Effects of coexpression of the LDL receptor and apoE on cholesterol metabolism and atherosclerosis in LDL receptor-deficient mice

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Abstract The low density lipoprotein receptor (LDLR) plays a major role in regulation of plasma cholesterol levels as a ligand for apolipoprotein B-100 and apolipoprotein E (apoE). Consequently, LDLR-deficient mice fed a Western-type diet develop significant hypercholesterolemia and atherosclerosis. ApoE not only mediates uptake of atherogenic lipoproteins via the LDLR and other cell-surface receptors, but also directly inhibits atherosclerosis. In this study, we examined the hypothesis that coexpression of the LDLR and apoE would have greater effects than either one alone on plasma cholesterol levels and the development of atherosclerosis in LDLR-deficient mice. LDLR-deficient mice fed a Western-type diet for 10 weeks were injected with recombinant adenoviral vectors encoding the genes for human LDLR, human apoE3, both LDLR and apoE3, or lacZ (control). Plasma lipids were analyzed at several time points after vector injection. Six weeks after injection, mice were analyzed for extent of atherosclerosis by two independent methods. As expected, LDLR expression alone induced a significant reduction in plasma cholesterol due to reduced VLDL and LDL cholesterol levels, whereas overexpression of apoE alone did not reduce plasma cholesterol levels. When the LDLR and apoE were coexpressed in this model, the effects on plasma cholesterol levels were no greater than with expression of the LDLR alone. However, coexpression did result in a substantial increase in large apoE-rich HDL particles. In addition, although the combination of cholesterol reduction and apoE expression significantly reduced atherosclerosis, its effects were no greater than with expression of the LDLR or apoE alone. In summary, in this LDLR-deficient mouse model fed a Western-type diet, there was no evidence of an additive effect of expression of the LDLR and apoE on cholesterol reduction or atherosclerosis.—Kawashiri, M-a., Y. Zhang, D. Usher, M. Reilly, E. Puré, and D. J. Rader.

The low density lipoprotein receptor (LDLR) mediates the removal of LDL and remnant lipoproteins from circulation by binding to apolipoprotein B-100 (apoB-100) and apolipoprotein E (apoE) and plays a major role in regulation of plasma cholesterol levels in humans (1). The LDLR-deficient mouse has modestly elevated levels of plasma cholesterol and, when fed a high fat, high cholesterol diet, develops much higher levels of cholesterol, xanthomas, and atherosclerosis (2). Liver-directed expression of human LDLR using an adenoviral vector in LDLR-deficient mice on a normal chow diet transiently reduced plasma cholesterol levels to normal (3). The effect of transient reduction of cholesterol on the development of atherosclerosis in the LDLR-deficient mouse has not been studied. ApoE is a 34-kD glycoprotein synthesized in variety of tissues, including liver and macrophages (4). As an integral surface component of lipoproteins, apoE mediates the uptake of chylomicron and VLDL remnants by acting as a ligand for LDLR (4), heparan sulfate proteoglycans (5), and the low density lipoprotein receptor related protein (LRP) (6). ApoE-deficient mice have high levels of remnant lipoproteins owing to defective clearance (7, 8). Liver-directed gene transfer of human apoE3 using adenoviral vectors decreased plasma cholesterol levels (9–11). Bone marrow transplantation of wild-type mouse bone marrow into apoE-deficient mice normalized the lipoprotein profile by providing a source of extrahepatic apoE (12, 13). Studies in apoE-deficient mice have established that approximately 1% of the normal plasma apoE con-
centration is adequate to normalize plasma cholesterol levels (14). The effects of overexpression of apoE on lipid levels in animal models other than apoE deficiency have also been studied. Overexpression of rat apoE in wild-type mice fed a high fat, high cholesterol diet reduced plasma cholesterol levels (15, 16). Overexpression of rat apoE in diabetic wild-type mice reduced plasma cholesterol and triglyceride levels (17). Overexpression of rat apoE in LDLR-deficient mice reduced VLDL cholesterol levels and accelerated VLDL clearance (18). Overexpression of human apoE3 in wild-type rabbits reduced VLDL cholesterol but increased LDL cholesterol levels (19). In fact, apoE-containing remnant lipoproteins have been shown to compete with LDL for binding to the LDL receptor (20).

