Adenoviral Delivery of Low-Density Lipoprotein Receptors to Hyperlipidemic Rabbits: Receptor Expression Modulates High-Density Lipoproteins

David R. Brown, Margaret E. Brousseau, Robert D. Shamburek, Glenda D. Talley, Susan Meyn, Stephen J. Demosky, Jr, Silvia Santamarina-Fojo, H. Bryan Brewer, Jr, and Jeffrey M. Hoeg

Plasma concentrations of low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) are inversely related in several dyslipoproteinemias. To elucidate the interactions between these lipoproteins, we used a recombinant adenovirus (hLDLR-rAdV) to express human LDL receptors (hLDLRs) in LDL receptor–deficient rabbits. hLDLR-rAdV administration resulted in hepatocyte expression and a reduction of total, intermediate-density lipoprotein (IDL), and LDL cholesterol. In addition, we found that hLDLR-rAdV treatment induced (1) increased very–low-density lipoprotein (VLDL) cholesterol, (2) increased VLDL, IDL and LDL triglycerides, (3) decreased α- and pre-β–migrating apolipoprotein E (apo E) and decreased pre-β–migrating apo A-I at 2 to 4 days posttreatment, and (4) increased total plasma apo A-I and pre-β–migrating apo A-I beginning 8 to 10 days posttreatment. Virtually all plasma apo A-I was present on α- and pre-β-HDL. Pre-β-HDL particles with size and electrophoretic properties consistent with nascent HDL demonstrated the greatest relative apo A-I enrichment following hLDLR-rAdV treatment. In summary, enhanced expression of hepatocyte LDLRs by hLDLR-rAdV treatment markedly altered apo A-I–containing lipoproteins and IDL and LDL. The use of recombinant viruses to express physiologically relevant genes in intact animals, analogous to transfection of cells in culture, provides a new strategy for the evaluation of effects of specific gene products on metabolic systems in vivo.

DISORDERS OF low-density lipoprotein (LDL) and high-density lipoprotein (HDL) metabolism underlie the pathogenesis of atherosclerotic vascular disease.[1-4] LDL and HDL metabolism are coupled in that various physiologic and pathologic conditions inversely affect plasma LDL and HDL levels. Since LDL and HDL correlations with cardiovascular endpoints are partially independent,[5-8] there exists the potential for additive or even synergistic effects on disease pathophysiology. Familial hypercholesterolemia (FH), a human disorder characterized by tendon xanthomas and premature atherosclerosis, results from a variety of mutations in the gene encoding the LDL receptor.[9] Patients homozygous for these mutations have LDL cholesterol levels elevated up to sixfold, as well as significant elevations in apolipoprotein B-100 (apo B-100), the protein component of LDL.[10] HDL cholesterol is markedly reduced and apo A-I, the principal structural protein of HDL, is also low.[11-13] In FH patients[14-18] and patients with polygenic dyslipidemias,[14,19,20] many therapeutic interventions that decrease LDL cholesterol are associated with HDL elevations.

This reciprocal relationship between LDL and HDL is reinforced by observations made in an animal model of FH, the Watanabe-heritable hyperlipidemic (WHHL) rabbit.[21-23] These rabbits carry a spontaneously arising mutation in the LDL receptor (LDLR) gene that encodes a 4–amino acid deletion in the cysteine-rich ligand-binding domain of the protein, severely disrupting receptor function.[24] Homozygotes suffer from spontaneous tendon xanthomas and atherosclerosis that exhibit remarkable pathological resemblance to human FH.[21] Most of the plasma cholesterol is present in intermediate-density lipoproteins (IDLs) and LDLs.[25] Plasma HDL and apo A-I levels are abnormally low.[26-28]

HDL is relevant to the pathogenesis of atherosclerosis in part because it plays a critical role in reverse cholesterol transport (reviewed by Hoeg and Remaley[29]). Human HDL can be separated into two major subfractions with different mobilities by agarose gel electrophoresis (α-HDL and pre-β-HDL).[30-33] It has been proposed that small pre-β-HDL particles (also referred to as nascent HDL), which are lipid-poor and contain apo A-I but no apo A-II, may initiate reverse cholesterol transport by accepting unesterified cellular cholesterol.[31,32,34-36] Larger pre-β-HDL particles may facilitate the esterification of free cholesterol and promote the flux of cholesteryl ester into the more lipid-rich α-HDL,[31,32] including a subpopulation that contains apo E[37-39] and lipoproteins containing apo B-100.[31] Thus, pre-β-HDL and α-HDL may be central to the mechanism by which cellular free cholesterol is removed, esterified, and ultimately transported back to the liver.

