Reversal of Hypercholesterolemia in Apolipoprotein E2 and Apolipoprotein E3-Leiden Transgenic Mice by Adenovirus-Mediated Gene Transfer of the VLDL Receptor

Ko Willems van Dijk, Bart J.M. van Vlijmen, Andre van der Zee, Belinda van’t Hof, Hans van der Boom, Kunihisa Kobayashi, Lawrence Chan, Louis M. Havekes, Marten H. Hofker

Abstract—We have investigated the interaction of apolipoprotein E2(Arg158-Cys) (apoE2) and apolipoprotein E3-Leiden (apoE3-Leiden) with the very low density lipoprotein (VLDL) receptor in vivo and in vitro to define the possible role of this receptor in lipoprotein metabolism and atherosclerosis. The in vivo binding specificity of the VLDL receptor for apoE2 and apoE3-Leiden was investigated by adenovirus-mediated gene transfer of the VLDL receptor in apoE2 and apoE3-Leiden transgenic mice lacking endogenous mouse apoE (Apoe−/−). Ectopic overexpression of the VLDL receptor gene in the liver resulted in a 50% decrease of plasma cholesterol levels in both apoE2 and apoE3-Leiden transgenic mice compared with liver expression of the β-galactosidase gene. This reduction in plasma cholesterol was mainly due to a reduction in the VLDL level. Overexpression of the VLDL receptor did not affect the hepatic VLDL triglyceride production, indicating that the hypocholesterolemic effect is due to an increased level of plasma clearance mediated by the VLDL receptor. In vitro binding analysis showed that both apoE2 and apoE3-Leiden VLDL compete efficiently with rabbit β-VLDL for binding to the VLDL receptor expressed on LDL receptor–deficient Chinese hamster ovary cells. We conclude from these data that both apoE2 and apoE3-Leiden function as proper ligands for the VLDL receptor in vitro and in vivo. This finding substantiates a possible role for the VLDL receptor in atherosclerosis in hyperlipidemic subjects homozygous for apoE2 or carrying apoE3-Leiden and indicates that the VLDL receptor expressed on the liver has therapeutic potential as an alternative route for clearance of binding-defective lipoproteins. (Arterioscler Thromb Vasc Biol. 1998;18:7-12.)

Key Words: VLDL receptor • apoE variants • atherosclerosis • gene therapy

The VLDL receptor gene was initially cloned from a rabbit heart cDNA library on the basis of homology to the LDL receptor gene. The protein encoded by this cDNA appeared highly homologous to the LDL receptor. An apparent difference between the LDL receptor and VLDL receptor proteins exists between the number of cysteine-rich repeat sequences in the N-terminal ligand binding domains. The LDL receptor ligand-binding domain consists of seven copies of an approximately 40–amino acid cysteine-rich sequence, whereas the VLDL receptor ligand binding domain consists of eight such copies. Both the LDL receptor and the VLDL receptor contain NPXY sequences within the cytoplasmic domain. For the LDL receptor, this sequence is required for clustering of the receptor into coated pits before internalization. However, it is not known whether this sequence in the VLDL receptor is responsible for internalization of the bound ligand in vivo.

The VLDL receptor is specific for apoE and binds β-VLDL derived from cholesterol-fed rabbits and VLDL and IDL derived from LDL receptor–deficient Watanabe rabbits with high affinity. Based on mRNA analysis of whole tissues, the VLDL receptor was found to be highly transcribed in the heart, muscle, and adipose tissue but not the liver. Because the VLDL receptor was found to be transcribed in tissues relying on fatty acids for energy supply and storage, these observations have led to the hypothesis that the VLDL receptor may be involved in the supply of fatty acids derived from triglyceride-rich lipoproteins to adjacent tissues. This hypothesis has been further strengthened by immunolocalization studies, which identified high levels of the VLDL receptor in the endothelium of capillaries and small arterioles; the site where the VLDL receptor would be expected to reside if it plays a role in this process. In addition, the amount of body fat is significantly reduced in the Vldl receptor–deficient mouse, which is in line with a role in fatty acid delivery to peripheral tissues for energy metabolism and storage.

