Reversal of Hypercholesterolemia in Low Density Lipoprotein Receptor Knockout Mice by Adenovirus-mediated Gene Transfer of the Very Low Density Lipoprotein Receptor*

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We have used the technique of adenovirus-mediated gene transfer to study the in vivo function of the very low density lipoprotein receptor (VLDLR) in low density lipoprotein receptor (LDLR) knockout mice. We generated a replication-defective adenovirus (AdmVLDLR) containing mouse VLDLR cDNA driven by a cytomegalovirus promoter. Transduction of cultured Hepa cells by the virus led to high-level expression of immunoreactive VLDLR proteins with molecular sizes of 143 kDa and 161 kDa. Digestion of the cell extract with the enzymes neuraminidase, G- and O-glycanase resulted in the stepwise lowering of the apparent size of the 161-kDa species to 135-kDa species. LDLR mice fed a 0.2% cholesterol diet were treated with a single intravenous injection of 3 × 109 plaque-forming units of AdmVLDLR. Control LDLR mice received either phosphate-buffered saline or AdLacZ, a similar adenovirus containing the LacZ cDNA instead of mVLDLR cDNA. Comparison of the plasma lipids in the 3 groups of mice indicates that in the AdmVLDL animals, total cholesterol is reduced by ~50% at days 4 and 9 and returned toward control values on day 21. In these animals, there was also a ~30% reduction in plasma apolipoprotein (apo) E accompanied by a 90% fall in apoB-100 on day 4 of treatment. By FPLC analysis, the major reduction in plasma cholesterol in the AdmVLDLR animals was accounted for by a marked reduction in the intermediate density lipoprotein/low density lipoprotein (IDL/LDL) fraction. Plasma VLDL, IDL/LDL, and HDL were isolated from the three groups of animals by ultracentrifugal flotation. In the AdmVLDL animals, there was substantial loss (~65%) of protein and cholesterol mainly in the IDL/LDL fraction on days 4 and 9. Nondenaturing gradient gel electrophoresis indicates a preferential loss of the IDL peak although the LDL peak was also reduced. When 125I-IDL was administered intravenously into animals on day 4, the AdmVLDLR animals cleared the 125I-IDL at a rate 5-10 times higher than the AdLacZ animals.

We conclude that adenovirus-mediated transfer of the VLDLR gene induces high-level hepatic expression of the VLDLR and results in a reversal of the hypercholesterolemia in 0.2% cholesterol diet-fed LDLR (~−/−) mice. The VLDLR overexpression appears to greatly enhance the ability of these animals to clear IDL, resulting in a marked lowering of the plasma IDL/LDL. Further testing of the use of the VLDLR gene as a therapeutic gene for the treatment of hypercholesterolemia is warranted.

Lipoprotein metabolism and cholesterol homeostasis are mediated in part by receptor-mediated uptake of lipoproteins from the circulation via specific cell-surface receptors. The very low density lipoprotein receptor (VLDLR) is the most recently identified apolipoprotein (apo) E receptor that belongs to the low density lipoprotein receptor (LDLR) gene family. This gene family includes LDLR, α2-macroglobulin receptor/LDLR-related protein, and glycoprotein 330, which all share some common structural features including: 1) cysteine-rich repeats consisting of ~40 amino acid residues in the ligand binding domain or in complement-type domain; 2) epidermal growth factor precursor-type repeats; 3) modules of ~50 amino acid residues with a consensus tetrapeptide, YWTD; 4) a single transmembrane domain; and 5) a cytoplasmic domain containing an NPXY sequence, thought to be important for the clustering of the receptor into coated pits (1−4). Structurally, the VLDLR is most similar to the LDLR. A major difference appears to be that the VLDLR N-terminal domain contains eight, instead of seven, cysteine-rich repeats as in LDLR. The similarities in the structure of VLDLR and LDLR are reflected in their genomic organization (5). The VLDLR is highly expressed in heart, muscle, and adipose tissue that are active in fatty acid metabolism. Interestingly, the amount of VLDLR mRNA in the liver is very small (3, 6–10), although the liver is a primary tissue for clearance of circulating lipoproteins. The tissue-specific expression of the VLDLR is very similar to, but not identical with that of lipoprotein lipase. Lipoprotein lipase resides on the endothelial surface of capillaries and hydrolyzes triglycerides of circulating triglyceride-rich lipoproteins such as VLDL, intermediate density lipoprotein (IDL), and chylomicrons, thus playing a pivotal role in lipid metabolism in vivo.

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role in lipoprotein metabolism. Given the structural features and ligand specificity of VLDLR, and the tissue distribution of the VLDLR mRNA, it was hypothesized that the primary role of the VLDLR is the delivery of triglycerides in triglyceride-rich apoE-containing lipoproteins to extracellular tissues for energy source or storage (3, 11).

Apart from its postulated physiological role, the VLDLR has been implicated in the pathogenesis of atherosclerosis. Incubation of β-VLDL with LDLR-deficient CHO-IdaA7 cells transfected with rabbit VLDLR enabled these cells to accumulate cholesteryl ester resulting in foam cell formation (12). Howbeit, in human monocytic leukemia cell line THP-1 and rabbit cholesteryl ester containing lipoproteins have been implicated in the pathogenesis of atherosclerosis. Incubation of β-VLDL (5, 9, 12) with LDLR 4% (w/w) animals was still viable and productively infected by intratotal virosis. The homogenates were centrifuged at 5000 × g for 60 min and the resulting pellets were resuspended in the same buffer. The VLDLR mRNA has been maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and incubated as described above except at a density of 4 × 10⁵ cells/well.