Expression of apoE also has significant effects on atherosclerosis. ApoE-deficient mice develop extensive atherosclerotic lesions on a chow diet (7, 8, 21). Bone marrow transplantation of wild-type mouse bone marrow into apoE-deficient mice reduced progression of atherosclerosis (12, 13). Gene transfer and hepatic overexpression of human apoE3 in apoE-deficient mice induced marked regression of pre-existing atherosclerosis (22, 23). In all of these experiments, apoE expression substantially reduced plasma cholesterol levels, making it difficult to determine the mechanism by which apoE reduced atherosclerosis. However, when WHHL rabbits were injected repeatedly with apoE for 8.5 months, there was no effect on plasma cholesterol levels but atherosclerosis was reduced (24). Gene transfer and hepatic overexpression of apoE in apoE-deficient mice inhibited the progression of early atherosclerotic lesions (25) and reduced regression of advanced complex atherosclerotic lesions (26) in LDLR-deficient mice fed a Western-type diet despite having no significant effect on plasma cholesterol levels. Expression of apoE by the adrenals at plasma levels below the threshold for reducing plasma cholesterol levels still reduced atherosclerosis in apoE-deficient mice (27). Therefore, extravascular expression of apoE has direct effects on atherosclerosis independent of its ability to mediate hepatic uptake of atherogenic lipoproteins.

In the current study, we tested the hypothesis that coexpression of the LDLR and apoE in LDLR-deficient mice fed a Western-type diet would have greater effects on cholesterol reduction and atherosclerosis than the expression of either gene alone.

MATERIALS AND METHODS

Mouse studies

A first-generation recombinant adeno-virus encoding the human LDLR cDNA (28) was kindly provided by Dr. Karen Kozarsky. A second-generation recombinant adeno-virus encoding the human apoE3 cDNA (22, 25) was used for apoE gene transfer. As control adeno-viruses, we used first- and second-generation vectors encoding beta galactosidase (AdlacZ and tsAdlacZ). A total of 40 LDLR-deficient male mice (back-crossed 10 times to C57BL/6 mice), 28 weeks old, were obtained from Jackson Labs (Bar Harbor, ME) and fed a Western-type diet (normal chow supplemented with 0.15% cholesterol and 20% butter fat) for 10 weeks before gene transfer. When the mice were 38 weeks of age, serum cholesterol was determined and the mice were divided into four groups such that the mean plasma cholesterol levels in each group were not different. The mice were injected intravenously with four different combinations of adenoviruses (1.0 × 10¹ⁱ particles of each virus, 2.0 × 10¹¹ particles total dose): 1) AdlacZ + tsAdlacZ, 2) AdLDLR + tsAdlacZ, 3) AdlacZ + tsAdapoE3, and 4) AdLDLR + tsAdapoE3. All the mice received the same total dose of virus. Each group contained 9–11 mice. Blood was obtained from the retroorbital plexus after a 4-h fast on days 0, 3, 7, 14, 21, 28, and 42 after adeno-virus injection for analysis of transgene expression and lipids. Aliquots of plasma were stored at −20°C until quantification. All mice were killed 6 weeks after virus injection to quantify the extent of atherosclerotic lesions.

Quantification of atherosclerotic lesions

The extent of atherosclerosis was analyzed by two independent methods, en face analysis of the whole aorta and cross-sectional analysis of the aortic root, as previously described (22, 29). Mice were anesthetized with xylazine/ketamine. After the aorta was gently perfused with ice-cold PBS via the left ventricle, the heart was severed just above the aortic root, cut at the base, then the upper half of the heart containing aortic root was embedded in OCT and frozen at −80°C. For the en face analysis, the rest of the aorta, from ascending aorta to common iliac artery, was removed and fixed in 10% formalin. After the adventitial and adipose tissue was removed, the aortic arches were cut open longitudinally, then stained with Sudan IV solution (0.5% Sudan IV, 35% ethanol, 50% acetone) for 8 min and destained with 80% ethanol for 5 min to eliminate background staining. Images were captured with a Leica MZ12 microscope and digitized, and Sudan IV-stained lesion area was quantified using Image Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD). All data capture and quantification was performed in a blinded fashion.

