Background—Familial hypercholesterolemia (FH) that results from LDL receptor (LDLR) deficiency affects ≈1 in 500 persons in the heterozygous state and ≈1 in 1 million persons in the homozygous state. We tested a novel gene therapy strategy for the treatment of FH in a mouse model.

Methods and Results—We delivered the VLDL receptor (VLDLR) to the liver of LDLR-deficient mice and compared the effect of a helper-dependent adenoviral vector with all viral coding sequences deleted (HD-Ad-mVLDLR) with a first-generation vector (FG-Ad-mVLDLR), an HD-Ad (HD-Ad-0) that contained no expression cassette, and dialysis buffer (DB). A single intravenous injection of HD-Ad-mVLDLR led to a lowering of plasma cholesterol that lasted 6 months. Acute liver toxicity (as measured with liver enzyme elevation) occurred after FG-Ad-mVLDLR but not after HD-Ad-mVLDLR, HD-Ad-0, or DB treatment. At 6 months, VLDLR was detected in the liver with Western blotting and with immunofluorescence staining only in HD-Ad-mVLDLR–treated mice. Aortic atherosclerosis was almost completely prevented in these animals.

Conclusions—HD-Ad–mediated intravenous delivery of VLDLR to hepatocytes is well tolerated. It produces long-term lowering of plasma cholesterol and prevents atherosclerosis development in LDLR-deficient mice. These data provide support for the feasibility and safety of this approach for therapy of human subjects. (Circulation. 2001;103:1274-1281.)

Key Words: genes ■ adenovirus ■ receptors ■ lipoproteins ■ hypercholesterolemia

Familial hypercholesterolemia (FH) is an autosomal dominant disorder that is caused by mutations in the LDL receptor (LDLR).1,2 With optimal pharmaceutical intervention, the plasma LDL cholesterol of the majority of patients with heterozygous FH can be maintained below 2.59 to 3.36 mmol/L (100 to 130 mg/dL). However, the majority of homozygous and a minority of heterozygous patients have proved to be resistant to treatment with conventional therapy. For these patients, the only other therapeutic option is LDL apheresis.3 However, there is rapid reaccumulation of the LDL, and the procedure must be repeated every 2 to 3 weeks to maintain the LDL cholesterol transiently below 2.59 to 3.36 mmol/L. Because of the difficulty in reversing the hypercholesterolemia of this subgroup of FH patients, somatic gene therapy has been examined as an alternative form of treatment.

The only clinical trial for somatic gene therapy of FH used a retroviral vector to deliver normal LDLR by transducing hepatocytes isolated from such patients and reimplanting them into the patients.4 Unfortunately, the therapeutic response in 5 patients treated by ex vivo gene transfer was only marginal, which “precludes a broader application of liver-directed gene therapy” via this approach.4

In vivo transfer of the LDLR gene with first-generation adenoviral vectors to LDLR-deficient mice5 and WHHL rabbits6-7 was highly effective in producing a transient lowering of the plasma cholesterol in these animals. Furthermore, the in vivo transfer of the VLDL receptor (VLDLR), a paralogue of the LDLR, to the liver of LDLR-deficient mice was also found to be effective in reversing the hypercholesterolemia of these animals.8,9
The VLDLR is a member of the LDLR gene family. It is normally expressed in heart, skeletal muscle, adipose, and other tissues but is essentially undetectable in liver. When expressed in cultured cells, the VLDLR binds to apoE-enriched lipoproteins but not to LDL, which contains only apoB-100. VLDLR gene therapy in LDLR-deficient mice produced a substantially more sustained hypocholesterolemic response than did LDLR. The superiority of the surrogate receptor (VLDLR) over the “natural” receptor (LDLR) was attributable to the fact that LDLR was recognized as a neoantigen by the LDLR-deficient animals, which mounted humoral and cellular immune responses to the LDLR, leading to the extinction of transgene expression, whereas ectopic expression of VLDLR did not induce an immune response. Therefore, VLDLR appears to be an excellent surrogate gene for the treatment of FH, especially in the context of complete LDLR deficiency.