Animals—Mice lacking LDLR (15) were maintained on a normal chow diet (Teklad 4% mouse chow 7001 from Harlan Teklad Premier Diets) that contained 4% (w/w) animal fat and <0.04% (w/w) cholesterol. Diets were switched to high cholesterol diets that contained 0.2% (w/w) cholesterol and 10% (w/w) coconut oil (15) 2 weeks before virus injection, and this diet was maintained throughout the experiments.

Recombinant adenovirus stock was diluted with phosphate-buffered saline (PBS) to the appropriate concentration, and 0.5 ml of diluted recombinant adenovirus was injected via tail vein. Following adenoviral transduction, animals were fasted for 6 h before blood was collected in a tube containing EDTA by puncturing the retro-orbital plexus. Plasma was stored at 4°C prior to lipid analysis or lipoprotein fractionation. At the times indicated, animals were anesthetized and killed by cervical dislocation. Prior to turnover studies, samples were frozen on dry ice. A retro-orbital vein was left open by a skin incision and the indicated ¹²⁵I-lipoproteins were slowly injected in a total volume of 200 μl. The wound was closed by stapling. Blood (50–100 μl) was obtained at the indicated times by puncture of the retro-orbital plexus from the anesthetized animals and collected into EDTA-treated Pasteur pipettes. Turnover studies were performed as described previously (10). The homogenates of VLDL, IDL, and LDL, plasma collected was diluted to 0.25 ml with saline containing human LDL (0.5 mg/ml) as carrier, and 0.25 ml of isopropyl alcohol was added to precipitate apoB. For turnover studies of HDL, the plasma content of triglyceric acid–precipitable ¹⁴C radioactivity was measured (20).

Expression of VLDL Receptor in Cell Lines—Chinese hamster ovary CHO-IdaA7 cells lacking LDLR (19) in 6-well culture dish were infected with the indicated amount of recombinant virus at density of 1 × 10⁶ cells/well in 1 ml of infection media (modified Eagle’s medium containing 2% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin) for 90 min. After removing the media, the cells were added 1 ml of Ham’s F-12 medium containing 5% fetal bovine serum and the inoculation was continued in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 0.5 ml of diluted recombinant adenovirus was injected via tail vein. Following adenoviral transduction, animals were fasted for 6 h before blood was collected in a tube containing EDTA by puncturing the retro-orbital plexus. Plasma was stored at 4°C prior to lipid analysis or lipoprotein fractionation. At the times indicated, animals were anesthetized and killed by cervical dislocation. Prior to turnover studies, samples were frozen on dry ice. A retro-orbital vein was left open by a skin incision and the indicated ¹²⁵I-lipoproteins were slowly injected in a total volume of 200 μl. The wound was closed by stapling. Blood (50–100 μl) was obtained at the indicated times by puncture of the retro-orbital plexus from the anesthetized animals and collected into EDTA-treated Pasteur pipettes. Turnover studies were performed as described previously (10). The homogenates of VLDL, IDL, and LDL, plasma collected was diluted to 0.25 ml with saline containing human LDL (0.5 mg/ml) as carrier, and 0.25 ml of isopropyl alcohol was added to precipitate apoB. For turnover studies of HDL, the plasma content of triglyceric acid–precipitable ¹⁴C radioactivity was measured (20).

Lipoprotein and Lipid Analysis—Plasma (0.2 ml) collected from individual mice was subjected to fast performance liquid chromatography (FPLC) gel filtration on two Superose 6 columns (Pharmacia Biotech Inc.) connected in series as described (21) and fifty 0.5 ml fractions were collected. The cholesterol and triglyceride content of each fraction and of plasma were determined enzymatically by using kits from Sigma. Plasma from PBS-treated, AdLacZ-treated, and AdmVLDLR-treated mice at the indicated days after toxin was obtained in 10 μl of phosphate buffer and centrifuged at density d = 1.006 (VLDL), 1.063 (IDL/LDL), and 1.21 g/ml (HDL). Lipoprotein fractions collected were dialyzed and used for denaturing gradient gel and SDS-polyacrylamide gel analyses.

Lipoproteins and Iodination—Blood was collected into EDTA by retro-orbital puncture from 80 LDLR (-/-) mice fed the high-cholesterol diet for 2 weeks. To separate VLDL, IDL, LDL, and HDL, plasma was fractionated by sequential ultracentrifugation at density d = 1.006 (VLDL), 1.060–1.019 (IDL), 1.019–1.063 (LDL), and 1.21 g/ml (HDL) as described (22). The floated lipoproteins were dialyzed against 150 μl NaCl, 1 ml EDTA, 1 ml NaOH, 10 μM phenylmethylsulfonyl fluoride (pH 7.4). Isolated lipoproteins were iodinated by the method of Bilheimer et al. (23).