Recombinant adenoviruses have emerged as the most efficient vectors for transient delivery of functional genes to the mammalian liver (reviewed by Li et al[40]). The advent of these vectors provides a new set of tools for the study of metabolic relationships among the lipoproteins in vivo. Previous studies have demonstrated that following retrovirally[41-43] or adenovirally[44] mediated transfer of the hLDLR gene to rabbits, the hLDLR interacts efficiently with rabbit LDL. We report the use of a recombinant adenovirus encoding the hLDLR gene for the analysis of lipoproteins in hyperlipidemic rabbits homozygous for the WHHL LDLR allele. We find that adenoviral-mediated transfer of the hLDLR gene to the rabbit liver causes reductions in IDL and LDL (IDL/LDL) cholesterol and associated changes in the cholesterol, triglyceride, and apoprotein composition of lipoproteins including α- and pre-β-HDL. In particular, pre-β–apo A-I levels decreased at 2 to 4 days posttreatment and increased above baseline beginning 8 to

From the Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

Submitted June 23, 1995; accepted July 1, 1996.

Address reprint requests to Jeffrey M. Hoeg, MD, National Institutes of Health, National Heart, Lung, and Blood Institute, Bldg 10, Room 7N115, 10 Center Dr, MSC 1666, Bethesda, MD 20892-1666.

0026-0495/96/4512-0002$03.00/0

10 days posttreatment. These studies demonstrate the utility of viral-mediated gene delivery in the analysis of complex metabolic systems in intact animals, and have important implications for reverse cholesterol transport in the setting of gene therapy for atherosclerosis.

MATERIALS AND METHODS

Animals

An outbred strain of hyperlipidemic rabbits homozygous for the defective LDLR allele of the WHHL rabbit[24] were used in this study and have been previously described.[45] All animals were adult males that were approximately 3 kg in weight, fed high-fiber rabbit diet (no. 5325; Purina Mills, St Louis, MO), and fasted for 12 hours before blood sampling for lipid and lipoprotein determinations.

Recombinant Adenoviruses

The recombinant adenoviruses hLDLR-rAdV and Luc-rAdV, containing the hLDLR[46] or the firefly luciferase (Luc)[47,48] cDNA, respectively, were constructed as described by McGrory et al.[49] Briefly, shuttle vectors were derived from the plasmid pXCX2 (a gift from Dr Frank Graham, McMaster University, Hamilton, Ontario, Canada; based on pXC1[50]). Transcription was under the control of the human cytomegalovirus (CMV) immediate-early enhancer and promoter, the simian virus 40 (SV40) splice donor and splice acceptor signals, and the SV40 bidirectional polyadenylation signal. cDNA inserts containing code for either the hLDLR or Luc transgenes were inserted immediately 3′ of the splice acceptor signal. The shuttle vectors were mixed with the plasmid pJM17[49] and cotransfected by the method of Chen and Okayama[51] into 293 cells (CRL 1573; American Type Culture Collection, Rockville, MD[52]) in Richter's CM medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Following an overnight incubation at 37°C under 3% CO_2, transfected cells were overlaid with 1% agarose in supplemented minimal essential medium (GIBCO BRL, Gaithersburg, MD) and viral plaques were isolated as previously described.[53] Recombinant viruses that had undergone E1a/E1b deletion and incorporation of the hLDLR or Luc transgenes were identified using a conventional polymerase chain reaction (PCR) amplification assay that scored for unique hLDLR or Luc sequences covalently joined to adenoviral DNA. The PCR sense primers with 3′ homology to the hLDLR and Luc cDNA transgenes were 5′-CCCAGAACCCTTCCTGAG-3′ and 5′-CTGTATTCAGCGATGACG-3′, respectively. The PCR antisense primer with homology to the adenovirus sequence 3′ of the SV40 polyadenylation signal was 5′-GTCTCATCGTACCTCAGC-3′. This method of recombinant virus construction was efficient: 90% to 100% of primary plaques were positive in PCR assays. Positive isolates were tested for the ability to confer hLDLR or Luc activity to infected cells (assays described later). Concordance between PCR assays and gene-product activity assays was 100%. Selected viruses were subjected to three cycles of plaque purification; after each cycle, the PCR assay was confirmed positive for the desired transgene. Amplified stocks were tested in the PCR and gene-product activity assays and used as inocula for large-scale virus production. Large-scale virus cultures were grown in 293-cell monolayers on 15-cm culture dishes, harvested 48 to 60 hours following infection, and subjected to four cycles of freeze/thaw lysis. Crude lysates were extracted with Freon (halocarbon 113; Matheson Gas, Bridgeport, NJ), banded twice through cesium chloride density gradients as previously described,[54] adjusted to 1 mg/mL rabbit serum albumin, dialyzed against physiologic saline supplemented with 10 mmol/L HEPES, pH 7.3, and 0.1 mmol/L magnesium chloride, divided into unit doses, and flash-frozen in liquid nitrogen. Titers of thawed virus aliquots were determined by plaque assay as described previously.[53] Unit-dose aliquots were thawed rapidly in a 37°C water bath and immediately injected intravenously via the marginal ear vein.