The VLDL receptor has also been implicated in the pathology of atherosclerosis. The VLDL receptor is present in...
primary cells involved in this process and binds lipoproteins that are known to be atherogenic. Foam cell formation can be mediated by the VLDL receptor in vitro by prolonged incubation of rabbit β-VLDL with LDL receptor–deficient CHO cells overexpressing the VLDL receptor. Moreover, VLDL receptor protein has been detected in atherosclerotic lesions. The role of the VLDL receptor in atherogenesis could be particularly pronounced in the presence of high levels of apoE-rich chylomicron and VLDL remnant lipoproteins, such as observed in patients with FD. FD is caused by mutations in apoE, leading to disturbed lipoprotein clearance and premature atherosclerosis (for review, see References 10 and 11).

Several variant forms of apoE leading to FD have been described, such as the common apoE2 variant that is inherited as a recessive trait and the rare apoE3-Leiden variant, which is inherited as a dominant trait. In vitro binding studies have revealed a severe binding defect of apoE2 and a moderate binding defect of apoE3-Leiden to the LDL receptor. A proposed role for the VLDL receptor in triglyceride metabolism and atherogenesis in the case of FD patients is thus dependent on the binding capacity of apoE2 and apoE3-Leiden to the VLDL receptor. To determine this, we investigated the specificity of the VLDL receptor for apoE2 and apoE3-Leiden, in vivo, in transgenic mouse models by overexpressing the VLDL receptor in the livers of apoE2 and apoE3-Leiden mice using adenovirus-mediated gene transfer. Our results indicate that the VLDL receptor recognizes both apoE2 and apoE3-Leiden VLDL and thus substantiate a possible role for the VLDL receptor in triglyceride metabolism and atherosclerosis in FD patients. In addition, because, in contrast to the endogenous LDL receptor, the VLDL receptor efficiently clears apoE2 and apoE3-Leiden–containing lipoproteins when expressed in the liver, a gene therapy strategy using the VLDL receptor as an alternative receptor for lipoprotein clearance has definite possibilities for application in FD patients.

Methods

Mice

Transgenic mice expressing human apoE2 and apoE3-Leiden in the absence of endogenous mouse apoE have been described previously. These mice are maintained by crossbreeding with Apoe-/- mice. Transgenic offspring were identified by ELISA for the presence of human apoE in the serum, and the endogenous Apoe-/- genotype was confirmed by PCR analysis on tail tip DNA, as described earlier. Adenovirus-mediated gene transfer of the VLDL Receptor in apoE2 and apoE3-Leiden Transgenic Mice

We previously reported plasma cholesterol levels for apoE2 and apoE3-Leiden mice on an endogenous Apoe-/- background.
Effect of Systemic Ad-VLDLR Administration to apoE2 ApoE−/− and apoE3-Leiden ApoE−/− Ldlr−/+ Mice on Serum Lipid and Human ApoE Levels*

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Ad-LacZ</th>
<th>Ad-VLDLR</th>
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<tr>
<td></td>
<td>n</td>
<td>TC, mmol/L</td>
<td>n</td>
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<tr>
<td>Ldlr−/−</td>
<td>8</td>
<td>9.8±1.8</td>
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<tr>
<td>ApoE−/−</td>
<td>7</td>
<td>19.7±3.5</td>
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<tr>
<td>apoE2</td>
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<tr>
<td>ApoE−/−</td>
<td>13</td>
<td>15.9±2.8</td>
<td>5</td>
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<tr>
<td>apoE3L</td>
<td>6</td>
<td>13.2±3.8</td>
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<tr>
<td>Ldlr−/+</td>
<td></td>
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</tr>
<tr>
<td>ApoE−/−</td>
<td>6</td>
<td>13.2±3.8</td>
<td>3</td>
</tr>
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|                      | Ad-LacZ | Ad-VLDLR | |
|----------------------|---------|----------|
|                      | ApoE, mg/dL | ApoE, mg/dL |
| ApoE−/−              |           |           |
| Ldlr−/−              |           |           |
| ApoE−/−              |           |           |
| apoE2                |           |           |
| apoE−/−              |           |           |

*Female mice, 10 to 15 weeks of age were administered 3×10⁹ PFU Ad-VLDLR or Ad-LacZ. Values are shown for fasted blood samples drawn from the tail vein 2 days before (preinjection) and 5 days after adenovirus injection. nd, not determined; TC, total cholesterol; TG, triglycerides; apoE, human apoE concentration.

