Effect of Macrophage-Derived Mouse ApoE, Human ApoE3-Leiden, and Human ApoE2 (Arg158→Cys) on Cholesterol Levels and Atherosclerosis in ApoE-Deficient Mice

Miranda Van Eck, Nicole Herijgers, Ko Willems Van Dijk, Louis M. Havekes, Marten H. Hofker, Pieter H.E. Groot, Theo J.C. Van Berkel

Abstract—The effect of monocyte/macrophage-derived wild-type mouse apolipoprotein E (apoE), human apoE3-Leiden, and human apoE2 on serum cholesterol levels and the development of atherosclerosis in apoE-deficient (apoE–/–) mice was investigated by using bone marrow transplantation (BMT). At 4 weeks after BMT, murine apoE+/+ bone marrow reduced serum cholesterol levels by 87% in apoE–/– mice, whereas macrophage-derived human apoE3-Leiden and human apoE2 induced a maximal, transient reduction of 35% and 48%, respectively. At 4 months after BMT, atherosclerosis was 23-fold (P<0.001) reduced in apoE+/+→apoE–/– mice, whereas no significant reduction in apoE3-Leiden.apoE+/+→apoE–/– and apoE2.apoE+/+→apoE–/– mice could be demonstrated. A highly significant decrease in serum cholesterol levels (78% reduction) and atherosclerosis (21-fold, P<0.001) was found in apoE3-Leiden.apoE+/– animals expressing high levels of apoE in multiple tissues, whereas apoE2 was ineffective even at high concentrations. Furthermore, in contrast to apoE-deficient macrophages, cholesterol efflux from apoE2 or apoE3-Leiden macrophages was not impaired. In conclusion, apoE3-Leiden as well as apoE2 are less effective in reducing cholesterol concentrations. Furthermore, in contrast to apoE-deficient macrophages, cholesterol efflux from apoE2 or apoE3-Leiden macrophages was not impaired. In conclusion, apoE3-Leiden as well as apoE2 are less effective in reducing cholesterol levels and atherosclerosis in apoE–/– animals, compared with apoE+/+, with apoE2<apoE3-Leiden<apoE+/+, irrespective of the observed adequate efflux of cholesterol from macrophages expressing apoE2 and apoE3-Leiden, indicating that normalization of cholesterol efflux by macrophages is not accompanied by measurable effects on lesion growth. (Arterioscler Thromb Vasc Biol. 2000;20:119-127.)

Key Words: apolipoprotein E ■ atherosclerosis ■ hyperlipidemia ■ macrophages ■ bone marrow transplantation

ApoE is a 34-kDa, arginine-rich protein that mediates the uptake of remnant lipoproteins by several receptor systems in the liver. Structural mutations in the apoE gene, including homozygosity for apoE2 (Arg158→Cys) or a complete deficiency of apoE, may lead to the development of type III hyperlipidemia. Type III hyperlipidemia is associated with the accumulation of VLDL and chylomicron remnants in the circulation due to impaired clearance of these lipoproteins, leading to the premature development of atherosclerosis. In 90% of cases, recessive inheritance of type III hyperlipidemia is associated with the apoE2 phenotype. Besides this recessive inheritance of type III hyperlipidemia, several other mutations of apoE are known that lead to dominant inheritance of this disease, including apoE3-Leiden. Compared with the recessive apoE2 variant, apoE3-Leiden demonstrates only mildly reduced in vitro binding efficiency to the LDL receptor apoE3-Leiden binding is 20% to 40% of normal apoE3 versus 1% to 2% for apoE2. Although other secondary genetic or environmental factors are necessary to induce type III hyperlipidemia in subjects homozygous for apoE2, almost all subjects with dominant apoE3-Leiden develop hyperlipidemia. Recently, Van den Maagdenberg et al and Van Vlijmen et al reported that transgenic mice expressing the apoE3-Leiden mutation develop hyperlipidemia in the presence of endogenous mouse apoE. In addition, atherosclerotic lesion development was observed to be dependent on the level of serum cholesterol. Huang et al demonstrated that in the presence of endogenous mouse apoE, low concentrations of apoE2 (<10 mg/dL) did not alter serum lipid levels, whereas intermediate concentrations of apoE2 (10 to 30 mg/dL) induced hypcholesterolemia, and high concentrations of apoE2 (>50 mg/dL) resulted in hyperlipidemia. Van Vlijmen et al additionally demonstrated that apoE2-transgenic mice develop hyperlipidemia only in the absence of the endogenous mouse apoE gene. As with humans, apoE2 and apoE3-Leiden in

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transgenic mice behave like a recessive and a dominant trait, respectively, in the expression of hyperlipidemia. Consequently, these transgenic mice can be considered suitable animal models for the further understanding of the development of type III hyperlipidemia. In addition, various groups have generated apoE-deficient mice by targeted inactivation of the apoE gene in embryonic stem cells.\textsuperscript{19–21} Inactivation of the apoE gene in these mice is also associated with a prominent increase in serum cholesterol levels and the development of atherosclerosis, further indicating the importance of apoE in the development of hyperlipidemia and atherosclerosis.