Detection of Human mRNA

Comparison of cDNA sequences of the human[46] and WHHL rabbit[24] LDL receptors revealed sequence identity at human cDNA residues 2470 to 2487 and the absence of rabbit sequence at human residues 2626 to 2643. Both the upstream primer, 5′-CAGCATCAACTTTGACAA-3′, and the downstream primer, 5′-AGGTCTCAGGAAGGGTTC-3′, were synthesized on an Applied Biosystems 380 B DNA synthesizer (Foster City, CA). Expression of hLDLR mRNA in cells isolated from control and hLDLR-AdV–treated animals was assessed using mRNA that had been purified by the method of Chirgwin et al[55] followed by treatment with DNAse I.[56] After reverse transcription (RT) using the downstream primer with the TaKaRa RNA PCR Kit (Oncor, Gaithersburg, MD), the upstream primer was added to the reaction mixture. After the initial 94°C 2-minute cycle, 40 cycles of PCR were conducted using the following sequence: 94°C for 30 seconds, 58°C for 40 seconds, 72°C for 40 seconds, and a final 5°C soak cycle. A 20-μL aliquot of the PCR mixture was then subjected to agarose gel electrophoresis, and the resultant 174-bp PCR product was detected by UV light after exposure to ethidium bromide.

Lipid Analysis

Cholesterol, phospholipid, and triglyceride were assayed on a Cobas Mira Plus autoanalyzer (Roche Diagnostic Systems, Branchburg, NJ) using the following commercially available reagent kits: cholesterol, Sigma Diagnostics (St Louis, MO) procedure No. 352[57]; triglycerides, Sigma Diagnostics procedure No. 339[58]; and phospholipids, Wako Pure Chemical Industries (Osaka, Japan) Phospholipids B reagent kit. Plasma HDL cholesterol was determined using modifications of previously published procedures.[59,60] Fresh plasma and plasma diluted 1:1 (vol/vol) with physiologic saline were processed in parallel. Plasma samples (both diluted and undiluted) were mixed 10:1 (vol/vol) with 1% dextran sulfate (MW 50,000)/0.5-mol/L magnesium sulfate (HDL-Cholesterol Precipitant; Ciba Corning Diagnostics, Palo Alto, CA) and incubated for 15 minutes at room temperature (diluted) or 60 minutes on ice (undiluted). After precipitation of lipoproteins containing apo B, samples were centrifuged at 8,000 × *g* for 15 minutes at room temperature (diluted) or 16,000 × *g* for 30 minutes at 4°C (undiluted). Supernatants were separated and clarified by incubation overnight at 4°C to allow the formation and settling of additional precipitate. Cholesterol concentrations were determined the following morning in the clarified supernatants as described.

