells were observed in any of the media or intima of WT or HE mice. This was confirmed by an independent and blinded pathologist, who was able to accurately distinguish between LE and HE mice on the basis of foam cell amount and distribution, with only 1 exception after examining 2 sections from each artery of every mouse in the study.

In contrast to the pattern of macrophage staining, \(\alpha\)-actin staining of SMCs showed a direct relationship with apoE expression levels (Figure 2, \(\alpha\)-actin panels). \(\alpha\)-Actin staining was present and layered in the media of WT arteries, less prominent in the HE arteries, sparse and dispersed throughout the neointima of the LE arteries, and barely detectable in the neointima of the apoE mice. The media of LE and apoE mice contained only traces of \(\alpha\)-actin stained cells.

For the sections shown from the HE and LE groups, the mice used were matched for similar cholesterol levels (HE 211 mg/dL; LE 276 mg/dL). Therefore, histological changes between these 2 mice illustrate the greater effect of apoE (versus cholesterol) on lesion characteristics.

### Table 2. Summary of Morphometric Data and Statistical Analyses

#### A. Morphometric Data

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>HE</th>
<th>LE</th>
<th>ApoE−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>11.19±2.8</td>
<td>27.65±5.04</td>
<td>36.27±5.4</td>
<td>47.91±7.07</td>
</tr>
<tr>
<td>MA</td>
<td>13.62±0.99</td>
<td>20.19±1.8</td>
<td>22.33±1.9</td>
<td>23.69±2.07</td>
</tr>
<tr>
<td>I/M</td>
<td>0.87±0.2</td>
<td>1.3±0.2</td>
<td>1.59±0.2</td>
<td>1.96±0.2</td>
</tr>
<tr>
<td>HS</td>
<td>21.0±5.5</td>
<td>29.9±5.1</td>
<td>50.5±7.0</td>
<td>59.3±7.2</td>
</tr>
</tbody>
</table>

#### B. One-Way ANOVA

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Overall P Value for Group Differences</th>
<th>Significantly Different Groups (P&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>P=0.0025</td>
<td>WT vs apoE−/−</td>
</tr>
<tr>
<td>MA</td>
<td>P=0.0162</td>
<td>WT vs apoE−/−</td>
</tr>
<tr>
<td>I/M ratio</td>
<td>P=0.0267</td>
<td>WT vs apoE−/−</td>
</tr>
<tr>
<td>HS</td>
<td>P=0.0006</td>
<td>WT vs apoE−/−; HE vs apoE−/−</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Units are mm², except for HS, which is percentage. One-way ANOVA with the Bonferroni Multiple Comparison Test was used to determine statistically significant effects of the different genotypes (WT, HE, LE, and apoE−/−) on the indicated dependent variables. IA indicates intimal area; MA, medial area; HS, histopathological stenosis.

### Table 3. Stepwise Regression Analysis of the I/M Ratio (Dependent Variable) and the Plasma Levels of ApoE and Cholesterol (Independent Variables)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Regression Coefficient for the ApoE Term</th>
<th>Standard Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE enters the model first</td>
<td>-0.11</td>
<td>0.048</td>
<td>-2.31</td>
<td>0.032</td>
</tr>
<tr>
<td>I/M Ratio</td>
<td>-0.19</td>
<td>0.080</td>
<td>-2.23</td>
<td>0.031</td>
</tr>
</tbody>
</table>

ApoE and cholesterol values were transformed logarithmically because they were not normally distributed. In both models, P values associated with the coefficient for the cholesterol term were nonsignificant (P>0.25).
Despite of the inverse relationship between apoE and cholesterol plasma levels, statistical testing indicated the plasma cholesterol level per se was not as strong a factor as apoE with regard to the amount of neointimal formation after arterial injury (as assessed by the I/M ratio). Notably, in a 2-step, forward multiple regression model, whether the level of apoE or cholesterol was entered first into the model, plasma cholesterol did not add statistical significance. In contrast, the inclusion of apoE in the model was consistently significant (Table 3).

Because of the presumed overlap in some of the mechanisms underlying restenosis and atherosclerosis, results from a number of mouse atherosclerosis studies may be relevant to our finding that the level of apoE but not plasma cholesterol was strongly associated with the I/M ratio. For example, in our previous studies, levels of apoE too low to result in reduced plasma cholesterol were still associated with retarded atherosclerosis progression. Chronic intravenous administration of apoE in the Watanabe heritable hyperlipidemic rabbit inhibited atherosclerosis without a reduction in total plasma cholesterol. Also, apoE overexpressed in the arterial wall by either transgenic means or retroviral transduction of bone marrow before transplantation protected the aorta from early atherosclerosis, although plasma cholesterol levels and lipoprotein profiles remained unchanged from those seen in control mice. Furthermore, Zhu et al were able to show that not only atherosclerosis but also neointimal formation after arterial injury was reduced in normocholesterolemic WT mice overexpressing apoE, independent of further cholesterol lowering. Except in our report, in all of these studies, supraphysiological apoE concentrations were used (either systemically or locally in the vessel wall). Notable, then, is our present finding that very low plasma apoE levels are sufficient to observe a beneficial effect on the I/M ratio after arterial injury.