Immunoblot Analysis—Crude cell membranes were prepared from cultured cells or tissues as described by Simonsen et al. (24). In brief, cells scraped off from 6-well plates or tissues were homogenized in 10 ml HEPES, 2.5 mM NaH₂PO₄, pH 7.4, 250 mM sucrose, 5 mM EDTA, and 0.5 M phenylmethylsulfonyl fluoride using 1 ml of buffer per well and about 1 g of tissue. Plasma was centrifuged at 500 × g for 5 min, 8,000 × g for 15 min, and then 100,000 × g for 60 min. The resulting pellets were resuspended in the same buffer and centrifuged at 100,000 × g for 60 min. Crude cell membranes were solubilized by homogenizing pellets in 20 μl HEPES, 2.5 mM NaH₂PO₄, pH 7.4, 124 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 0.25% Nonidet P-40, and 0.05% CHAPS. Total cell extracts were prepared by homogenizing PBS-washed cells in solubilization buffer described above. Crude membrane protein was separated on a 7.5% SDS-polyacrylamide gel (Bio-Rad) and transferred electrophoretically to an Optitran nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with PBS containing 0.1% Tween 20 and 10% nonfat dry milk for 15 min at room temperature and then incubated with rabbit anti-mouse LDLR C-terminus peptide antibody. The C-terminal peptide antibody was generated by immunizing rabbits with keyhole limpet hemocyanin-conjugated synthetic peptide.
VLDLR Gene Therapy in LDLR Knockout Mice

CTYPASSVSTDDDLA, which corresponds to a conserved intracytoplasmic C-terminal domain of mouse, rabbit, and human VLDLR plus a Cys residue at the N terminus for conjugation. The VLDLR protein was detected by chemiluminescence using an ECL kit (Amer sham). Analysis of VLDLR Receptor Protein Glycosylation—Crude cell membranes were prepared as described above, and membrane proteins were solubilized in 15 mM sodium phosphate, pH 7.2, 0.1% (w/v) SDS, 1% Triton X-100 buffer containing 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 20 μg/ml aprotinin. A total of 5 μg of crude membrane protein was treated with N-glycanase, O-glycanase (Genzyme), or neuraminidase (Boehringer Mannheim) (8). The samples were then analyzed by immunoblot as described.

Northern Blot Analysis—Total cellular RNA was prepared by the guanidinium/phenol extraction method (25), using Ultraspec RNA Isolation System (Biotec Laboratories). 20 μg of RNA was electrophoresed on a 1% formaldehyde/agarose gel, and transferred to a Hybond N+ membrane, and hybridized to a 5′-1.6-kilobase mouse VLDLR cDNA probe described above. The same membrane was then hybridized to mouse glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Ambion).

Plasma Apolipoprotein Quantification—Apolipoproteins A-I and E were determined in EDTA-plasma samples by radial immunodiffusion essentially as described previously (27) employing monospecific rabbit antiserum raised against purified apolipoproteins. The samplediluent for the assay of apoE was altered to 10% normolipidemic human plasma (v/v), 1% Triton X-100 (v/v) in Sal EN buffer (150 mM NaCl, 1 mM EDTA (pH 7.4), 0.05% NaN3 (w/v)).

Apolipoprotein B-48 and B-100 were analyzed by quantitative scanning densitometry of Coomassie Blue stained polyacrylamide gels. ApoB-containing lipoproteins were isolated by density gradient ultracentrifugation (Beckman 42.2 Ti rotor, 40,000 rpm, 10°C, 8 h) after adjusting 25-μl plasma samples to a density of 1.063 g/ml with a KBr solution (d = 1.35 g/ml) in tubes containing a KBr overlay solution (d = 1.063 g/ml). The top 50-μl lipoprotein fractions were dialyzed against Sal EN and enzymatically assayed for cholesterol. Cholesterol recoveries were compared to VLDL + LDL cholesterol measurements of identical plasma samples treated with polyethylene glycol (28). For analysis, purified lipoproteins (2-10 μg of cholesterol) were solubilized in SDS sample buffer at 60°C for 30 min. ApoB-48 and -B-100 were resolved in a 2-20% linear polyacrylamide gel containing dilutions of purified mouse β-VLDL previously quantified for apoB-48 and apoB-100 protein content by densitometry to purified human LDL apoB-100. Scanned peak areas in the range of 0.125 to 2.00 μg were linear (r = 0.989, n = 12), and chromogenicities were similar for both proteins.

Lipoprotein Size and Distribution Analysis—Isolated lipoprotein fractions (VLDL, IDL/LDL, and HDL) were examined by non-denaturing polyacrylamide gradient gel electrophoresis to determine both the size and distribution of major components within each lipoprotein fraction (29). VLDL and IDL/LDL were electrophoresed on 2-16% gels and stained with Oil Red O and Coomassie G-250, respectively. HDL distribution and size as determined on 4-30% gels stained with Coomassie G-250. Scans were obtained with the RFT Spectrophotometer.

RESULTS

Efficiency of Expression of Recombinant Adenovirus Following Transduction in Vitro—Recombinant adenoviral vector AdmVLDLR was used to infect a mouse hepatoma cells, Hepa, and LDLR-deficient CHO-Ida7 cells. Twenty-four h after transduction with AdmVLDLR or AdlacZ cells were harvested and total cell extracts were prepared. VLDLR protein expression was determined by Western blot analysis using a monospecific antibody directed against a conserved C-terminal peptide in the cytoplasmic domain of VLDLR. In uninfected cells, the major endogenous VLDLR protein migrated as two bands of apparent molecular masses of ~161 kDa and ~143 kDa, respectively. The ratio of these two bands differed in the two cell lines: in Hepa, the 143-kDa band occurred almost exclusively, whereas in CHO-Ida7, the 161-kDa band was much more prominent. The VLDLR proteins with molecular masses of 161 kDa and 143 kDa were induced in a dose-dependent manner in both cell lines (Fig. 1, A and B). In addition to the 143-kDa and 161-kDa bands, there was a ~50.2-kDa band in uninduced and induced cells that was also detected by this antibody. It ac-
counted for <5% of the total immunoreactive signal and was not investigated further. To determine if the difference in the molecular sizes of the 143-kDa and 161-kDa VLDLR forms was due to post-translational glycosylation of the VLDLR, total cell extracts and crude membrane preparations were treated with neuraminidase to remove sialic acids and N- and O-glycanase to remove the N- and O-linked oligosaccharides. The apparent size of the 161-kDa protein was reduced in a stepwise fashion by treatment with these three enzymes (Fig. 1C), suggesting that the 161-kDa band represents fully glycosylated processed VLDLR, containing sialic acids, N- and O-linked oligosaccharides. In contrast, the protein with a molecular mass of 143 kDa was not affected by enzyme digestion. This pattern was not different in total cell extracts or crude membrane preparations, suggesting that the 143-kDa protein is present in membrane fraction and does not have fully processed oligosaccharides. Its size also approximates that of the triple glycosidase-treated 161-kDa VLDLR protein.