Although the liver is the major source of apoE synthesis, macrophages in different organs are also active in secreting large quantities of apoE.\textsuperscript{22–25} Because foam cells in atherosclerotic lesions are derived from macrophages, modulation of macrophage apoE synthesis might influence the development of atherosclerosis. Recently, we\textsuperscript{26} and others,\textsuperscript{27–29} using the technique of bone marrow transplantation (BMT), have demonstrated that macrophage-derived, wild-type mouse apoE nearly normalizes serum cholesterol levels in apoE-deficient mice, leading to a marked decrease in atherosclerosis.

In the present study, the effects of apoE3-Leiden and apoE2 expression in macrophages or by the liver on serum cholesterol levels and atherosclerosis development were studied against an endogenous mouse apoE-knockout background. Our results indicate that macrophage-derived human apoE3-Leiden and human apoE2 can only transiently reduce serum cholesterol levels in apoE-deficient mice without significantly reducing the susceptibility to atherosclerosis, irrespective of an adequate efflux of cholesterol from macrophages. Only high serum levels of apoE3-Leiden, achieved by production in the liver, reduced serum cholesterol levels and atherosclerosis development dramatically, whereas high levels of apoE2 were ineffective. In conclusion, apoE3-Leiden as well as apoE2 is less effective in reducing cholesterol levels and atherosclerosis in apoE–/– mice compared with wild-type apoE, irrespective of an adequate efflux of cholesterol. This finding indicates that normalization of cholesterol efflux from macrophages is not accompanied by measurable effects on lesion growth.

\section*{Methods}

\section*{Animals}

ApoE-deficient (apoE–/–) mice and transgenic mice without endogenous mouse apoE but expressing either human apoE3-Leiden (apoE3-Leiden.apoe–/–) or human apoE2 (apoE2.apoe–/–) were generated as described previously.\textsuperscript{18,20,30} All mice were hybrids of the C57BL/6 and 129Sv strains. The animals were housed and bred at the animal facility of the Sylvius Laboratories in Leiden, The Netherlands. Mice used for BMT experiments were housed in airtight cages at 24°C with a 12-hour light-dark cycle.

\section*{Irradiation and BMT}

To induce bone marrow aplasia, female (aged 5 to 6 weeks) apoE–/– (nontransgenic littermates), apoE3-Leiden.apoe–/–, and apoE2.apoe–/– mice were exposed to a single dose of 13 Gy (0.28 Gy/min, 200 kV, 4 mA) total-body irradiation by using an Andrex Smart 225 Ro¨ntgen source (Andrex Radiation Products AS) with a 4-mm aluminum filter 1 day before transplantation. Bone marrow cell suspensions were isolated by flushing the femurs and tibias from male apoE–/–, apoE3-Leiden.apoe–/–, apoE2.apoe–/–, and C57BL/6 mice with PBS. Single-cell suspensions were prepared by passing the cells through a 30-µm nylon gauze. Irradiated recipients received 10\textsuperscript{6} bone marrow cells by intravenous injection into the tail vein.

\section*{Serum Cholesterol and Triglyceride Analysis}

After an overnight fast, \~100 µL of blood was drawn from each individual mouse by tail bleeding. The concentrations of total cholesterol, free cholesterol, and triglycerides in serum were determined by using enzymatic procedures (Boehringer Mannheim). Precipath (standardized serum, Boehringer Mannheim) was used as an internal standard.

The distribution of cholesterol and triglycerides over the different lipoprotein classes in serum was determined by loading 30 µL of serum from each mouse onto a Superox 6 column (3.2 x 30 mm, Smart system, Pharmacia). Serum was fractionated at a constant flow rate of 50 µL/min with PBS. Total cholesterol content in the effluent was determined enzymatically.

\section*{Quantitation of ApoE}

Both human and murine apoEs were measured by using a sandwich ELISA. For determination of human apoE, purified goat anti-human apoE polyclonal antibody was used as a primary antibody, whereas purified goat anti-human apoE polyclonal antibody, conjugated to horseradish peroxidase, was used as a secondary antibody. For determination of mouse apoE, a rabbit anti-mouse apoE polyclonal antibody (SB rabbit 67-AH) was used as a primary antibody, biotinylated rabbit anti-mouse apoE polyclonal antibody was used as a secondary antibody (SB rabbit 67-AH–biotin), and finally biotinylated horseradish peroxidase–conjugated streptavidin was used.\textsuperscript{26} Horseradish peroxidase was detected by incubation with 3,3',5,5'-tetramethyлензidine (Pierce Chemical Co) for 30 minutes at room temperature. The reaction was stopped with addition of 2 mol/L H\textsubscript{2}SO\textsubscript{4}, and the absorbance was read at 450 nm. Pooled serum from healthy, human volunteers and C57BL mice with known apoE levels was used as the standard for the human apoE ELISA and the murine apoE ELISA, respectively.