Despite the much more protracted transgene expression, adenovirus-mediated VLDLR gene transfer resulted in metabolic correction that lasted only 3 to 9 weeks. This was not unexpected, because the vector that was used, a first-generation adenoviral vector (FG-Ad) vector, had been shown to induce inflammation and an immune response.

HD-Ad vectors devoid of all viral coding regions were developed in an attempt to reduce the immunogenicity of the vector and to prolong transgene expression. The recent development of helper-dependent systems that use a FG-Ad helper virus to provide the necessary viral proteins in trans for efficient and stable packaging. The resulting plasmid was linearized by Pmel digestion before transfection into 293Cre4 cells. Rescue and amplification of the HD vector were performed with 293Cre4 cells through coinfection with AdLC8cLuc1 helper-virus according to the method of Parks et al. The recombinant HD-Ad was purified twice through CsCl density ultracentrifugation and dialyzed against 10 mmol/L Tris, pH 7.4, 1 mmol/L MgCl2, 10% (v/v) glycerol (DB) at 4°C, and aliquots of purified vectors were stored at −85°C. FG-Ad-mVLDLR was prepared as described previously. The ratio of particle to infectious unit was ~40:1 with FG-Ad-mVLDLR.

**Animals**

Female LDLR-deficient mice (6 to 8 weeks of age) on a C57BL/6 background were fed a diet containing 0.2% (wt/wt) cholesterol and 10% (v/v) coconut oil, which maintained the plasma cholesterol level at 9.05 to 14.22 mmol/L. After 4 weeks of the diet, various vectors in dialysis buffer were injected into mice through the tail vein. On the days indicated, mice were anesthetized with Methofane (mefotame) and blood was collected into tubes containing EDTA from the retro-orbital plexus.

**Polymersase Chain Reaction Analysis of Vector DNA and Reverse Transcription–Polymerase Chain Reaction Analysis of Vector-Derived mVLDLR Transcripts in Various Mouse Tissues**

The presence of adenoviral ITR in various tissues was detected with polymerase chain reaction (PCR). Primers used were 5'-AGCGATATCGATATGAGGGCGTG-3' and 5'-TACGCTCCA-TGAGCTACAAACA-3'. The apo e gene was used as an internal control. PCR primers for apo e were exon 3 forward, 5'-GAACCGCTTCTGGGATTTACCAT-3', and exon 4 reverse, 5'-GGCCTCACAGGATGCACCTCAC-3'. To analyze for vector-specific VLDLR mRNA, we used vector-specific PCR primers to perform reverse transcription (RT)-PCR. Total RNA was isolated from mouse tissues with an RNeasy kit (QIAGEN Inc); residual DNA was removed with DNase I (Promega Corp) digestion. The forward primer was derived from the human apoA-I exon sequences 5' to the mVLDLR cDNA (5'-GAAGGAAGGTCCCCACCGGC-3'), and the reverse primer was complementary to mouse VLDLR cDNA (5'-TTCACACCTGACATCTCCAGAC-3'). The endogenous GAPDH mRNA was used as an internal PCR control with primers 5'-CCCCCTATTGACCTCAATCACTAGGC-3' and 5'-CCTGCTTACAACCTTCTTGAC-3'. The amplified products were fractionated on 1.5% agarose gel.
Liver tissue was frozen in tissue freezing medium (Triangle Biomedical Sciences) in liquid nitrogen. Cryosections (5 μm thick) were fixed in cold acetone at -20°C and used for immunofluorescence staining. The primary antibody was a rabbit anti-mouse C-terminal peptide purified over a peptide affinity column. The sections were incubated with a purified antibody at a concentration of 2 to 10 μg/mL for 1 hour at 37°C. The FITC-conjugated goat anti-rabbit immunoglobulin diluted to 1 to 5 μg/mL in saline was used for detection.

Other Procedures

Lipoprotein measurements, lipid and FPLC analyses, and rate of 125I-IDL disappearance in plasma were determined as previously described. Southern blotting and immunoblot analyses were performed as described previously with probes and restriction enzymes and antibodies specified in the figure legends. Liver sections were prepared for histological examination as previously described. Quantitative analyses of aortic atherosclerotic lesion areas were performed as previously described. Statistical analyses were made with ANOVA with SigmaStat (SPSS).