Atherosclerosis was also quantified in the aortic root cross-sections from the fresh-frozen OCT-embedded hearts (22, 29). Serial 8 μm sections of the aortic root were cut and mounted on slides, then fixed in acetone, rehydrated in PBS containing 0.02% NaNO₃, and blocked with 1% BSA in PBS/NaNO₃. For detection of macrophages, sections were immunostained with monoclonal rat anti-murine MAC-1 antibody, monoclonal hamster anti-CD18, and monoclonal hamster anti-CD11c, followed by incubation with mouse anti-rat or goat anti-hamster IgG in the presence of 200-μg/ml normal mouse IgG. Antibody reactivity was detected using HRP-conjugated biotin-streptavidin complexes and developed with diaminobenzidine tetrahydrochloride (DAB) as substrate. Images of immunostained aortic root sections, captured digitally with a video camera connected to a Leica microscope, were analyzed using computerized image analysis (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Total lesion area in aortic root sections was measured by manually tracing entire lesions in 4 equally spaced aortic root sections per mouse. Macrophage lesion area was quantified in the same sections by determination of area that stained positively for macrophage markers. The percent of lesion occupied by macrophage-derived foam cells was calculated by dividing the macrophage lesion area by the total lesion area. The acquisition of images and analysis of lesions were performed in a blinded fashion.

Analytical methods

The plasma total cholesterol levels were measured in individual mice at each time point with an enzymatic assay on a Cobas Fara II (Roche Diagnostic System, Inc., Indianapolis, IN) with the use of Sigma reagents (Sigma, St. Louis, MO). Plasma human-specific apoE levels were quantified in pooled plasma at each time point by enzyme-linked immunosorbent assay. Pooled plasma on days 0, 3, and 14 from each group was subjected to fast protein
liquid chromatography (FPLC) gel filtration (Pharmacia LKB Biotechnology) on two Superose 6 columns as previously described (11). Cholesterol concentrations in the fractions were determined with an enzymatic assay (Wako Pure Chemical Industries, Ltd.), and human-specific apoE levels in each fraction were quantified by the same method as above. The amount of cholesterol in each class was calculated using FPLC fractions #3–#10 for VLDL, #11–#24 for IDL/LDL, and #25–#40 for HDL. After separating the pooled plasma samples on FPLC, 6-μl samples from three adjacent fractions were pooled and subjected to 3–20% gradient SDS-PAGE. Mouse apoA-I and mouse apoB-48 and apoB-100 were detected using rabbit anti-mouse apoA-I and apoB (Biodesign International). Peroxidase-labeled goat anti-rabbit antibody (Jackson Immuno Research) was used for detection.

Statistical analysis
Atherosclerotic lesion area data and lipids data on each day were subjected to ANOVA with the Kruskal-Wallis test, followed by the Dunn’s multiple comparison test. Statistical significance for all comparisons was assigned at $P < 0.05$. Graphs and tables represent mean values ± SEM.

RESULTS
Effects on plasma lipid levels
Male LDLR-deficient mice on a Western-type diet were injected with adenoviral vectors encoding the LDLR alone, apoE3 alone, LDLR plus apoE3, or control LacZ. Mean plasma levels of human apoE in mice coexpressing the LDLR (250 mg/dl) were 51% higher than in the mice injected with the apoE3 vector alone (166 mg/dl) on day 7, and 806% higher (LDLR+apoE3; 154 mg/dl, apoE3 alone; 17 mg/dl) on day 14. Human apoE could be detected in both groups of mice injected with apoE vector throughout the experimental period but remained significantly higher in mice coexpressing the LDLR even after 6 weeks (LDLR+apoE3; 22 mg/dl, apoE3 alone; 12 mg/dl).