\section*{LDL Isolation, Acetylation, and Labeling}

Human LDL was isolated from healthy volunteers as described by Redgrave et al.\textsuperscript{31} After density ultracentrifugation, LDL (1.019<d<1.063 g/mL) and lipoprotein-deficient serum (d>1.21 g/mL) were collected and dialyzed against PBS/1 mmol/L EDTA. Protein content was determined according to Lowry et al.\textsuperscript{32} With BSA as an internal standard. LDL was acetylated according to Basu et al\textsuperscript{33} and subsequently labeled with 25 µCi of \textsuperscript{[14C]cholesteryl oleate according to Blomhoff et al\textsuperscript{34} in the presence of human lipoprotein-deficient serum as the source of cholesteryl ester transfer protein. Radiolabeled acetylated LDL was subsequently isolated by density ultracentrifugation. Hydrolysis of the cholesteryl ester label was tested by lipid extraction followed by thin-layer chromatography, and the electrophoretic mobility was examined by agarose gel electrophoresis. The specific activity of the \textsuperscript{[14C]cholesteryl oleate–radiolaabeled acetylated LDL, after density ultracentrifugation, was 37±7 disintegrations per minute per nanogram protein (n=5, mean±SEM).

\section*{Histological Analysis of Hearts and Aortas for Atherosclerosis}

To analyze the development of atherosclerosis throughout the aortic tree, transplanted mice were killed 4 months after BMT. Hearts and aortas were perfused in situ with oxygenated Krebs’ buffer (37°C, 100 mm Hg) for 20 to 30 minutes via a cannula in the left ventricle, followed by perfusion with 3.7% neutral buffered formalin (Formal-

\section*{densities, Shandon Scientific Ltd) for 30 minutes. Hearts and aortas were excised and stored in formalin. To evaluate the development of atherosclerotic lesions, the aortas were separated from the hearts. Hearts were bisected at the level of the atria, and the base of the heart plus aortic root were taken for analysis. Cryostat 10-µm cross sections of the aortic root were made and stained with oil red O (BDH Ltd). The atherosclerotic lesion area

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in the sections was quantified by using a light microscope connected to a 24-bit, full-color video camera and Optimas 6.1 image analysis software (BioScan). Mean lesion area was calculated (in μm²) from 10 sections, starting at the appearance of the tricuspid valves as described previously.16,35

Isolation of Murine Peritoneal Macrophages

Five days after intraperitoneal injection of 3% Brewer’s thiglycollate medium, macrophages were harvested from control apoE+/+, apoE–/–, and transgenic apoE3-Leiden apoE–/– and apoE2/Leiden animals by lavage of the peritoneal cavity with 10 mL of PBS. The isolated macrophages were washed 3 times with sterilized PBS and plated in 24-well plates at a density of 0.5 x 10⁶ cells/500 μL in Dulbecco’s modified Eagle’s medium supplemented with 2% (wt/vol) BSA, 2 mmol/L l-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin. After 4 hours, nonadhering cells were removed by washing. At 1 day after isolation, the cells were loaded with 50 μCi of [3H-1-cholesterol oleate according to Blomhoff et al34 for 45 hours at 37°C. Subsequently the supernatant was removed, and the cells were washed 2 times with washing buffer (0.9% wt/vol NaCl, 1 mmol/L EDTA, 0.05 mol/L Tris-HCl, 5 mmol/L CaCl₂, and 0.2% wt/vol BSA, pH 7.4) and 2 times with washing buffer without BSA to determine the 100% value. Cells were lysed with 0.1N NaOH, radioactivity was measured with a scintillation counter; and protein content was determined according to Lowry et al.32 No significant difference in cholesterol loading was observed between the different groups. The specific activity of the cells after being loaded with [3H-1-cholesterol oleate–labeled acetylated LDL was 9.0 ± 0.6, 9.4 ± 1.1, 11.4 ± 0.9, and 9.0 ± 1.3 μg/mg cell protein (n = 5 or 6, mean ± SEM) for apoE–/–, apoE+/+, apoE3-Leiden, and apoE2/Leiden and apoE2 macrophages, respectively. Cholesterol efflux was studied in the absence of cholesterol acceptors in Dulbecco’s modified Eagle’s medium supplemented with 2% wt/vol BSA for 5 hours after extensive washing of the cells with Dulbecco’s modified Eagle’s medium and 2% wt/vol BSA. After incubation, cholesterol efflux was determined as described by Zhu et al.30 In brief, the supernatant was removed and cell debris was pelleted by centrifugation at 16 000g for 10 minutes. After removal of the supernatant, a 200-μL aliquot was counted for radioactivity with a scintillation counter to determine the 100% value. The cells were washed 2 times with washing buffer with BSA and 2 times with washing buffer without BSA and lysed with 0.1N NaOH; radioactivity was measured with a scintillation counter; and protein content was determined according to Lowry et al.32 The percent cholesterol efflux was calculated as (dpm/ng/nadir)/(dpm/ng/efflux * 1000ng/10² dpm/ng/nadir).