†P<.05, significantly different from pretreatment values, using nonparametric Mann-Whitney tests.

In Vivo Binding of apoE2 and apoE3-Leiden VLDL to the VLDL Receptor

The interaction of VLDL derived from apoE2 ApoE−/− and apoE3-Leiden ApoE−/− Ldlr−/+ mice with the VLDL receptor was investigated in vitro using competition for binding of 125I-labeled rabbit β-VLDL to LDL receptor–deficient CHO cells (ldlA-7) stably expressing the human VLDL receptor. Figure 2 shows that both apoE2 and apoE3-Leiden VLDL compete efficiently with 125I-labeled rabbit β-VLDL for binding with these cells, whereas VLDL derived from ApoE−/− mice was very poor in this respect. ApoE3-Leiden VLDL seems more efficient than apoE2 VLDL in this competition assay.

Hepatic VLDL Triglyceride Production in Virus-Injected Mice

The in vitro binding studies show that apoE2 and apoE3-Leiden VLDLs bind efficiently to the VLDL receptor. To determine whether (part of) the hypocholesterolemic effect of Ad-VLDLR administration can be attributed to a decrease in VLDL production, the hepatic VLDL triglyceride production was determined. apoE2 ApoE−/− mice treated with either Ad-LacZ (n=5) or Ad-VLDLR (n=5) were injected with Triton WR1339 at day 5 postinjection and the plasma triglyceride increase was measured. The plasma triglyceride increase did not differ significantly between Ad-LacZ or Ad-VLDLR treated apoE2 mice (respectively, 45±21 and 49±28 μmol·kg⁻¹·h⁻¹), indicating that VLDL production is not affected by Ad-VLDLR administration.
In the present study, we demonstrate that ectopic overexpression of the VLDL receptor in the liver of apoE2 and apoE3-Leiden transgenic mice results in a 50% reduction of hypercholesterolemia. This reduction in cholesterol is mainly due to a reduction of the VLDL fraction. To confirm the binding of apoE2 and apoE3-Leiden to the VLDL receptor, in vitro binding studies were performed showing that both apoE variants effectively compete with β-VLDL for binding to the VLDL receptor expressed in LDL receptor–deficient CHO cells. Because production of VLDL is not affected by liver expression of the VLDL receptor, we conclude from these data that the hypocholesterolemic effect of liver expression of the VLDL receptor in apoE2 and apoE3-Leiden transgenic mice is due to an increase in lipoprotein clearance.

Our data showing that apoE2 binds to the VLDL receptor are in line with previously reported results. Takahashi et al. investigated the binding of VLDL derived from normolipemic human subjects, homozygous for either apoE3 or apoE2, by competition for binding with β-VLDL to LDL receptor–deficient CHO cells expressing the VLDL receptor. It was found that both apoE3 VLDL and apoE2 VLDL compete equally well with β-VLDL for binding to the VLDL receptor. Our data indicate that the VLDL receptor is also capable of internalizing lipoproteins in vivo via apoE2 and apoE3-Leiden when expressed in the liver. This latter property is in line with the presence of an NPXY internalization sequence within the cytoplasmic domain of the VLDL receptor. However, internalization of bound ligand by the VLDL receptor could be a function of ectopic liver expression.

**Figure 1.** Distribution of serum cholesterol and triglycerides among lipoprotein fractions. Lipoprotein fractions were separated by FPLC permeation chromatography using two 25-mL Superose 6B columns in series, and fractions were analyzed for cholesterol (solid line) and triglycerides (dashed line). Lipoprotein profiles are shown of: Apoe-/- mice before injection (A) and 5 days after Ad-VLDLR injection (B); Ldlr-/- mice before injection (C) and 5 days after Ad-VLDLR injection (D); apoE2 Apoe-/- mice 5 days after Ad-LacZ injection (E) and 5 days after Ad-VLDLR injection (F); and apoE3-Leiden Apoe-/- Ldlr-/- mice 5 days after Ad-LacZ injection (G) and 5 days after Ad-VLDLR injection (H). Each run is performed with a fasted serum pool of 3 to 8 mice. The horizontal lines in D indicate the fractions in which the various lipoproteins (VLDL, IDL, LDL and HDL) are present.