Results

Construction and Amplification of VLDLR and Control Vectors

Three types of adenoviral vectors were constructed: a first-generation VLDLR vector (FG-Ad-mVLDLR), a helper-dependent VLDLR vector (HD-Ad-mVLDLR), and an empty HD-Ad vector (HD-Ad-0). During the production of HD-Ad-mVLDLR and HD-Ad-0, possible DNA rearrangement was monitored throughout the process. As shown in Figure 2a, for HD-Ad-mVLDLR, we did not detect any rearrangement of the DNA by restriction mapping with the 3 different enzymes. Helper virus contamination was analyzed through Southern blotting with an ITR probe. The PstI digestion of the helper virus produces a banding pattern that is distinct from that of the HD-Ad-mVLDLR or HD-Ad-0. We did not detect any helper-virus bands in the HD-Ad-mVLDLR (Figure 2b) or HD-AD-0 preparations (data not shown). TaqMan PCR analysis indicated that helper virus contamination was <0.2%. We performed two independent experiments with different doses of HD-Ad-mVLDLR. We used a cytomegalovirus promoter–driven FG-Ad-mVLDLR as a FG-Ad control, because a PEPCK promoter–driven FG-Ad-mVLDLR was ineffective in reducing plasma cholesterol in LDLR-deficient mice (Oka et al, unpublished results).

Comparison of HD-Ad-mVLDLR, FG-Ad-mVLDLR, and DB Injection in LDLR-Deficient Mice

In the first experiment (low dose), an equivalent dose of HD-Ad-mVLDLR and FG-Ad-mVLDLR (1×1011 particles per mouse or 5×1012 particles/kg) or an equal volume of DB was injected intravenously via the tail vein into LDLR-deficient mice. Liver enzymes were monitored weekly after treatment. As shown in Figure 3 (left), marked elevations of plasma AST and ALT levels were observed with FG-Ad-mVLDLR, but enzyme levels remained unchanged after HD-Ad-mVLDLR or DB treatment. DB treatment was associated with a small dip in plasma cholesterol levels during the first month, which we believe was caused by the substantial blood loss that resulted from the substantial blood loss that resulted from...
the repeated phlebotomies in all 3 groups of animals (Figure 4, top). The cholesterol levels in DB-treated mice remained at 7.76 to 10.34 mmol/L (mean ± SEM, n = 10) thereafter. FG-Ad-mVLDLR treatment caused an early drop in plasma cholesterol levels that occurred within 1 week after vector injection. Levels gradually returned toward pretreatment values thereafter and did not differ from those of the DB-treated control animals after day 28. HD-Ad-mVLDLR injection led to a more gradual but sustained lowering of the plasma cholesterol level. The hypolipidemic effect was evident in 7 days, but cholesterol levels did not reach a nadir until 28 days after treatment. The cholesterol levels remained significantly lower than those in DB-treated animals at 161 days (6.28 ± 0.98 and 8.26 ± 1.37 mmol/L, respectively, for HD-Ad-mVLDLR- and DB-treated animals, P < 0.01). The significant difference was also evident at 196 days (P < 0.001, Figure 4, bottom).

Comparison of HD-Ad-mVLDLR and HD-AD-0 Treatment in LDLR-Deficient Mice
In the second experiment, we administered 3 × 10^{11} particles per mouse of HD-AD-0 or HD-Ad-mVLDLR. There was no change in plasma ALT or AST activities in either treatment group (Figure 3, right). The basal plasma cholesterol level (Figure 4, bottom) was the same in the 2 groups (mean ± SEM, 11.82 ± 1.63 and 11.72 ± 1.58 mmol/L, respectively, for the HD-AD-0 and HD-Ad-mVLDLR groups). For the HD-AD-0–treated mice, the level increased slightly before it showed a slight decline, leveling off at 8.53 ± 1.37 mmol/L (n = 12) at 161 days. In the HD-Ad-mVLDLR–treated mice, there was a sharp decline in plasma cholesterol from 11.72 ± 1.58 to 3.08 ± 0.52 mmol/L (n = 11) at 28 days. It then displayed a gradual upward trend; at 161 days, the plasma cholesterol level in HD-Ad-mVLDLR–treated mice was 5.74 ± 0.54 mmol/L, still significantly lower than that of HD-AD-0–treated control animals (P < 0.001).