Statistical Analysis

Statistically significant differences among the means of the different populations were tested by ANOVA. The Student-Newman-Keuls multiple comparison test was performed after ANOVA.

Results

Peritoneal Macrophage ApoE Production in ApoE2 apoE–/– and ApoE3-Leiden apoE–/– Transgenic Mice

Analysis of apoE secretion by thioglycollate-elicited macrophages demonstrated that apoE secretion was higher in wild-type macrophages (1059 ± 204 ng of mouse apoE secreted per milligram of cell protein per 24 hours, n = 5) compared with macrophages expressing human apoE (208 ± 75, n = 4 and 246 ± 64, n = 4 ng · mg⁻¹ · 24 h⁻¹ for apoE3-Leiden and apoE2, respectively).

Effect of Macrophage or Liver ApoE2 apoE–/– and ApoE3-Leiden apoE–/– Production on Serum Lipids and ApoE Levels

To study the effect of murine apoE, human apoE2, and human apoE3-Leiden production by macrophages in an apoE-deficient background, bone marrow from wild-type (apoE+/+), apoE3-Leiden apoE–/–, and apoE2 apoE–/– animals was transplanted into apoE-deficient recipients. Control groups of apoE3-Leiden apoE–/–, apoE2 apoE–/–, and apoE–/– mice receiving homologous bone marrow were also included.

At 4 weeks after BMT, the concentration of apoE as measured in the circulation of apoE–/– mice transplanted with apoE+/+, apoE2 apoE–/–, or apoE3-Leiden apoE–/– bone marrow was 131 ± 28, 25 ± 2.9, and 19 ± 1.6 μg/dL (n = 6 or 7), respectively. High concentrations of circulating apoE3-Leiden and apoE2 were found in the apoE3-Leiden apoE–/– and apoE2 apoE–/– mice expressing the mutant apoE forms in multiple tissues (macrophages, liver, brain, and muscles)18 but mainly in the liver. The circulating human apoE levels in these animals were 8392 ± 386 and 657 ± 38 μg/dL in the apoE2 apoE–/– and the apoE3-Leiden apoE–/– mice, respectively. At 10 weeks after BMT, the human serum apoE concentration was further increased to 39 ± 4.9 and 83 ± 4.1 μg/dL in the apoE–/– mice transplanted with apoE2 apoE–/– and apoE3-Leiden apoE–/– bone marrow, whereas apoE levels in the control transplanted apoE2 apoE–/– and the apoE3-Leiden apoE–/– animals were 4408 ± 277 and 436 ± 86 μg/dL, respectively.

In the weeks after BMT, serum cholesterol levels were analyzed (Figure 1). As previously reported,26 transplantation of wild-type bone marrow to apoE–/– animals results in an almost-complete correction of their hypercholesterolemia within 4 weeks after BMT (87% reduction, Figure 1A). Transplantation of apoE2 apoE–/– or apoE3-Leiden apoE–/– bone marrow to apoE–/– animals resulted in an initial transient decrease in total serum cholesterol levels (Figures 1B and 1C). Analysis of the effect of transplantation of apoE2 apoE–/– or apoE3-Leiden apoE–/– bone marrow to mice lacking both apoE and the LDL receptor demonstrated that this initial decrease was mediated by the LDL receptor (data not shown). At 4 weeks after BMT, the distribution of cholesterol over the different serum lipoproteins at 12 weeks after BMT indicated that the secondary increase in serum cholesterol levels was not due to a loss of apoE-producing bone marrow cells, which was also confirmed by the increase in serum apoE2 and apoE3-Leiden levels at 10 weeks after BMT. Fractionation of serum lipoproteins at 12 weeks after BMT indicated that the secondary increase in serum cholesterol levels was caused by an increase in cholesterol associated with the VLDL fraction (data not shown). No significant effect of BMT on serum triglyceride levels could be demonstrated.