Fast Protein Liquid Chromatography

Plasma samples were fractionated by Superose 6 HR 10/30 gel filtration (Pharmacia, Uppsala, Sweden) as previously described.[61] Lipoprotein elution volumes were as follows: VLDL, 14.5 to 16.5 mL; IDL/LDL, 20.0 to 24.0 mL; HDL, 29.0 to 31.0 mL; and nascent (pre-β) HDL, 32.5 to 34.5 mL. Peak fractions were pooled and analyzed by lipoprotein agarose gel electrophoresis.

Lipoprotein Agarose Gel Electrophoresis

The Lipoprotein System (Ciba Corning Kit/8) was used as directed. Samples (4 μL) of plasma or pooled fast protein liquid chromatography (FPLC) fractions (concentrated 15-fold by ultrafiltration in Ultrafree-MC filter units, MW cutoff 10,000; Millipore,

Bedford, MA) were electrophoresed and stained for lipids (Fat Red 7B) or transferred to Immobilon-P (Millipore).

SDS–Polyacrylamide Gel Electrophoresis

FPLC column fractions were subjected to SDS–polyacrylamide gel electrophoresis through 4% to 20% gradient gels in 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS, pH 8.3, and transferred to Immobilon-P.

Immunostaining

Immobilon-P membranes were coupled to polyclonal goat anti-human apo E (Biodesign International, Kennebunkport, ME) or monoclonal mouse antirabbit apo A-I (clone no. 2C3; gift from Dr David Usher, University of Delaware, Newark, DE) and stained with horseradish peroxidase using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA).

Apo A-I Competitive Enyzme-Linked Immunosorbent Assay

Plastic microtiter plates were coated with purified rabbit apo A-I (gift from R. Ronan, Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) in 50 mmol/L sodium carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were blocked with 1% gelatin in fresh sodium carbonate-bicarbonate buffer for 1 hour and washed with phosphate-buffered saline ([PBS] pH 7.4, supplemented with 0.05% Tween 20; Sigma). Plasma samples or purified rabbit apo A-I standards were diluted in PBS/Tween 20, and 100-μL aliquots were transferred to the microtiter wells. Monoclonal mouse antirabbit apo A-I was added to each well. Plates were incubated overnight at 4°C and washed with PBS/Tween 20, and alkaline phosphatase–conjugated goat antimouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. Plates were incubated for 2 hours at room temperature and washed with PBS/Tween 20, and 0.2 mL *p*-nitrophenylphosphate (1 mg/mL in 1.0 mol/L diethanolamine; Kirkegaard and Perry Laboratories) was added to each well. Plates were incubated at room temperature, and optical density at 405 nm was monitored. Final readings were taken when the optical density of blank wells was approximately 2.0.

In Vitro LDL Uptake Assay

Human LDL was labeled with DiI (3,3′-dioctadecylindocarbocyanine; Molecular Probes, Eugene, OR) as previously described.[62] Human fibroblasts were obtained from a skin biopsy of a patient with FH under a protocol approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute. Cells were expanded in Eagle's minimal essential medium (Biofluids, Rockville, MD; supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine), frozen, and stored under liquid nitrogen until use. Fibroblast monolayers were grown to 50% confluence, infected with recombinant adenovirus, washed and fed with fresh media, and incubated for 2 days. Cells were then washed, incubated for 3 hours with DiI-LDL, processed, and photographed as previously described.[62]