Treatment of LDLR (−/−) Mice by Intravenous Injection of AdmVLDLR—LDLR (−/−) mice fed with high-cholesterol diet for 2 weeks were injected with AdLacZ, AdmVLDLR (3 × 10⁹ pfu per animal), or PBS ("Mock" control) in a volume of 0.5 ml via tail veins. Tissue and blood samples were obtained at the indicated times. The amount of AdmVLDLR DNA in the transduced liver was detected by Southern blot analysis. The highest amount of AdmVLDLR DNA taken up by the liver was detected at day 4. It decreased thereafter but was readily detected on day 9 and became barely detectable at day 21 (Fig. 2A). The VLDLR mRNA expression in the liver paralleled the amount of AdmVLDLR DNA in this tissue (Fig. 2B). The highest mRNA level was detected at day 4; substantial levels were detected on day 9, and a low but significant mRNA level was found at day 21. On more prolonged exposure, the day 21 samples revealed a strong band, while the endogenous VLDLR mRNA in Mock or AdLacZ controls was still undetectable. This extremely low hepatic expression of VLDLR mRNA has been reported previously (10). The different levels of VLDLR mRNA expression were not the result of uneven loading because the glyceraldehyde phosphate dehydrogenase mRNA control showed only minor variation in expression.

To determine whether the transduced mVLDLR mRNA was efficiently translated into VLDLR protein, we performed immunoblot analysis of hepatic membrane preparations from mice treated with AdmVLDLR at days 4, 9, and 21 following virus administration. Control samples consist of preparations from day 4 Mock (PBS-injected) and AdLacZ-injected mice. Twenty μg of total membrane protein was separated on a 7.5% SDS-polyacrylamide gel and transferred to an Optitran membrane, and immunoreactive VLDLR protein was detected by chemiluminescence (see "Experimental Procedures"). As shown in Fig. 3, PBS-treated and AdLacZ-treated day 4 samples failed to show the immunoreactive VLDLR bands, whereas in the AdmVLDLR-treated animals, there was a very high level expression of the VLDLR bands in the day 4, and to a lesser extent, in the day 9 samples. Even on day 21, when the VLDLR bands had largely disappeared, they were still somewhat higher than the control samples. Some lower molecular weight bands were also detected in the day 4 samples which account for <1% of the total and were not investigated further.

Effect of AdmVLDLR Transduction in Vivo on Plasma Apolipoproteins—Plasma apolipoproteins were measured on days 4, 9, and 21 (Table I). A lowering of the major apolipoproteins...
was noted on all 3 days. Compared with AdLacZ controls, AdmVLDLR animals responded with a lowering of the plasma apoE by 35% on day 4, 17% on day 9, and 18% on day 21. The reduction in apoE was more even of magnitude; it amounted to a drop of 90% on day 4, 49% on day 9, but no significant change on day 21. A marked lowering of apoB-48 was noted on day 4 (74%) and day 9 (50%). Mild reductions of apoA-I of 21% and 24% were noted on days 4 and 9.

Effect of AdmVLDLR Transduction on Plasma Lipids—Plasma cholesterol and triglyceride were measured in mice treated with AdmVLDLR, AdLacZ, or PBS (Mock) (Table II). It is evident that on days 4 and 9, AdmVLDLR treatment produced a highly significant reduction in total plasma cholesterol which went down to 50% of control. At day 21, the AdmVLDLR-treated samples remained at 55%–76% of controls but were statistically not different from the latter because of a wide variability in response. The effect of AdmVLDLR treatment on plasma triglyceride was much less marked than the treated samples showing a 23–34% increase at day 4. Considerable spontaneous fluctuations in plasma triglyceride were noted when levels at days 4, 9, and 21 were compared.

Effect of AdmVLDLR Transduction on Plasma Lipoproteins Fractionated by FPLC—Ishibashi et al. (15) documented by FPLC fractionation that LDLR (−/−) mice develop hypercholesterolemia involving the IDL/LDL class upon feeding a 0.2% cholesterol diet. We analyzed the plasma lipoprotein profile by the same technique on day 4 of treatment when there was maximal hepatic expression of VLDLR in AdmVLDLR-transduced animals. As shown in Fig. 4, PBS-treated controls had a very prominent IDL/LDL peak and a relatively low HDL peak as reported by Ishibashi et al. (15). AdmVLDLR treatment caused a marked and specific reduction in the IDL/LDL peak without significant change in the VLDL and HDL peaks. The AdLacZ-treated controls did not show any significantly different lipoprotein profile compared with the PBS-treated (Mock) controls.