At 140 days after treatment, plasma lipoproteins were fractionated with FPLC (Figure 5). In HD-AD-0 control animals, there is a minor VLDL peak, a prominent IDL/LDL peak, and a moderate HDL peak. In HD-Ad-mVLDLR–treated animals there was a decrease in all 3 peaks. The lowering was most marked in the IDL/LDL peak. Although considerable lowering of the HDL peak was also evident, there was a major reduction in the ratio of VLDL/IDL/LDL to HDL, suggesting that the HD-Ad-mVLDLR treatment led to a marked reduction in the atherogenic potential of the originally highly atherogenic plasma lipoproteins in these animals. Such a reduction in HDL cholesterol associated with
a decreased plasma apoAI level has been observed in mice treated with a first-generation virus. This may be secondary to the marked reduction of IDL, which results in insufficient transfer of the surface components for HDL assembly by triglyceride lipase-mediated remodeling of IDL particles.

We showed previously that induced hepatic VLDLR overexpression specifically targets the removal of IDL compared with other lipoproteins. At 63 days after HD-Ad-0 or HD-Ad-mVLDLR treatment, we measured the rate of disappearance of $^{125}$I-IDL (Figure 6). In the HD-Ad-0–treated mice, $^{125}$I-IDL disappeared from plasma with a half-life of $57\pm 2$ minutes (mean $\pm$ SD for 4 mice). In the HD-Ad-mVLDLR–treated mice, $^{125}$I-IDL disappeared with a half-life of $18\pm 1$ minutes. Therefore, HD-Ad-mVLDLR treatment accelerates IDL removal from the circulation by 300%. Because IDL is a precursor of LDL, both IDL and LDL are lowered as a result of the therapy.

Tissue Distribution of Vector and Transgene Expression

We tested the distribution of HD-Ad-mVLDLR vector DNA in the various mouse tissues at the end of the second experiment. Through PCR analysis with vector-specific primers and with the endogenous apoe locus used as a single-copy gene control, HD-Ad-mVLDLR DNA was detected mainly in the liver. It was also detected, albeit at much lower levels (substantially $<1$ copy per cell), in lung, adipose tissue, and spleen. It was also present, but barely detectable, in small intestine and muscle (data not shown). We performed RT-PCR on RNA isolated from these tissues using vector transcript-specific primers and found that of all of the tissues that harbored vector DNA, only the liver expressed vector-derived mVLDLR transcripts (Figure 7a).

VLDLR transgene expression was further examined in liver samples through Western blotting (Figure 7b). At the end of 196 days (experiment 1), VLDLR protein expression was undetectable in DB or FG-Ad-mVLDLR animals. It was clearly detected in the HD-Ad-mVLDLR–treated animals. In experiment 2, at the end of 168 days, VLDLR protein expression was not detected in HD-Ad-0 animals but was strongly positive in HD-Ad-mVLDLR animals. We further analyzed for VLDLR protein expression through immunofluorescence staining in liver sections from the second experiment (Figure 7c). We found that at 28 days after HD-Ad-mVLDLR treatment, $>95\%$ of hepatocytes expressed VLDLR protein, and at 168 days, 30% to 35% of the liver cells displayed immunoreactive VLDLR detectable with the monospecific polyclonal antibody. VLDLR was undetectable in the liver of HD-Ad-0–treated mice.