Luciferase Assay

Luciferase enzyme activities were measured in 293-cell culture media 48 hours following Luc-rAdV infection, or in extracts from rabbit cells, organs, and tissues harvested 40 hours after intravenous injection of Luc-rAdV. Rabbits received virus as 5×10^9 plaque-forming units (PFU)/kg via the marginal ear vein, and tissue necropsies (aorta, duodenum, jejunum, ileum, large intestine, skeletal muscle, skin, and fat) and whole organs (cerebrum, cerebellum, thymus, lung, heart, adrenals, kidneys, spleen, liver, gallbladder, stomach, appendix, cecum, urinary bladder, and testes) were harvested, weighed, and frozen in liquid nitrogen. Frozen samples were ground to a fine powder under liquid nitrogen, combined 1:1 (wt/vol) with ice-cold extraction buffer (100 mmol/L potassium phosphate, pH 7.4, 1 mmol/L dithiothreitol, 2 mmol/L EDTA, and 1% Triton X-100), thawed, and sonicated. Lysates were centrifuged at 16,000 × *g* for 1 hour at 4°C. Supernatants from rabbit samples and infected 293-cell culture media were assayed for Luc activity using the Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Total activities recovered from individual whole organs were measured directly. Total activities in tissues were estimated from the activities recovered from necropsies, the necropsy wet weights, and the estimated total tissue wet weights. Total activities recovered from all organs and tissues tested were summed, and the percent contribution from each organ or tissue was calculated.

Hepatocyte/Nonhepatocyte Fractionation

Forty hours following infusion of Luc-rAdV (5 to 7×10^9 PFU/kg) via the marginal ear vein, hepatocytes and nonhepatocytes were isolated from liver lobes as previously described.[63,64] Briefly, liver lobes were dissected from the animals, perfused with collagenase, dispersed in Eagle's minimal essential medium, and filtered through gauze. Cells were subjected to differential centrifugation as previously described[64]; cells that pelleted at 50 × *g* after 3 minutes were primarily hepatocytes, and cells that remained in the supernatant were primarily nonhepatocytes. Fractions were washed four times, inspected by phase-contrast microscopy, and judged to be approximately 90% pure. Cell pellets were resuspended in extraction buffer and lysed by sonication, and soluble supernatants were assayed for Luc activity as described.

Statistical Analysis

Data were analyzed with Student's nonpaired two-tailed *t* test and expressed as the mean ± SEM.

RESULTS

The genomes of recombinant adenoviruses contain cDNA inserts coding for either the hLDLR (hLDLR-rAdV) or firefly Luc (Luc-rAdV). The two viruses are replication-defective and isogeneic except for the cDNA code inserted between the splicing signals and the termination/polyadenylation signal. Viruses were administered to LDLR-deficient rabbits by peripheral intravenous infusion via the marginal ear vein at doses of 5 to 7×10^9 PFU/kg. At this dose, no toxicity was evident and there were no significant alterations in liver function tests (data not shown).

To determine the locations of adenoviral gene expression in vivo, both the hLDLR-AdV and Luc-rAdV were used. The Luc-rAdV was used to test the activity of adenovirus as a gene-delivery vector after infusion into the peripheral circulation of the rabbit, and to assess the organ distribution of gene expression. Multiple organs and tissues were surveyed for Luc activity 40 hours following virus administration; the percent contribution from each organ or tissue to the total recovered activity was determined. Of all organs and tissues tested, significant Luc activities were found only in the liver (84%), spleen (9%), and testes (3% to 4% each). The sum of Luc activities from all other tested organs and tissues combined was less than 1% of the total. Low but detectable activities were recovered from the lung, adrenals, thymus, and intestinal tract. Activities were undetect-

able from the cerebrum, cerebellum, heart, aorta, kidneys, gallbladder, stomach, urinary bladder, fat, skin, and skeletal muscle. To further characterize the hepatic expression, hepatocyte and nonhepatocyte fractions were isolated from collagenase-perfused liver lobes of rabbits 40 hours after either Luc-rAdV or hLDLR-rAdV treatment. Hepatocyte and nonhepatocyte fractions contained 79% and 21%, respectively, of the total recovered hepatic Luc activity. Similarly, RT-PCR analysis of mRNA isolated from hepatocytes and nonhepatocytes indicated that most of the hLDLR mRNA was present in the hepatocytes (Fig 1). Some detectable message was also in the fraction containing Kupffer cells, but the hepatocyte fractional activity represented a minimum estimate, since cross-contamination of the nonhepatocyte fraction with visible hepatocytes was approximately 10%. Nevertheless, the fractionation procedure demonstrated the remarkable tropism of recombinant adenoviral vectors for hepatocytes, an observation consistent with previous studies in mice,[65-67] rats,[68,69] and rabbits.[44]

The ability of hLDLR-rAdV to confer the expression of functional LDLRs was tested in vitro by evaluating the uptake of fluorescent (DiI-labeled) LDL by LDLR–negative fibroblasts (primary skin fibroblasts from a patient homozygous for FH). Before infection, no uptake of fluorescent LDL by the fibroblasts was detected. Two days following infection, multiple fluorescent endocytic vesicles characteristic of LDLR function[62] were detected in every cell of the culture. No viral cytotoxicity was detected (data not shown).