Effect of AdmVLDLR on Size and Distribution of Lipoproteins Prepared by Ultracentrifugal Flotation—The greatest impact of AdmVLDLR expression on size and distribution of lipoproteins appears to be on the IDL/LDL fraction as indicated in Fig. 5. Total staining mass of IDL/LDL from AdmVLDLR-treated mice is greatly reduced at 4 days compared with control and AdLacZ mice and remains somewhat reduced at day 9. At day 9 there is preferential loss of IDL (30–35 nm region) in mice expressing the VLDL receptor; this loss of IDL particles appears to persist through day 21. Expression of the VLDL receptor at days 4 and 9 is also associated with the appearance of smaller sized particles (16–19 nm) in the size interval between LDL and HDL.

In a separate experiment, IDL (d 1.006–1.019 g/ml) and LDL (d 1.019–1.063 g/ml) were isolated from LDLR (−/−) mice ultracentrifugally to establish the size boundaries for each. The non-denaturing gradient gel scan of each fraction (Fig. 6A) indicates that there is little or no overlap of particle size between these two fractions. LDL banded between 30 and 35 nm, whereas LDLR had a major component at approximately 28–29 nm and minor components between 25 and 27 nm. The apolipoprotein composition of the LDL and LDLR fractions is shown in Fig. 6B where it is apparent that apoB-100, apoB-48, and apoE are the major proteins of each; however, the gel also indicates that apoB-100 is more pronounced in the LDLR fraction than in the LDL fraction.

The major VLDL species in control mice is a particle which peaks at 38–39 nm (Fig. 7); the size and distribution were little altered in the AdLacZ-treated mice. AdmVLDLR-treated mice have a major peak in this region, but at day 4 and 9 this fraction also possesses a small quantity of smaller sized particles seen as a shoulder at 35 nm. At these time points, there is also an apparent decrease in total mass of VLDL in the AdmVLDLR-treated mice. At day 21, the VLDL profile of the AdmVLDLR-treated mice, with the appearance of an intermediate component at 37.7 nm, begins to show signs of reversing to that of AdLacZ-treated or control mice.

Unlike the less dense lipoproteins, HDL size distribution, as determined by non-denaturing gradient gel electrophoresis, is not altered by the expression of the VLDL receptor. The HDL

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**Table I**

Effect of VLDLR on plasma apolipoprotein levels

|          | Day 4   | Day 9   | Day 21  |
|----------|---------|---------|---------|
|          | B-48    | B-100   | A-1     |
| Mock     | 10 ± 4  | 15 ± 4  | 15 ± 4  |
| LacZ     | 15 ± 8  | 18 ± 8  | 13 ± 8  |
| AdmVLDLR | 9 ± 2   | 5 ± 2   | 7 ± 2   |

Values are in mg/dl ± S.D.

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**Table II**

Total cholesterol and triglyceride in Mock-, AdLacZ-, and AdmVLDLR-treated LDLR (−/−) mice

| Days    | Total cholesterol (mg/dl) | Total triglyceride (mg/dl) |
|---------|--------------------------|---------------------------|
|         | B-48                     | B-100                     |
| Mock    | 398 ± 93 (9)             | 444 ± 64 (6)              |
| LacZ    | 391 ± 127 (9)            | 401 ± 53 (6)              |
| AdmVLDLR| 204 ± 150±5 (5)          | 182 ± 58±4 (5)            |
|         | 440 ± 120 (4)            | 316 ± 90 (3)              |
|         | 316 ± 90 (3)             | 113 ± 33 (2)              |
|         | 242 ± 143 (3)            | 140 ± 135 (5)             |
|         | 140 ± 135 (5)            | 156 ± 16 (4)              |
|         | 140 ± 135 (5)            | 156 ± 16 (4)              |
|         | 140 ± 135 (5)            | 156 ± 16 (4)              |
|         | 140 ± 135 (5)            | 156 ± 16 (4)              |

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*a* p < 0.05 vs. Mock.

*b* p < 0.01 vs. Mock.

*c* p < 0.001 vs. Mock.

*d* p < 0.001 vs. LacZ.

*e* p < 0.05 vs. Mock.

*f* p < 0.01 vs. LacZ.
for control, AdLacZ, and AdmVLDLR mice are homogeneous in size and have peaks at 10.2, 10.3, and 10.0 nm, respectively (data not shown).

Composition of VLDL and IDL/LDL Fractions—The pooled fractions used to generate the nondenaturing gradient gel profiles were used to quantitate total protein, cholesterol, and triglyceride in each fraction. The summary of the data in Table III suggests that there is a substantial loss of both protein and cholesterol in the IDL/LDL fraction at 4 and 9 days of VLDLR expression; this is consistent with the distribution data seen in Fig. 5, suggesting a loss of mass in the IDL/LDL fraction. Triglyceride, unlike cholesterol, does not appear to be affected, indicating a selective removal of cholesterol-containing particles. The composition of VLDL from VLDLR expressing mice was more variable; however, compared with controls, there was a trend toward a decrease in protein and cholesterol at day 4 which was more pronounced at days 9 and 21. Unlike the IDL/LDL fraction, the VLDL showed a decrease in triglyceride at days 9 and 21; this decrease parallels the disappearance of VLDL noted in the nondenaturing gradient gel profiles (compare Fig. 7).