Liver Histopathology of HD-Ad-mVLDLR–Treated and Control Mice

Liver pathology was examined by a pathologist (M.J.F.) who was blinded to the treatment the mice received. Five groups of mice were examined in the 2 sets of experiments. All except the DB control animals displayed focal portal tract inflammation with minimal differences among the groups. None of the livers displayed architectural disturbances, and there was minimal hepatocellular necrosis, consisting of focal cell dropout in association with lymphocytic infiltrates. The latter were frequent among all groups (all mice receiving vectors, 63% of DB controls in experiment 1, and $\sim 55\%$ of mice in experiment 2), possibly reflecting the endemic presence of mouse pathogens in the mouse room used. Some livers in all groups of mice also displayed focal proliferation of bile ductules in the lobules, and in a few livers of each group, there was mild hepatocyte anisocytosis. The histolog-
ical alterations in some of the mice exposed to Ad vectors suggest the possibility that entry of the vector, whether FG-Ad or HD-Ad, may produce some injury to the liver that is still evident after several months. Finally, the average amount of hepatocellular steatosis in the groups was slightly less in the controls than in the VLDLR vector recipients, which may have been caused by efficient uptake of lipoprotein. However, this was not statistically significant.

**Induced Hepatic VLDLR Expression Prevents Aortic Atherosclerosis Development in LDLR-Deficient Mice**

We measured the extent of aortic atherosclerotic lesion involvement by quantitative morphometry. At 196 days (Figure 8, left), the DB-treated control group had the most extensive lesions, averaging 7.77 ± 1.50 mm² (mean ± SD, n=9). The FG-Ad-mVLDLR group, at 5.07 ± 2.29 mm² (n=7), displayed a mild (35%) but significant reduction in lesion area. The HD-Ad-mVLDLR group had an even smaller lesion area of 3.38 ± 1.10 mm² (n=9), a 57% protection. In the second experiment (Figure 8, right), at the end of 168 days, the aortas of the HD-Ad-0 group displayed an average lesion area of 5.41 ± 1.03 mm² (n=12). In the HD-Ad-mVLDLR group, atherosclerosis lesion development was almost completely prevented. The lesion area of 0.69 ± 0.29 mm² (n=11) in our experience is only slightly above that seen in wild-type mice and represented an 87% protection compared with the extensive lesions seen in HD-Ad-0 animals.

**Discussion**

Liver-directed LDLR gene transfer in vivo was found to be an effective means to reverse the hypercholesterolemia in 2 FH animal models: the WHHL rabbit and the LDLR-deficient mouse. However, the effect of in vivo LDLR gene transfer was transient because a FG-Ad was used in these studies. In addition, the LDLR induced de novo is recognized as a neoantigen in LDLR-deficient mice, and the resultant humoral and cellular immune response led to the extinction of transgene expression. The use of VLDLR instead of LDLR circumvents this problem. Another potential advantage of VLDLR over LDLR in specific clinical situations is that VLDLR binds to apoE and some dominant apoE mutants with higher affinity than does LDLR. It may be more effective than the latter in reversing the abnormal lipids in the dyslipidemias associated with some apoE mutants. Unlike humans, mice have substantial hepatic apoB mRNA editing activity, resulting in IDL that contains both apoB100 and apoB48. Hepatic expression of the VLDLR was found to be more effective in lowering apoB100 than in lowering apoB48; thus, VLDLR gene therapy may be more effective in humans who produce only apoB-100 in the liver. Our observations, and those in 2 previous reports, support the efficacy of the VLDLR as an effective surrogate lipoprotein receptor gene for the treatment of FH.

As a liver-directed gene-transfer vector, Ad offers some advantages as well as drawbacks. The major advantages include the high efficiency of transduction and the propensity of the vector administered intravenously to reach hepatocytes. The liver selectivity is not absolute, however, as evidenced by the presence of small amounts of vector DNA in tissues other than the liver. The PEPCK promoter used for the HD-Ad-mVLDLR construct confers an additional level of specificity. In HD-Ad-mVLDLR–treated mice, we could detect by RT-PCR vector-directed VLDLR mRNA exclusively in the liver (Figure 7a) and not in any other tissues tested. These data
indicate that the combination of liver-specific uptake and the promoter-mediated tissue specificity led to an essentially absolute liver specificity of HD-Ad transgene expression. All previously reported studies on FH animal models have used ubiquitously expressed promoters. We believe that use of a liver-specific promoter such as PEPCK would avoid some of the problems, such as enhanced immune stimulation, that are associated with promiscuous transgene expression.