The effects on plasma cholesterol levels are shown in Table 1. Mice injected with control vector (AdlacZ) experienced transiently decreased levels of plasma cholesterol, which returned to baseline by day 14. Mice injected with the LDLR vector had a significant 75% decrease in cholesterol that was transient, similar to that previously reported in chow-fed mice (3), and cholesterol levels returned to the level of the control mice by day 14. Mice injected with the apoE3 vector did not show a decrease in cholesterol relative to the control group, consistent with our previous report (25). Interestingly, coexpression of apoE3 with

### Table 1. Changes in plasma total cholesterol after vector injection (mg/dl)

<table>
<thead>
<tr>
<th></th>
<th>Day 9</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,946</td>
<td>1,442</td>
<td>1,331</td>
<td>1,653</td>
<td>1,497</td>
<td>1,572</td>
<td>1,909</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(373)</td>
<td>(299)</td>
<td>(217)</td>
<td>(363)</td>
<td>(290)</td>
<td>(340)</td>
<td>(318)</td>
</tr>
<tr>
<td>LDLR</td>
<td>2,061</td>
<td>525a</td>
<td>501a</td>
<td>1,344</td>
<td>1,335</td>
<td>1,342</td>
<td>1,745</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(352)</td>
<td>(278)</td>
<td>(162)</td>
<td>(299)</td>
<td>(264)</td>
<td>(240)</td>
<td>(385)</td>
</tr>
<tr>
<td>ApoE3</td>
<td>1,937</td>
<td>1,869b</td>
<td>1,322b</td>
<td>1,257</td>
<td>1,353</td>
<td>1,441</td>
<td>1,933</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(336)</td>
<td>(234)</td>
<td>(142)</td>
<td>(244)</td>
<td>(287)</td>
<td>(240)</td>
<td></td>
</tr>
<tr>
<td>LDLR+apoE3</td>
<td>1,931</td>
<td>644a</td>
<td>628a</td>
<td>1,120</td>
<td>1,342</td>
<td>1,616</td>
<td>1,939</td>
</tr>
<tr>
<td>(n = 11)</td>
<td>(308)</td>
<td>(258)</td>
<td>(102)</td>
<td>(170)</td>
<td>(301)</td>
<td>(325)</td>
<td>(423)</td>
</tr>
</tbody>
</table>

Values are given as mean (SEM).

a Significantly different from control, $P < 0.05$.

b Significantly different from LDLR, $P < 0.05$.

c Significantly different from apoE3, $P < 0.05$.
LDLR did not reduce cholesterol levels to any greater degree than expression of the LDLR alone. Thus, hepatic overexpression of apoE3 in LDLR-deficient mice had minimal effects on plasma cholesterol levels either alone or in coexpression with the LDLR.

Effects on lipoprotein distribution

To gain insight into the effects of apoE3 and LDLR expression on plasma lipoprotein, we separated lipoproteins by FPLC gel filtration. Prior to vector injection (Fig. 1A), there were no significant differences among the four groups. On day 3 after injection (Fig. 1B), expression of the LDLR alone markedly reduced the VLDL and IDL/IDL/LDL fractions. In contrast, expression of apoE alone increased VLDL and IDL cholesterol compared with the control group. Coexpression of apoE3 with the LDLR resulted in a higher VLDL peak than expression of the LDLR alone and had no effect on IDL and LDL com-

Fig. 2. Distribution of murine apoA-I (A) and murine apoB (B) among lipoprotein classes. Six-μl samples from three adjacent FPLC fractions were pooled and subjected to SDS-PAGE, followed by Western blot analysis with the antibody against murine apoA-I and apoB. C: Agarose electrophoresis of pooled FPLC fractions (VLDL #5–#7, LDL #16–#18, New peak #24–#26, and HDL #34–#36) of plasma obtained 3 days after injection from control and apoE3+LDLR vector-injected mice. The new cholesterol peak is noted to run in the alpha region. D: ApoE concentrations in FPLC fractions. Pooled plasma samples were subjected to gel filtration using Superose 6 columns, and human-specific apoE levels were measured by ELISA. Squares: mice injected with control vector; open triangles, mice injected with apoE3 vector alone; filled triangles, mice injected with LDLR+apoE3 vectors.
pared with expression of LDLR alone. In addition, coexpression of apoE3 and LDLR resulted in the appearance of a new peak (fractions #22–#33) between the LDL and HDL peaks. This peak is unlikely to be an artifact caused by the adenoviral vector itself because it is not seen in control, LDLR, or apoE vector-injected mice, but only in mice expressing both the LDLR and apoE. On day 7 after injection (Fig. 1C), expression of LDLR alone continued to result in substantially reduced VLDL, IDL, and LDL, whereas expression of apoE3 alone increased VLDL but modestly reduced LDL. The new cholesterol peak between LDL and HDL was still present on day 7 in the mice coexpressing the LDLR plus apoE.