Effect of AdmVLDLR Transduction in Vivo on the Ability of LDLR (−/−) Mice to Clear 125I-labeled Lipoproteins—To examine the functional consequence of VLDLR gene delivery on lipoprotein catabolism in LDLR (−/−) mice, we compared the ability of AdmVLDLR- and AdLacZ-treated LDLR (−/−) mice to clear 125I-labeled lipoproteins from the circulation. We isolated the lipoprotein fractions, VLDL, IDL, LDL, and HDL by ultracentrifugal flotation of pooled plasma of LDLR (−/−) mice. After the lipoproteins were labeled with 125I, they were injected into LDLR (−/−) mice which had received a single dose of 3 × 10⁹ pfu of AdmVLDLR or AdLacZ 4 days previously. As shown in Fig. 8B (right panel), there was no difference in the clearance rate of HDL between the two groups of animals. For 125I-VLDL, the AdmVLDLR-treated animals showed some variability in response but generally the difference between AdmVLDLR- and AdLacZ-treated mice was minor (Fig. 8A, left panel). For 125I-IDL clearance, we note that there was some variability among the five AdmVLDLR-treated animals studied (Fig. 8B, left panel). Three of them showed a response identical with that of AdLacZ-treated animals. Two AdmVLDLR-treated animals cleared the 125I-IDL more efficiently than controls. We note that the one animal showing the most efficient 125I-IDL clearance expressed the highest amount of VLDLR by Western blot analysis (data not shown). The greatest difference in lipoprotein clearance between animals treated with AdmVLDLR and those treated with AdLacZ was observed for 125I-IDL (Fig. 8B, right panel).
animals. The AdLacZ-treated mice eliminated 50% of the $^{125}$I-IDL in 1.5–5 h whereas the AdmVLDLR-treated animals uniformly cleared the same amount in <30 min. At the end of the 8-h experiment, in AdLacZ animals, some 30–40% of the $^{125}$I-IDL remained in the circulation compared to only 2–5% in the AdmVLDLR animals.

DISCUSSION

The physiological role of the VLDLR in normal lipoprotein metabolism is unclear. Recently, Frykman et al. (30) showed that mice with targeted disruption of the VLDLR gene have normal plasma lipoproteins. Homozygous VLDLR (−/−) mice had normal lipids and lipoproteins when they were fed normal, high carbohydrate, or high fat diets. Therefore, if the VLDLR plays a significant role in lipoprotein metabolism, its role must be subtle and can be filled by adaptation in these VLDLR (−/−) animals.

To date, the ligand-binding function of the VLDLR has been tested only in vitro systems using transfected cells (3, 13, 31). Although non-lipoprotein ligands have been identified (24, 31, 32), most of the studies have concentrated on identifying possible lipoprotein ligands. These experiments demonstrate that the VLDLR recognizes apoE-containing lipoproteins. VLDL artificially enriched with apoE in vitro (13) as well as diet-induced apoE-rich β-VLDL (3) appear to serve as effective ligands for VLDLR-expressing cells in culture. In contrast, apoB-100-only LDL is poorly recognized by these cells (3, 11). The gene transfer experiments described in this study allow us to determine what lipoprotein ligands are recognized by the VLDLR in vivo.

Transfer of the mVLDLR gene to cultured mouse hepatoma cells and LDLR-deficient CHO-ldlA7 cells induced the expression of two species of immunoreactive VLDLR (Fig. 1). By selective glycosidase digestion, we showed that the high molecular mass (161 kDa) species represents a fully processed glycosylated and the low molecular mass (143 kDa) species, the unglycosylated mVLDLR. These results corroborate and extend the observations of Jokinen et al. (8) in rats.

We have chosen LDLR (−/−) mice as the experimental model for a number of reasons. The LDLR knockout mice are a useful model of familial hypercholesterolemia (15). When they are put on a 0.2% cholesterol diet, they develop significant hypercholesterolemia involving mainly the IDL/LDL fraction (15). In these animals, any effect of gene transfer on the plasma lipoproteins can be attributed to VLDLR expression because the animals do not express LDLR.

We used the technique of adenovirus-mediated gene transfer to deliver the VLDLR to the liver of LDLR (−/−) mice. In agreement with previous experiments from this laboratory (16) and other laboratories (14, 15), most of the transgene ends up in the liver. The mouse liver normally expresses very low amounts of VLDLR (7, 10). Within 4 days of in vivo gene transfer, Western blots (Fig. 3) indicate that VLDLR was expressed at a high level in the liver. This was accompanied by a lowering in the plasma apoE concentration, an observation consistent with apoE-enriched lipoproteins being the preferred ligand for the VLDLR. Interestingly, there was an even more pronounced drop in the plasma level of apoB-100. Since apoB-100-only lipoproteins are poorly recognized by the VLDLR, the clearance of apoB-100 must involve particles that contain both apoB-100 and apoE. The fact that the decrease in apoB-100 was substantially greater than that of apoE suggests that a significant portion of apoE was in a form (in lipoprotein particles or as the free soluble protein) not recognized by the VLDLR or, a high production rate of apoE relative to apoB-100 was able to partially compensate for its greatly enhanced clearance. In any case, the reduction in apoE and apoB-100 was accompanied by a ∼50% fall in plasma cholesterol. The plasma lipoprotein fraction most affected by the treatment was in the IDL/LDL class, which lost much of its protein and cholesterol content in response to treatment (Table III).

![Fig. 7. Profiles of VLDL fractions from control, AdLacZ, and AdmVLDLR mice at days 4, 9, and 21 obtained from non-denaturing gradient gel scans.](image)

The numbers over the peaks and shoulders indicate particle size in nanometers: an equal volume of each fraction was applied to the gel. It is apparent that in all cases the major VLDL particle is approximately 38–39 nm in diameter. In the AdmVLDLR-treated mice, there is a decrease in mass of the 39 nm component compared to controls and there is a concomitant appearance of a smaller sized particle at 35 nm. At day 21, it appears that the VLDL profile may be normalizing since a larger particle at 37.7 nm is also present.