As demonstrated in Figure 1, after an initial decrease the serum cholesterol levels in the apoE–/– animals transplanted with apoE2 apoE–/– or apoE3-Leiden apoE–/– bone marrow did not persist and increased to a level slightly lower than that in control transplanted apoE–/– animals. This result contrasts with that in animals transplanted with wild-type bone marrow, indicating that the secondary increase in serum cholesterol levels was not due to a loss of apoE-producing bone marrow cells, which was also confirmed by the increase in serum apoE2 and apoE3-Leiden levels at 10 weeks after BMT. Fractionation of serum lipoproteins at 12 weeks after BMT indicated that the secondary increase in serum cholesterol levels was caused by an increase in cholesterol associated with the VLDL fraction (data not shown). No significant effect of BMT on serum triglyceride levels could be demonstrated.

As presented in the Table, in animals expressing human apoE solely in macrophages, all lipoprotein subclasses contained significantly less apoE compared with those from the
control apoE2, apoE3-Leiden, and apoE2 apoE3-Leiden. Furthermore, a preferential association of apoE2 compared with apoE3-Leiden to HDL could be demonstrated in the apoE2 apoE3-Leiden animals compared with apoE3-Leiden mice, which is in accordance with data from Tsukamoto et al. 37

Effect of Macrophage or Liver ApoE2 and ApoE3-Leiden Production on Atherosclerosis

To investigate whether the ability of macrophages in the arterial wall to produce human apoE2 or apoE3-Leiden would influence lipid deposition and the formation of foam cells, atherosclerosis was quantified in all transplanted groups at 4 months after BMT. The measured mean lesion area for each mouse is depicted in Figure 3, and representative photomicrographs of lesions in cross sections are shown in Figure 4. Cross sections of control apoE2 apoE3-Leiden animals transplanted with apoE2 apoE3-Leiden bone marrow showed extensive lipid-rich lesions, even 1 with an aneurysm in the vascular wall (Figure 4A). The mean lesion area measured in these animals was 5.85 ± 0.91 × 10^3 μm^2 (Figure 3A). Although atherosclerotic lesion development in the apoE2 apoE3-Leiden animals transplanted with apoE3-Leiden apoE2 apoE3-Leiden bone marrow appeared slightly less dramatic, no significant decrease in lesion area could be demonstrated (4.21 ± 1.27 × 10^3 and 4.18 ± 0.16 × 10^3 μm^2, respectively). Also, no significant de-
crease in lesion area of control transplanted apoE-deficient mice (3.50 ± 0.53 × 10^6 μm²) was found. In contrast, apoE-deficient animals transplanted with wild-type apoE+/+ bone marrow (0.25 ± 0.16 × 10^6 μm²) and the control transplanted apoE3-Leiden bone marrow did show a dramatic decrease in atherosclerosis. The inability of macrophage-derived apoE and apoE3-Leiden to reduce atherosclerosis was confirmed by data from mice lacking both apoE and the LDL receptor (apoE−/−LDLr−/−; Figure 3B).

**Table 1. Amount of Human ApoE in VLDL, LDL, and HDL Determined 4 Weeks After BMT**

<table>
<thead>
<tr>
<th>BMT Type</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>HDL/LDL Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE3-Leiden to apoE3-Leiden</td>
<td>2.83 ± 0.23</td>
<td>5.03 ± 2.10</td>
<td>3.53 ± 1.25</td>
<td>0.73 ± 0.14</td>
</tr>
<tr>
<td>ApoE3-Leiden to apoE−/−</td>
<td>0.15 ± 0.07</td>
<td>0.27 ± 0.13</td>
<td>0.37 ± 0.23</td>
<td>1.37 ± 0.60</td>
</tr>
<tr>
<td>ApoE2(158) to apoE2(158)</td>
<td>1.81 ± 0.68</td>
<td>3.46 ± 1.08</td>
<td>2.29 ± 0.54</td>
<td>0.74 ± 0.41</td>
</tr>
<tr>
<td>ApoE2(158) to apoE−/−</td>
<td>0.10 ± 0.05</td>
<td>0.16 ± 0.04</td>
<td>0.71 ± 0.45</td>
<td>4.27 ± 1.92†</td>
</tr>
</tbody>
</table>

†P<0.01, compared with apoE−/− mice transplanted with apoE3-Leiden bone marrow.

**Figure 3. Effect of macrophage-derived wild-type murine apoE, human apoE3-Leiden, and human apoE2 on atherosclerotic lesion development in apoE-deficient mice**

A. Transplantation of apoE+/+, apoE3-Leiden, apoE−/−, and apoE2 apoE−/− bone marrow to apoE−/− mice, as well as the control transplanted apoE3-Leiden apoE−/− and apoE3-Leiden apoE−/− to apoE3-Leiden apoE−/− mice. B. Effect on atherosclerotic lesion development of transplantation of apoE−/− LDLr−/−, apoE3-Leiden apoE−/− LDLr−/−, and apoE2 apoE−/− LDLr−/− bone marrow to apoE−/− LDLr−/− mice. Mean lesion area was calculated from oil red O-stained cross sections of the aortic root at the level of the tricuspid valves. Values indicate mean lesion area of 10 sections from each mouse.