The high efficiency of transduction is another desirable property of Ad vectors. We found that the use of HD-Ad-mVLDLR at a dose of $3 \times 10^{11}$ particles per mouse produced VLDLR transduction in $>$95% of the hepatocytes at 28 days. At the end of a 6-month period, $\approx$30% to 35% of the liver cells still expressed immunoreactive VLDLR. This contrasts with $\leq$5% hepatocyte transduction after the direct intraportal administration of an adenov-associated viral vector in mice.29

Many of the drawbacks of FG-Ad vectors have been circumvented by the generation of HD-Ad vectors.14 Despite the relatively prolonged expression of the VLDLR after a single injection of HD-Ad-mVLDLR, there was a gradual decline in both the level of expression (with decreasing proportion of liver cells expressing the VLDLR with time, from $>$95% at 28 days to 30% to 35% at 168 days) and the plasma lipid-lowering effect. A major reason for this finite period of transgene expression is the fact that the Ad chromosomal integration is extremely low.30 Reinjection of the same HD-Ad vector is generally ineffectual because of the induction of neutralizing antibodies to the Ad vector. However, this problem can be circumvented by the use of HD-Ad generated with a helper Ad virus of a different serotype, because neutralizing antibodies to Ad are serotype specific. In fact, Parks et al31 recently demonstrated the feasibility of such an approach. Theoretically, if each HD-Ad vector injection produces a therapeutic effect that lasts $\geq$6 months, reinjections of HD-Ad vectors of different serotypes should enable the same HD-Ad vector to be readministered multiple times (only the helper virus has to be changed; the therapeutic vector HD-Ad remains the same) and the maintenance of a therapeutic response that lasts for years. The use of different helper serotypes might also permit reinjection in patients who received subtherapeutic doses of vectors early in clinical trials to later receive higher doses of HD-Ad vectors.

In conclusion, we have shown that HD-Ad–mediated gene transfer of VLDLR to the liver of a mouse model of FH produces long-term correction of the hypercholesterolemia and prevention of aortic atherosclerosis. The use of HD-Ad in this study greatly attenuated the acute liver injury associated with FG-Ad.32 The results are encouraging, and further evaluation of this therapeutic strategy for the treatment of FH is warranted.

While this article was under review, Chen et al33 reported delivering the VLDLR to the liver of LDLR-deficient mice using an adeno-associated viral vector. They injected intra-portal via the spleen 1 $\times 10^{12}$ particles per mouse. The plasma cholesterol levels were as follows (mean $\pm$SEM): preinjection, 1404 $\pm$ 166 mg/dL; 3 months after injection, 1126 $\pm$ 486 mg/dL; and 6 months after injection, 864 $\pm$ 204 mg/dL. Immunocytochemical staining showed that 2% to 5% of the liver cells expressed VLDLR at 3 and 6 months. After 6 months, aortic atherosclerotic lesion area was reduced 33% compared with PBS controls.

**Acknowledgments**

This work was supported by grant HL-59314 from the National Institutes of Health. Dr Lee was supported by KOSEF (Korean Science and Engineering Foundation). Dr Merched was supported by ARCOL (French Committee for the Coordination of Research on Atherosclerosis and Cholesterol). We thank Merck & Co. for providing reagents developed by Dr Frank Graham of McMaster University, Hamilton, Canada; Dr Guojun Bu of Washington University, St Louis, Mo, for providing a monospecific rabbit anti-VLDLR antibody (used for Western blotting); Ekaterina Antonova for technical assistance; and Dr Lanny Haverkamp for assistance with capture of the immunohistochemical images.

**References**


Long-Term Stable Correction of Low-Density Lipoprotein Receptor–Deficient Mice With a Helper-Dependent Adenoviral Vector Expressing the Very Low-Density Lipoprotein Receptor
Kazuhiro Oka, Lucio Pastore, In-Hoo Kim, Aksam Merched, Shuichi Nomura, Hye-Jeong Lee, Maria Merched-Sauvage, Celeste Arden-Riley, Brendan Lee, Milton Finegold, Arthur Beaudet and Lawrence Chan

_Circulation_. 2001;103:1274-1281
doi: 10.1161/01.CIR.103.9.1274

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/103/9/1274

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