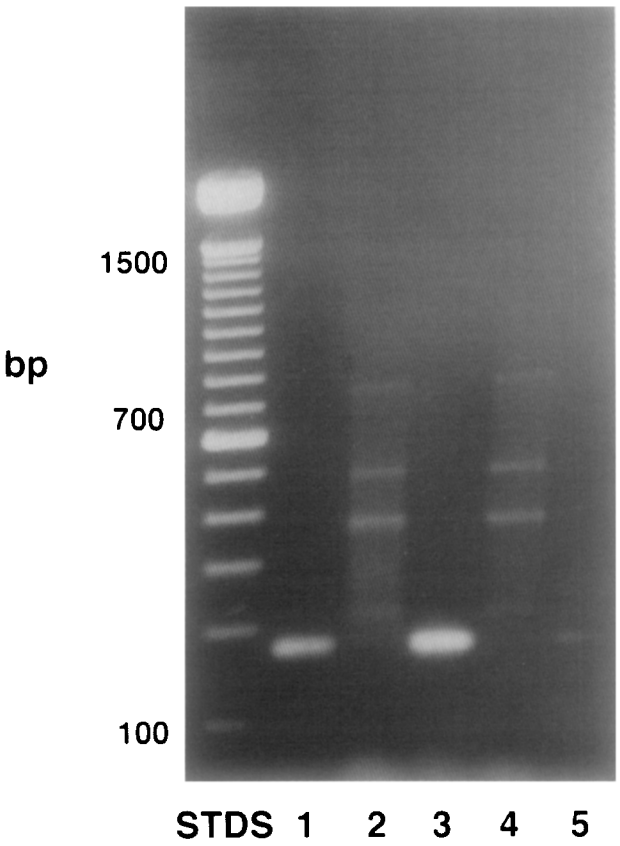

Fig 1. Expression of human LDLR in hepatocytes and Kupffer cells isolated from control and hLDLR-rAdV–treated WHHL rabbits. Lane STDS, DNA size standards. The predicted 174-bp product was revealed from RT-PCR of human hepatic mRNA (lane 1) and mRNA isolated from hepatocytes from a hLDLR-rAdV–treated WHHL rabbit (lane 3) but not mRNA from hepatocytes isolated from a control WHHL rabbit (lane 2). Although no hLDLR mRNA was detected in mRNA in control Kupffer cells (lane 4), a trace of hRT-PCR product was detected in Kupffer cells isolated after hLDLR-rAdV treatment.

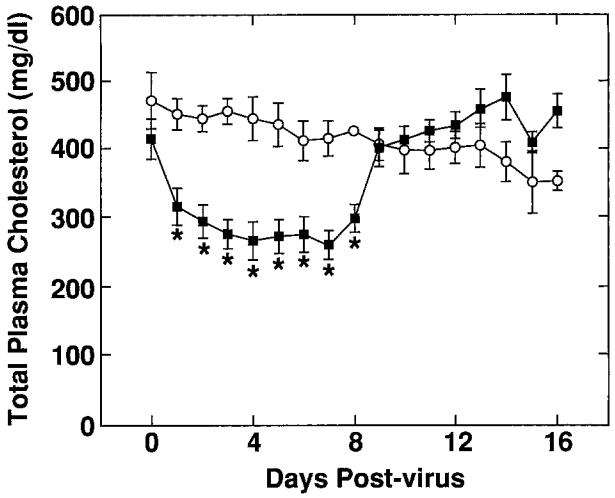

Fig 2. Total plasma cholesterol response to rAdV treatment. Hyperlipidemic adult male rabbits were treated at doses of 5 to 7 × 10^9 PFU/kg with either Luc-rAdV (○, n = 3) or hLDLR-rAdV (■, n = 6) on day 0. Plasma total cholesterol levels were measured daily for 16 days. Data are the mean ± SEM (*P < .01).