### Table III

| Lipoprotein Fraction | Protein (mg/dl) | Cholesterol (mg/dl) | Triglyceride (mg/dl) |
|---------------------|----------------|---------------------|---------------------|
| **Day 4**           |                |                     |                     |
| IDL/LDL  Mock       | 1.24           | 2.24                | 0.136               |
| LacZ               | 1.49           | 2.26                | 0.217               |
| VLDLR              | 0.42           | 1.44                | 0.188               |
| VLDL  Mock          | 0.39           | 0.184               | 0.324               |
| LacZ               | 0.42           | 0.215               | 0.279               |
| VLDLR              | 0.33           | 0.137               | 0.444               |
| **Day 9**           |                |                     |                     |
| IDL/LDL  Mock       | 0.71           | 1.36                | 0.104               |
| LacZ               | 0.85           | 1.88                | 0.202               |
| VLDLR              | 0.28           | 0.616               | 0.124               |
| VLDL  Mock          | 0.42           | 0.207               | 0.304               |
| LacZ               | 0.34           | 0.169               | 0.323               |
| VLDLR              | 0.24           | 0.097               | 0.188               |
| **Day 21**          |                |                     |                     |
| IDL/LDL  Mock       | 1.07           | 2.17                | 0.111               |
| LacZ               | 1.04           | 1.87                | 0.261               |
| VLDLR              | 0.76           | 1.26                | 0.219               |
| VLDL  Mock          | 0.67           | 0.394               | 0.734               |
| LacZ               | 0.39           | 0.154               | 0.269               |
| VLDLR              | 0.32           | 0.107               | 0.284               |

Each sample represents plasma pooled from three animals and was prepared by ultracentrifugal flotation as described under “Experimental Procedures.”

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To determine what lipoprotein ligands are recognized by the VLDLR (−/−) animals, any effect of gene transfer on the plasma lipoproteins can be attributed to VLDLR expression because the animals do not express LDLR.

We used the technique of adenovirus-mediated gene transfer to deliver the VLDLR to the liver of LDLR (−/−) mice. In agreement with previous experiments from this laboratory (16) and other laboratories (14, 15), most of the transgene ends up in the liver. The mouse liver normally expresses very low amounts of VLDLR (7, 10). Within 4 days of in vivo gene transfer, Western blots (Fig. 3) indicate that VLDLR was expressed at a high level in the liver. This was accompanied by a lowering in the plasma apoE concentration, an observation consistent with apoE-enriched lipoproteins being the preferred ligand for the VLDLR. Interestingly, there was an even more pronounced drop in the plasma level of apoB-100. Since apoB-100-only lipoproteins are poorly recognized by the VLDLR, the clearance of apoB-100 must involve particles that contain both apoB-100 and apoE. The fact that the decrease in apoB-100 was substantially greater than that of apoE suggests that a significant portion of apoE was in a form (in lipoprotein particles or as the free soluble protein) not recognized by the VLDLR or, a high production rate of apoE relative to apoB-100 was able to partially compensate for its greatly enhanced clearance. In any case, the reduction in apoE and apoB-100 was accompanied by a ∼50% fall in plasma cholesterol. The plasma lipoprotein fraction most affected by the treatment was in the IDL/LDL class, which lost much of its protein and cholesterol content in response to treatment (Table III).

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### Table III

| Lipoprotein Fraction | Day 4 | Day 9 | Day 21 |
|---------------------|-------|-------|--------|
| IDL/LDL  Mock       |       |       |        |
| LacZ               |       |       |        |
| VLDLR              |       |       |        |
| VLDL  Mock          |       |       |        |
| LacZ               |       |       |        |
| VLDLR              |       |       |        |

Each sample represents plasma pooled from three animals and was prepared by ultracentrifugal flotation as described under “Experimental Procedures.”
The size distribution of the IDL/LDL on day 4 was very informative (Fig. 5). There was a selective lowering of IDL (30–35 nm range), which suggests that IDL is the preferred ligand for the VLDLR. This interpretation was supported by the 125I-lipoprotein disappearance curves (Fig. 8). In comparing AdmVLDLR- and AdLaclZ-treated animals, the greatest difference in clearance rate of the injected 125I-labeled lipoprotein was with IDL. The VLDLR animals cleared the 125I-IDL at least 5–10-fold faster than the LacZ animals. There was no difference between these animals in their ability to clear 125I-HDL and only minor differences in their ability to clear 125I-LDL. We believe that the slightly increased clearance of 125I-IDL in two of the five animals tested (Fig. 8B, left panel) was possibly caused by contamination of the LDL by IDL. The LDL preparation used for the study contained significant amounts of apoE which could not be removed by repeated ultracentrifugal flotation (Fig. 6). The animal that had the fastest clearance of the injected 125I-LDL also expressed the highest amount of LDLR protein. The VLDLR, in contrast, is nonimmunogenic, because VLDLR is normally expressed in various tissues of the LDLR (−/−) patient and is not recognized by the host immune system as a foreign protein. Additional experiments comparing the long-term expression of LDLR and VLDLR in LDLR (−/−) animals, and eventually in LDL (−/−) patients, will be needed to determine if this is a valid concern. In any case, VLDLR appears to be a good alternative to LDLR in the treatment of hypercholesterolemia. Further testing of its use in somatic gene therapy is warranted.