Western blotting for murine apoA-I (Fig. 2A) and apoB (Fig. 2B) in the same FPLC fractions were performed to determine which class of lipoprotein contributed in the new cholesterol peak. The distribution of mouse apoA-I did not differ among the four groups around fractions #22–#33. Expression of the LDLR alone or apoE3 alone did not change apoB distribution around fractions #22–#33. The peak between LDL and HDL was noted to contain substantial mouse apoA-I but little mouse apoB, suggesting that it is a large HDL particle. We performed agarose electrophoresis on the FPLC-isolated fractions including this new peak and confirmed that it migrated in the alpha region, consistent with it being an HDL particle (Fig. 2C).

The amount of human apoE in FPLC fractions was quantitated by ELISA (Fig. 2D). When apoE3 was expressed alone, human apoE was found mainly in VLDL peak and fractions #22–#30 representing large HDL. When apoE3 was coexpressed with the LDLR, less human apoE3 was found in VLDL and, interestingly, greater than 2-fold more apoE3 was found in large HDL compared with apoE3 expression alone. This suggests that the reduction in VLDL brought about by LDLR expression resulted in a shift of human apoE to apoE-rich large HDL particles.

The amount of cholesterol in each lipoprotein fraction was calculated using the FPLC fraction cholesterol data. After control virus injection, both VLDL (Fig. 3A) and IDL/LDL cholesterol (Fig. 3B) decreased but HDL cholesterol (Fig. 3C) did not change. Expression of the LDLR alone decreased both the VLDL (90%) and IDL/LDL cholesterol (60%) levels, but did not change the HDL cholesterol level. Expression of apoE alone had little effect on the lipoprotein classes compared with controls. Interestingly, coexpression of apoE3 with LDLR decreased VLDL and IDL/LDL cholesterol similar to that of LDLR expression alone, but increased HDL cholesterol level to a greater extent than expression of either LDLR or apoE3 alone.

Effects on atherosclerosis

Atherosclerotic lesion area was quantified using two independent methods, en face analysis of the lipid-stained aorta and cross-sectional analysis of the aortic root. Both analytical methods produced similar results (Table 2). In the en face analysis, compared with mice injected with control vector, the mice expressing the LDLR alone had 43% reduced atherosclerosis ($P = 0.01$), mice expressing apoE alone had 21% reduced atherosclerosis ($P = 0.19$),
and mice coexpressing the LDLR and apoE had 38% reduced atherosclerosis ($P = 0.02$). In the aortic root analysis, compared with controls, the mice expressing the LDLR alone had 44% reduced atherosclerosis ($P = 0.01$), mice expressing apoE alone had 27% reduced atherosclerosis ($P = 0.16$), and mice co-expressing the LDLR and apoE had 44% reduced atherosclerosis ($P = 0.007$). By both methods of quantitation, coexpression of the LDLR and apoE did not reduce atherosclerosis to a greater extent than expression of the LDLR alone.

We also determined the effects of LDLR and apoE expression on the composition of lesions by staining for macrophages and quantitating the lesion area (Fig. 4A) and the percent of the total lesion occupied by macrophage-derived foam cells (Fig. 4B). Expression of LDLR alone and apoE alone each significantly reduced the macrophage lesion area and percent lesion area. However, coexpression of the LDLR and apoE did not reduce macrophage lesion area to a greater extent than either one alone.

**DISCUSSION**

In these studies, we used somatic gene transfer to test the additive effects of coexpression of the LDLR and apoE compared with either one alone on cholesterol metabolism and atherosclerosis in LDLR-deficient mice fed a Western-type diet. Previous studies (3) had confirmed that expression of the LDLR using gene transfer in LDLR-deficient mice effectively reduced plasma cholesterol levels (25). We hypothesized that hepatic gene transfer and coexpression of apoE and the LDLR in LDLR-deficient mice would result in a more substantial reduction in plasma cholesterol than either one alone.