The second major finding of our present studies was the altered quantity and distribution of cell-specific immunostaining in the neointima and media of the LE and apoE-/- mice. In apoE-/- and LE mice, foam cells positive for the macrophage marker MOMA-2 were present in the intima and media of the LE and apoE-/- mice. Arrows indicate some of the macrophage foam cells in the peri-IEL and medial regions discussed in the text.

**Discussion**

There were 2 major findings in the present study, namely (1) the I/M ratio after femoral arterial injury was reduced at subphysiological levels of plasma apoE and was statistically associated with changes in the plasma apoE but not cholesterol concentration; and (2) the lesional foam cell content and distribution were also influenced negatively by the apoE plasma level. A potential contributing factor to both findings is the effect of apoE on plasma cholesterol levels. As summarized in Results, even low apoE plasma levels were associated with reductions in plasma cholesterol. For example, at 0.1% of WT apoE levels, plasma cholesterol in LE mice was half of the average level of their apoE-/- littermates (but still 6-fold above normal). When plasma apoE was 2% to 3% of the WT level, plasma cholesterol in HE mice was ≈2-fold above normal. This is in agreement with previous reports. For example, Linton et al. showed that at 5% of the WT apoE level, plasma cholesterol levels were lower by 55% in apoE-/- mice after bone marrow transplantation, and at 12.5% of WT apoE, plasma cholesterol levels were almost normal. In another study, the amount of human apoE expressed in macrophages of apoE-/- mice was related inversely to the plasma cholesterol concentration; at 8% of the WT apoE level, cholesterol levels were lower by 60%. Hasty et al. reported similar results using apoE-/- mice after transplantation with WT marrow. A threshold of 1% of WT apoE was necessary to observe changes in cholesterol; at 3% of WT apoE, cholesterol levels were nearly normal. Similarly, in our previous studies of apoE-/- mice having an apoE transgene expressed in the adrenal gland, at 2% to 4% of the WT apoE level, plasma cholesterol levels were nearly normal.13

**Figure 2.** Macrophage and SMC content of lesions from mouse femoral arteries 4 weeks after injury. Arterial sections were immunostained with the macrophage marker MOMA-2 (brown staining) and the SMC marker α-actin (red staining). EKO indicates sections from the apoE-/- mouse. Differences in immunohistochemistry staining between HE and LE mice were observed despite similar cholesterol levels (211 mg/dL [HE] and 276 mg/dL [LE]). Arrows indicate some of the macrophage foam cells in the peri-IEL and medial regions discussed in the text. Magnification is ×200.
for example, when cells shift from a “contractile” to a proliferative phenotype, which would precede their migration into the neointima. Thus, given the larger neointima formed in the apoE−/− and LE mice, perhaps the loss of α-actin staining represents a high degree of recruitment of medial SMCs to the neointima.

There have been several suggestions about the molecular mechanisms by which apoE protects against atherosclerosis and restenosis. Similar to our observation that apoE may influence the cellular composition of lesions, other authors have demonstrated inhibition of early monocyte invasion and, therefore, reduction in foam cell deposition and atherogenesis. Previous studies showed that apoE has a variety of cytokine-like or hormonal effects in steroidogenic cells, platelets, and lymphocytes. More recently, Hu et al showed that apoE inhibits the platelet-derived growth factor (PDGF) stimulation of SMC proliferation and migration.

Inhibition of PDGF-stimulated cell migration appears to be mediated via suppression of signal transduction through the LDL receptor–related protein (LRP). LRP participates in signal transduction pathways in the brain and other cells. LRP is tyrosine phosphorylated on its cytoplasmic tail, leading to recruitment of downstream signaling components. LRP tyrosine phosphorylation is stimulated by PDGF, and this activity is inhibited by apoE. These findings suggest that low apoE levels might inhibit neointima formation after arterial injury via suppression of vascular SMC migration and proliferation. In support of this hypothesis, inactivation of the LRP gene in vascular SMCs caused activation of PDGF signaling, SMC proliferation, and enhanced aortic lesion development in LDL receptor–deficient mice.

We find it interesting that very little circulating apoE is apparently needed to reduce the cholesterol plasma level or to defend postinjury arterial morphology in apoE−/− mice. Why then is apoE expressed in such abundance? There is evidence that apoE plays a part in many normal and pathological processes beyond its traditional role in lipoprotein metabolism, such as in cerebral and cognitive functions, nerve regeneration, tumor cell proliferation, steroidogenesis, Alzheimer’s disease, gallstone formation, and proximal colon carcinoma. This suggests that a level of apoE effective in one context may not be relevant to another.

In summary, our results demonstrate that a very low apoE level provided systemically was associated with a major beneficial impact on the quantity (as assessed by the I/M ratio) and quality of the neointima after arterial injury in mice. Furthermore, the quantitative effects were statistically independent of changes in plasma cholesterol levels. Taken with the beneficial effects of apoE on atherosclerosis progression, the data suggest that apoE has a generally important protective function in the arterial wall.

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