**Discussion**

Inactivation of the gene for mouse apoE is associated with a prominent increase in serum cholesterol levels and the premature development of atherosclerosis. ApoE displays antiatherogenic properties, due not only to its role as a receptor ligand that mediates removal of cholesterol-rich lipoproteins by the liver but also to its contribution to reverse cholesterol transport. Although the liver produces the majority of apoE in the circulation, macrophages also secrete this important protein. Recently, we and others demonstrated that introduction of apoE-producing monocytes/macrophages into apoE-deficient mice by BMT reduces circulating cholesterol levels and atherosclerosis, even though the circulating apoE levels were only 3.5% of normal. Several isoforms of apoE, including apoE2 and apoE3-Leiden, differ in their affinity for several binding sites on the liver, like the LDL receptor, proteoglycans, and/or the remnant receptor. The purpose of this study was to compare the effects of macrophage-derived wild-type apoE, human apoE2, and human apoE3-Leiden on circulating cholesterol levels and atherosclerosis in the absence of endogenous mouse apoE by using the technique of BMT.

It appears that macrophage-derived human apoE3-Leiden and human apoE2, in contrast to wild-type murine apoE, only transiently reduced serum cholesterol levels. The reduction in...
serum cholesterol levels on transplantation of wild-type murine bone marrow into apoE-deficient recipients is caused by a reduction in VLDL and LDL levels. Previously, we demonstrated that this reduction in VLDL levels is induced by increased recognition by the liver owing to the association of macrophage-derived apoE with these lipoproteins. In contrast to wild-type apoe, the reduction in circulating cholesterol levels on transplantation of apoE3-Leiden or apoE2 bone marrow into apoE-deficient animals, though mainly confined to VLDL, was only transient. At 4 to 6 weeks after transplantation, a secondary increase in serum cholesterol levels was observed, as reflected by an increase in remnant lipoproteins compared with a sustained decrease when wild-type murine apoE was used. This secondary increase in serum cholesterol levels in apoE3-Leiden-apoe–/– and apoE2-apoe–/– animals was not associated with a decrease in circulating apoE levels, because its concentration even increased slightly over time. Furthermore, it is unlikely that the increase in serum cholesterol was caused by generation of antibodies against apoE, because the cells in the donor bone marrow form the new immune system in the reconstituted animal. Another mechanism underlying this observation might be downregulation of the LDL receptor, a classic sterol-responsive-gene. Srivastava et al demonstrated in vivo that downregulation of the LDL receptor occurs within 2 weeks of feeding the animals a cholesterol-rich diet. Recently, it has also been demonstrated in vivo that the LDL receptor on macrophages can be downregulated after transplantation of wild-type murine bone marrow to LDL receptor-deficient animals owing to a sustained increase in serum cholesterol levels. Indeed, the transient reduction in serum cholesterol levels on transplantation of apoE3-Leiden or apoE2 bone marrow to apoE-deficient mice appeared to be mediated by the LDL receptor, because no such reduction was observed after transplantation to mice lacking both apoE and the LDL receptor. However, at present it is not clear why this effect is observed only with macrophage-derived human apoE3-Leiden and human apoE2 and not with wild-type mouse apoE.

A permanent reduction of serum cholesterol levels in apoE-deficient mice could only be achieved by inducing high concentrations of circulating apoE3-Leiden (657 ± 38 μg/dL) by cross-breeding apoE3-Leiden mice against an apoE–/– background, leading to the expression of mutant apoE3-Leiden in multiple tissues, including macrophages, liver,
brain, and muscles. This is in accordance with the data of Bellosta et al., who demonstrated that levels of macrophage-derived human apoE3 must exceed 800 μg/dL to correct the hyperlipidemia in apoE-deficient mice.

Human apoE2 appears to be ineffective in reducing serum cholesterol concentrations, even at circulating concentrations of 8392 ± 386 μg/dL. This difference in response is most likely caused by the fact that the in vivo binding of apoE2 to the LDL receptor is severely defective, whereas the binding of apoE3-Leiden is only mildly affected. Furthermore, the higher level of human apoE3 macrophage expression necessary to reduce serum cholesterol levels as demonstrated by Bellosta et al. compared with murine wild-type apoE as found in our studies indicates that murine apoE more effectively facilitates the clearance of mouse lipoproteins than does human apoE3. A further increase in macrophage human apoE3-Leiden production may induce a permanent reduction of lipid levels in apoE-deficient mice. Because human apoE2 concentrations as extreme as 8392 ± 386 μg/dL were unable to reduce atherosclerosis, no effect of a further increase of macrophage human apoE2 production on atherosclerosis is expected to be beneficial.