As expected, expression of the LDLR alone had a substantial effect in reducing plasma cholesterol levels in this mouse model. However, in contrast to our expectations, coexpression of apoE with the LDLR did not result in a greater reduction of plasma cholesterol than expression of the LDLR alone. However, coexpression did result in a substantial increase in large apoE-rich HDL particles compared with expression of apoE alone. This may be because when apoE was expressed alone, a substantial amount of apoE associated with VLDL and IDL particles; however, coexpression of the LDLR resulted in clearance of the majority of apoB-containing lipoproteins, with the apoE then preferentially distributed to large HDL particles. As apoE in HDL is catabolized more slowly than apoE in apoB-containing lipoproteins (30, 31), this might also explain the seemingly paradoxical finding that human apoE levels were higher in mice coexpressing the LDLR than in those expressing apoE alone.

The failure of apoE expression alone to reduce cholesterol levels in the absence of the LDLR is consistent with other published data. Plasma cholesterol levels in apoE-deficient mice can be normalized by very low levels of apoE expression amounting to only about 1% of normal apoE levels (14). However, when apoE-deficient mice lacking the LDLR were transplanted with wild-type bone marrow (a source of macrophage-derived apoE) (32) or injected with an adenoviral vector expressing human apoE3 (33), there was no effect on plasma cholesterol levels. In fact, apoE-containing remnant lipoproteins may compete with LDL for uptake (20), and apoE inhibits lipolysis and catabolism of remnant lipoproteins (33). Finally, hepatic apoE promotes the hepatic secretion of VLDL triglycerides (34–37) and VLDL apoB (38). Therefore any effect of apoE in promoting clearance of VLDL may be offset by its effect in promoting VLDL production. In the current study, LDLR expression alone effectively decreased both VLDL and IDL/LDL. ApoE3 expression alone increased VLDL and IDL, probably due to increased VLDL production and/or reduced VLDL lipolysis. Coexpression of apoE3 and LDLR decreased VLDL less effectively compared with expression of the LDLR alone, and had a similar effect on LDL compared with LDLR alone.

Another goal of this study was to test the hypothesis that the effects of cholesterol reduction using LDLR gene transfer and apoE expression would be additive with regard to reduction in atherosclerosis. Abundant data in animals and humans indicate that long-term cholesterol reduction reduces atherosclerosis. A recombinant adeno-associated viral vector encoding the VLDL receptor reduced plasma cholesterol levels for up to 7 months and reduced atherosclerosis in LDLR-deficient mice fed a Western-type diet (39). ApoE has antiatherogenic effects that extend beyond...
its ability to reduce plasma cholesterol levels. Macrophage-derived apoE directly inhibits progression of atherosclerosis (12, 13, 40). Expression of apoE at levels that did not reduce plasma cholesterol levels reduced atherosclerosis in apoE-deficient mice (27). We previously showed that hepatic gene transfer of apoE to LDLR-deficient mice fed a Western-type diet reduced the progression of atherosclerosis despite having no effect on plasma cholesterol levels (25). Thus, cholesterol lowering alone and apoE expression in the absence of cholesterol reduction each have the ability individually to reduce progression of atherosclerosis in mice.

In this study, even transient reduction in plasma cholesterol brought about by LDLR expression was associated with a significant reduction in atherosclerosis. However, our results indicate that in this experimental model there were not additive effects of cholesterol reduction and apoE expression in inhibiting the progression of atherosclerosis. However, these were short-term studies, and it is possible that longer term combined expression could have greater effects on lipoprotein metabolism and/or atherosclerosis.

In summary, apoE3 and/or LDLR were expressed in the livers of LDLR-deficient mice fed a Western-type diet to test the additive effects on lipoprotein metabolism and the development of atherosclerosis. Overexpression of the LDLR alone reduced cholesterol levels transiently and had a significant effect on atherosclerosis, but the addition of apoE expression to LDLR did not reduce cholesterol levels or atherosclerosis to a greater extent than the LDLR alone.

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