Six hyperlipidemic rabbits homozygous for the defective WHHL LDLR allele[21,22,24] were used to evaluate the effects of hLDLR-rAdV–directed LDLR expression in vivo. Following virus infusion via the marginal ear vein, total plasma cholesterol levels were determined daily for 16 days (Fig 2). Statistically significant reductions in total plasma cholesterol of up to 56% were transiently observed, with levels returning to baseline at 9 to 11 days posttreatment. Since IDL and LDL cholesterol comprised the bulk of total plasma cholesterol in these animals, this observation was consistent with robust hLDLR function following adenoviral-mediated gene transfer. A separate group of control animals received an equivalent dose of Luc-rAdV. No statistically significant effects on plasma total cholesterol concentrations were observed following Luc-rAdV treatment. The slight and insignificant reduction of cholesterol in control animals over the course of the experiment reflected the effects of daily blood sampling.

HDL cholesterol levels in hLDLR-rAdV–treated rabbits were measured with an indirect method based on the differential precipitation of HDL and non-HDL cholesterol. The method, used widely with human samples in clinical practice, involved the precipitation of apo B–containing lipoproteins (VLDL, IDL, and LDL) with dextran sulfate and magnesium sulfate, followed by determination of residual soluble lipoprotein cholesterol.[59,60] This procedure has not been optimized for normal or hyperlipidemic rabbits, and when we used it with rabbit plasma, we often obtained results that did not correlate with HDL peak

fractions obtained by Superose 6B gel filtration. The procedure was therefore modified by plasma dilution; results obtained using diluted plasma correlated well with the results of gel filtration. Figure 3A shows mean nonprecipitable plasma cholesterol levels determined using undiluted plasma, following treatment with either hLDLR-rAdV or Luc-rAdV. Mean nonprecipitable lipoprotein cholesterol increased by 630% following hLDLR-rAdV treatment. Levels peaked at day 8 postvirus, and returned to baseline by day 13. This change in nonprecipitable cholesterol indicated that hLDLR-rAdV treatment caused a transient but significant change in the behavior and therefore the composition of plasma lipoproteins. The results shown in Fig 3B demonstrated that this change was not explained by an increase in plasma HDL cholesterol; no peak in nonprecipitable cholesterol was observed when diluted plasma was used in the precipitations. Taken together, the results shown in Fig 3A and B indicated that hLDLR-rAdV treatment altered the composition of lipoprotein particles but not the absolute levels of HDL cholesterol.

To better characterize changes in lipoprotein particle composition associated with hLDLR-rAdV treatment, pretreatment and posttreatment plasma samples were analyzed by Superose 6B gel filtration; profiles are shown in Fig 4. Lipoprotein fractions were pooled and lipid contents determined. Compared with pretherapy levels, VLDL and IDL/LDL cholesterol changed by +840% and −30%, respectively, by day 6 after treatment (Fig 4B); triglycerides changed by +290% and +120%, respectively. The triglyceride to cholesterol ratio in the IDL/LDL pool increased from 0.33 on day 0 to 1.05 on day 6; the total plasma triglyceride to cholesterol ratio increased from 0.29 to 1.17 during this interval. Not only did hLDLR-rAdV treatment reduce IDL/LDL cholesterol, but increases were observed for VLDL cholesterol, VLDL and IDL/LDL triglyceride, and the triglyceride to cholesterol ratios for both total plasma and IDL/LDL pools. Cholesterol reductions specifically in the IDL/LDL pools provided additional evidence of hLDLR function following hLDLR gene transfer. The increased VLDL cholesterol and the triglyceride enrichment of VLDL, IDL, and LDL suggested that increased hLDLR activity was associated with increased hepatic VLDL production.