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REFERENCES
1. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., and Russell, D. W. (1984) Cell 39, 27–38
2. Herz, J., Hamann, U., Ronge, S., Myklebost, O., Gaussepol, H., and Stanley, K. (1988) EMBO J. 7, 4119–4127
3. Takahashi, S., Kawarabayashi, Y., Nakai, T., Sakai, J., and Yamamoto, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9252–9256
4. Saigo, A., Pietromonaco, S., Loo, A. K.-C., and Farquhar, M. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9725–9729
5. Sakai, J., Koshino, A., Takahashi, S., Miura, Y., Ishii, H., Suzuki, H., Kawarabayashi, Y., and Yamamoto, T. (1994) J. Biol. Chem. 269, 2173–2182
6. Gäfve, M. E., Caid, M., Britt, D., Jackson, C. L., Patterson, D., and Strauss, J. F., III (1993) Somat. Cell Genet. Mol. Biol. 19, 557–569
7. Gäfve, M. E., Paavola, L. G., Boyd, C. O., Nolau, P. M., Wittemma, F., Chawla, A., Lazar, M. A., Bucan, M., Angelin, B., and Strauss, J. F., III (1994) Endocrinology 135, 397–394
8. Jokinen, E. V., Landschulz, K. T., Pyne, K. L., Ho, Y. Y., Frykman, P. K., and Hobbs, H. H. (1994) J. Biol. Chem. 269, 26401–26418
9. Webb, J. C., Patel, D. D., Jones, M. D., Knight, B. L., and Soutar, A. K. (1994) Human Mol. Genet. 3, 531–537
10. Oka, K., Ishimura-Oka, K., Chu, M.-J., Sullivan, M., Krushkal, J., Li, W.-H., and Chan, L. (1994) Eur. J. Biochem. 224, 975–982
11. Yamamoto, T., Takahashi, S., Nakai, T., and Kawarabayashi, Y. (1993) Trends Cardiovasc. Med. 3, 144–148
12. Suzuki, J., Takahashi, S., Oida, K., Shimada, A., Kohno, M., Tanai, T., Miyabo, S., Yamamoto, T., and Nakai, T. (1995) Biochem. Biophys. Res. Commun. 206, 835–842
13. Takahashi, S., Suzuki, J., Kohno, M., Oida, K., Tanai, T., Miyabo, S.
14. Herz, J., and Gerard, R. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2812–2816
15. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E., and Herz, J. (1993) J. Clin. Invest. 92, 883–893
16. Teng, B., Blumenthal, S., Forst, T., Navaratnam, N., Scott, J., Gotto, A. M., Jr., and Chan, L. (1994) J. Biol. Chem. 269, 29395–29404
17. McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988) Virology 163, 614–617
18. Graham, F. L., Smiley, J., Russel, W. C., and Norin, R. (1977) J. Gen. Virol. 36, 59–72
19. Krieger, M., Brown, M. S., and Goldstein, J. L. (1981) J. Mol. Biol. 150, 167–184
20. Shimano, H., Yamada, N., Katsuki, M., Yamamoto, K., Gotoda, T., Harada, K., Shimada, M., and Yazaki, Y. (1992) J. Clin. Invest. 90, 2084–2091
21. Cole, T., Kitchens, R., Daugherty, A., and Schonfeld, G. (1988) FPLC BioCommun. 4, 4–6
22. Havel, R. J., Edel, H. A., and Bragdon, J. H. (1955) J. Clin. Invest. 34, 1345–1353
23. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) Biochim. Biophys. Acta 260, 212–221
24. Simonsen, A. C. W., Heegaard, C. W., Rasmussen, L. K., Ellgaard, L., Kjoller, L., Christensen, A., Etzerodt, M., and Andreassen, P. A. (1994) FEBS Lett. 354, 279–283
25. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
26. Kroczeć, R. A. (1989) Nucleic Acids Res. 17, 9407
27. Ishida, B. Y., and Paigen, B. (1992) J. Lipid Res. 33, 1073–1078
28. Dias, V. C., Parsons, H. G., Boyd, N. D., and Keane, P. (1988) Clin. Chem. 34, 2322–2327
29. Nichols, A. V., Krauss, R. M., and Musliner, T. A. (1986) Methods Enzymol. 128, 417–431
30. Frykman, P. K., Brown, M. S., Yamamoto, T., Goldstein, J. L., and Herz, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8453–8457
31. Heegaard, C. W., Simonsen, A. C. W., Oka, K., Kjoller, L., Christensen, A., Madsen, B., Ellgaard, L., Chan, L., and Andreassen, P. A. (1995) J. Biol. Chem. 270, 20855–20861
32. Battey, F. D., Gáveles, M. E., FitzGerald, D. J., Argraves, W. S., Chappell, D. A., Strauss, J. F., III and Strickland, D. K. (1994) J. Biol. Chem. 269, 23268–23273
33. Kozarsky, K. F., McKinley, D. R., Austin, L. L., Raper, S. E., Stratford-Perricaudet, L. R., and Wilson, J. M. (1994) J. Biol. Chem. 269, 13695–13702
34. Li, J., Fang, B., Eisenmicht, R. C., Li, X. H. C., Nasonkin, I., Lin-Lee, Y.-C., Mims, M. P., Hughes, A., Montgomery, C. D., Roberts, J. D., Parker, T. S., Levine, D. M., and Woo, S. L. C. (1995) J. Clin. Invest. 95, 768–773
Reversal of Hypercholesterolemia in Low Density Lipoprotein Receptor Knockout Mice by Adenovirus-mediated Gene Transfer of the Very Low Density Lipoprotein Receptor

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