Macrophages are cells that actively secrete large quantities of apoE. The type of apoE secreted and the regulation of apoE synthesis by this cell type may be important in maintaining the balance between cholesterol influx and efflux. Macrophage foam cell formation and thus, atherosclerosis, will occur when either cholesterol influx is increased or cholesterol efflux is decreased or both. Several possible roles for macrophage-derived apoE in atherogenesis have been postulated. According to 1 hypothesis, apoE secretion by macrophages is proatherosclerotic by its association with lipoproteins in the extracellular space of the vessel wall, thereby enriching them with a high-affinity ligand for receptor-mediated uptake of lipoproteins and subsequently inducing foam cell formation. Ishibashi et al. demonstrated that macrophage-derived apoE facilitates the uptake of VLDL by these macrophages. Furthermore, it appears that apoE3 macrophages incorporated more VLDL particles than did macrophages expressing the receptor binding–defective form apoE2. Alternatively, macrophage-derived apoE may facilitate reverse cholesterol transport, thereby reducing the formation of foam cells. The role of macrophage-derived apoE in reverse cholesterol transport was confirmed in vitro by the observation that J774 macrophages, which do not synthesize endogenous apoE, have impaired efflux in the presence of an exogenous cholesterol acceptor. Subsequent stable expression of human apoE3 cDNA in these macrophages restored cholesterol efflux. According to the first model, as described by Basu et al., apoE and free cholesterol are secreted from macrophages by 2 independent mechanisms, leading to apoE-mediated facilitation of HDL-induced cholesterol efflux. This model was recently confirmed by data from Hayek et al., who demonstrated that peritoneal macrophages from apoE-deficient mice have similar rates of cholesterol efflux compared with wild-type macrophages. However, the efflux of cholesterol from wild-type macrophages was 40% lower when induced by HDL from apoE-deficient mice compared with HDL from wild-type mice. Addition of exogenous apoE to the apoE-deficient HDL restored the cholesterol efflux capacity, suggesting that apoE on HDL, rather than macrophage apoE, is responsible for cholesterol efflux. In contrast, addition of exogenous apoE3 to J774 macrophages failed to facilitate cholesterol efflux to HDL, as does endogenous expression in transfected J774 cells. Furthermore, in human monocyte–derived macrophages, it was demonstrated that they can release cholesterol without exogenously added cholesterol acceptors. From these in vitro studies, it can be concluded that macrophage-derived apoE may facilitate reverse cholesterol transport by 2 major pathways: (1) HDL-dependent secretion of cholesterol in apoE discoidal lipid particles and (2) an apoE-mediated facilitation of HDL-dependent release of cholesterol.

Recent in vivo data on the effect of selective expression of apoE in the vessel wall support the antiatherosclerotic function of locally produced apoE. To test whether apoE in the arterial wall has a local effect on atherogenesis, Shimano et al. established transgenic mice expressing human apoE under control of the mouse H2 Ld promoter. These mice, with high expression of human apoE in the vessel wall, showed no difference in plasma cholesterol levels compared with controls when fed either a control or an atherogenic diet, whereas the formation of fatty streaks was markedly inhibited. In addition, Bellosta et al. studied the effect of macrophage-specific expression of human apoE3 in apoE-deficient mice under control of the visna virus long-terminal repeat. Macrophage-specific expression of human apoE3 markedly reduced atherosclerotic lesion development even in the presence of high levels of atherogenic lipoproteins, indicating that macrophage-derived human apoE3 prevents atherosclerosis by promoting cholesterol efflux from the arterial wall.