**Figure 2.** Competition for binding of 125I-labeled rabbit β-VLDL to ldlA-7 cells expressing the human VLDL receptor by VLDL derived from Apoe-/-, apoE2 Apoe-/-, and apoE3-Leiden Apoe-/- Ldlr-/- mice. Competition studies were performed by incubating VLDL receptor–expressing ldlA-7 cells with 10 μg/mL of 125I-labeled β-VLDL derived from cholesterol-fed rabbits for 3 hours at 4°C and the indicated amounts of β-VLDL (+) and VLDL derived from Apoe-/- (open circles), apoE2 Apoe-/- (filled triangles), and apoE3-Leiden Apoe-/- Ldlr-/- (open squares) mice. The binding is expressed as a percentage of the value in the absence of a competitor as described in the methods section. Values represent the mean±SD of 4 measurements.
If the internalization of lipoproteins via the VLDL receptor does occur in vivo, the VLDL receptor provides a possible mechanism for the formation of foam cells from smooth muscle cells and macrophages, one of the hallmarks of atherosclerosis. In addition to a direct role in the formation of foam cells, the VLDL receptor expressed in the endothelium could also play an indirect role in the transfer of lipoproteins or its constituents to the (pre)atherosclerotic lesion, by functioning as a docking protein.

Patients with FD due to mutations in apoE have increased plasma levels of chylomicron and VLDL remnant lipoproteins and are prone to develop premature atherosclerosis. By demonstrating that the apoE variants apoE2 and apoE3-Leiden do bind to the VLDL receptor, we have substantiated the possibility that the VLDL receptor also plays a role in atherogenesis in FD patients. We are currently in the process of directly testing this hypothesis by generating mice overexpressing the VLDL receptor in tissues involved in atherosclerosis and crossing breeding them with apoE2 and apoE3-Leiden mice.

We have previously reported in vitro analyses of the binding of apoE2 and apoE3-Leiden to the LDL receptor and the LRP.14 Binding of apoE2 to the LDL receptor is severely disturbed, whereas binding of apoE3-Leiden to the LDL receptor is somewhat less affected. The binding specificity of the LRP for apoE2 and apoE3-Leiden is similar to the binding specificity of the VLDL receptor for these apoE variants.14 This finding has been determined in vivo by adenovirus-mediated gene transfer of the RAP, which is a potent inhibitor of ligand-LRP interaction. Administration of Ad-RAP to apoE2 and apoE3-Leiden transgenic mice resulted in a dramatic increase of plasma cholesterol levels in both transgenic mouse lines, indicating that the LRP plays a role as a backup receptor in the clearance of both apoE2 and apoE3-Leiden.

The apparent efficient binding of apoE2 and apoE3-Leiden to the VLDL receptor thus provides novel opportunities for the development of gene therapy strategies for the treatment of FD. Because increased expression of the LDL receptor may not be effective for some of the FD patients due to lack of binding to the apoE variant, the introduction of an alternative route for liver-mediated lipoprotein clearance via ectopic expression of the VLDL receptor seems a promising possibility. However, clinical application of gene therapy for hyperlipidemia using adenovirus-mediated gene transfer is currently hampered by the relatively short term of gene expression, mainly caused by the immune clearance of adenovirus-transfected cells. A primary target for the immune reactions consists of the late adenoviral genes that are expressed at low levels in first-generation vectors.26 In this respect, the recent demonstration that second-generation adenoviral vectors containing additional inactivating mutations give prolonged rescue in a hyperlipidemic mouse model looks particularly promising.27 The VLDL receptor has also been applied in the murine model for familial hypercholesterolemia, the Ldlr−−deficient mouse.16,24 In this model, the VLDL receptor as an alternative clearance route was used to avoid immune problems associated with the expression of the LDL receptor, which may be recognized as a foreign protein in these mice. In conclusion, our data extend the possibilities of the VLDL receptor to function as an alternative lipoprotein clearance receptor for gene therapy for hyperlipidemias.

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