BMT will lead to replacement of monocytes/macrophages in the recipient by cells of donor origin, including arterial wall macrophages. Using this technique, we studied the ability of macrophage-derived human apoE2 and human apoE3-Leiden to reduce atherosclerosis in apoE3-Leiden and apoE2 were unable to reduce atherosclerosis in apoE-/- mice. High concentrations of systemic apoE2 were ineffective in significantly reducing the severity of atherosclerosis, although there might have been a tendency to less dramatic lesions. These results are in accordance with recent ones of Tsukamoto et al., who investigated the effect of human apoE3, apoE2, and apoE4 on atherosclerosis by expressing these different isoforms in the livers of apoE-/- mice. Quantification of atherosclerosis indicated that apoE3, but not apoE2 and apoE4, was able to induce regression of atherosclerosis in apoE-/- animals. However, Sullivan et al. demonstrated, by using targeted replacement of the mouse apoE gene by either human apoE3 or human apoE2, that apoE2 could induce a 2-fold reduction in lesion area compared with apoE-/- mice. Using this same technique of targeted replacement of the mouse apoE gene, they also demonstrated that mice expressing human apoE3 are more susceptible to diet-induced atherosclerosis compared with wild-type mice. In addition, comparison of our transplantation data with the published data of Bellosta et al. indicated that higher levels of macrophage-derived human apoE3 were
required to reduce serum cholesterol levels compared with wild-type murine apoe. Nevertheless, macrophage-derived human apoE3 inhibited atherogenesis in these apoE-deficient animals despite high serum cholesterol levels. In this study, we demonstrated that macrophage-derived human apoE3-Leiden and human apoE2 were unable to reduce atherosclerosis at comparably high levels of cholesterol. Because the accumulation of cholesterol in macrophages and the subsequent formation of foam cells are the net effects of cholesterol influx and efflux, it may be expected that cholesterol efflux from macrophages expressing these mutant forms of apoE is impaired. To study cholesterol efflux induced by these mutant human apoE forms compared with apoE+/+ and apoE−/− macrophages, thiglycolate-elicited macrophages were isolated and loaded with [3H-1α,2α(n)]cholesterol oleate–labeled acetylated LDL. Cholesterol efflux was subsequently studied in the absence of specific cholesterol acceptors to deduce the direct effect of apoE secretion on cholesterol efflux, instead of apoE-facilitated HDL-dependent release. Compared with apoE+/+ macrophages, efflux from apoE−/− macrophages was reduced by ~50%. This reduction of cholesterol efflux from apoE−/− macrophages is in accordance with results obtained by Mazzone et al., who demonstrated that transfection of J774 macrophages with the gene for human apoE enhances cholesterol efflux from these cells. Recently, we (Van Eck et al, unpublished results, 1999) and Fazio et al. also found that transplantation of apoE−/− bone marrow to wild-type animals increased the sensitivity to atherosclerosis, indicating that wild-type mouse apoE promotes cholesterol efflux in vitro as well as in vivo.

Both human apoE2- and human apoE3-Leiden–expressing macrophages were equally capable of inducing cholesterol efflux compared with wild-type murine apoE. Because both apoE3-Leiden and apoE2 macrophages secrete low amounts of apoE compared with wild-type murine apoE and their efflux capacity is equal, it may be concluded that both apoE3-Leiden and apoE2 are very efficient in inducing cholesterol efflux. This view is in accordance with recent data from Cullen et al., who demonstrated that apoE2 macrophages secrete low amounts of apoE2, which is very efficient in inducing cholesterol efflux, thereby protecting these cells from cholesterol storage. In addition, apoE3 macrophages secrete very large amounts of apoE, thus protecting these cells from cholesterol accumulation despite their lower cholesterol efflux efficiency compared with that of apoE2 macrophages. In contrast, apoE4 macrophages were demonstrated to secrete the highest amounts of apoE, but they lack effective efflux capacity. Although human apoE3-Leiden and human apoE2 production by macrophages was very efficient in inducing cholesterol efflux in vitro, no significant effect was found on in vivo atherosclerotic lesion development. Thus, either normalization of cholesterol efflux in macrophages is not accompanied by measurable effects on arterial lesion growth under these conditions or the in vitro conditions do not completely reflect the in vivo situation with its complex interaction between various cell types and multiple factors.

In the present study, we demonstrated that the efficiency in reducing atherosclerosis in apoE−/− mice decreases in the order murine apoE > apoE3-Leiden > apoE2. A similar order was demonstrated for the relation between the type of apoE and serum cholesterol levels, indicating that accumulation of atherogenic, cholesterol-rich lipoproteins in the circulation is an important determinant for the development of atherosclerosis in these mice. Because we did not observe a difference in cholesterol efflux between wild-type mouse apoE, human apoE2, and human apoE3-Leiden in isolated peritoneal macrophages, we suggest that normalization of cholesterol efflux in macrophages is not accompanied by measurable effects on arterial lesion growth under these conditions and that the higher level of atherosclerosis in apoE2– apoE−/− and apoE3-Leiden apoE−/− mice, compared with apoE+/+ apoE−/− animals, is caused by a difference in cholesterol influx due to differences in serum cholesterol levels. Under circumstances of high circulating cholesterol levels, adequate efflux of cholesterol from macrophages does not prevent foam cell formation and the development of atherosclerosis, and it appears that the efficiency in decreasing serum cholesterol levels is a more important parameter for apoE, apoE3-Leiden, and apoE2.

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Apolipoprotein E Polymorphisms and Atherosclerosis


Effect of Macrophage-Derived Mouse ApoE, Human ApoE3-Leiden, and Human ApoE2 (Arg158 → Cys) on Cholesterol Levels and Atherosclerosis in ApoE-Deficient Mice

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