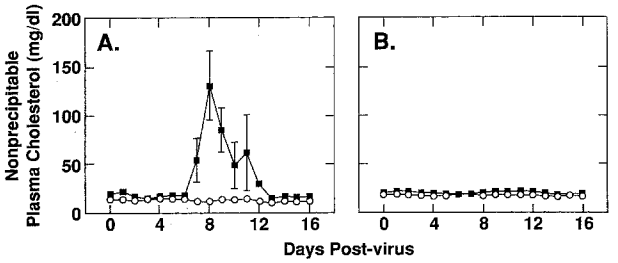

Fig 3. Precipitation of lipoproteins by dextran sulfate/magnesium sulfate: response to rAdV treatment. Plasma samples from rabbits treated at doses of 5 to 7 × 10^9 PFU/kg with either Luc-rAdV (○, n = 3) or hLDLR-rAdV (■, n = 6) on day 0 were collected daily for 16 days. Following precipitation of apo B–containing lipoproteins, cholesterol levels were quantified in supernatants. Precipitation procedure was performed using (A) undiluted plasma[59,60] and (B) diluted plasma. Data are the mean ± SEM.

The effects of Luc-rAdV or hLDLR-rAdV treatments on plasma apo A-I levels as measured by competitive ELISA were determined (Fig 5). By 10 days postvirus, the mean plasma apo A-I level of the hLDLR-rAdV group was increased 85% relative to the pretreatment value and by nearly fivefold compared with the value on day 2.5. In contrast, no statistically significant effects of Luc-rAdV treatment were observed. The mean plasma apo A-I level of the hLDLR-rAdV group was statistically significantly higher than that of the Luc-rAdV group on days 10, 11, and 12 following virus infusion. Thus, hLDLR-rAdV treatment resulted in an increase in apo A-I that was not associated with a parallel increase in HDL cholesterol.

Figure 6 illustrates the distribution of apo A-I and apo E within lipoprotein species before and at various time points after hLDLR-rAdV treatment. Plasma samples were evaluated by lipoprotein agarose gel electrophoresis and immunoblot analysis. Apo A-I was detected in lipoproteins with α and pre-β electrophoretic mobilities. Apo A-I in pre-β–migrating lipoproteins was reduced at 2.5 and 4.5 days posttreatment. There was little or no change in α-migrating apo A-I during this interval. Apo A-I was notably increased in pre-β species beginning at days 8 to 10 posttreatment, but α-migrating apo A-I demonstrated only minimal change. The early-phase decrease and late-phase increase in pre-β–apo A-I correlated temporally with the changes in total plasma apo A-I levels as measured by competitive ELISA (Fig 5). Although total plasma apo A-I levels returned to baseline on days 14 and 16 posttreatment (competitive ELISA; Fig 5), pre-β–apo A-I remained elevated due to a persistent shift in the distribution of apo A-I from α to pre-β lipoproteins (Fig 6). Apo E in pre-β– and α-migrating lipoproteins was also reduced at 2.5 and 4.5 days posttreatment, consistent with the known LDLR ligand function of apo E. Later in the response, after total plasma cholesterol had returned to baseline (9 to 16 days posttreatment), variability was observed in apo E levels of pre-β– and α-migrating lipoproteins. In all cases, apo E levels returned to or surpassed baseline levels. Reasons for the variability were not investigated further; they probably relate to the fact that our hyperlipidemic rabbit colony is genetically outbred.

We determined the sizes of the pre-β–apo A-I lipoproteins that were increased following hLDLR-rAdV treatment. Plasma samples both before and 10 days following hLDLR-rAdV treatment were fractionated by Superose 6B gel filtration. Elution volumes corresponding to peak VLDL, IDL/LDL, HDL, and nascent HDL were pooled, concentrated, and evaluated for apo A-I content by lipoprotein agarose gel electrophoresis and apo A-I immunoblot analysis. Results from a representative rabbit are shown in Fig 7. Pre-β–apo A-I lipoproteins from all rabbits were found only in nascent HDL pools. Apo A-I was not detected in pools containing particles the size of VLDL, IDL, or LDL, thus ruling out VLDL, IDL, or LDL as the source of pre-β–apo A-I. The HDL pools contained only lipoproteins with α-electrophoretic mobility. These HDL particles contained the majority of apo A-I detected; however, they were not

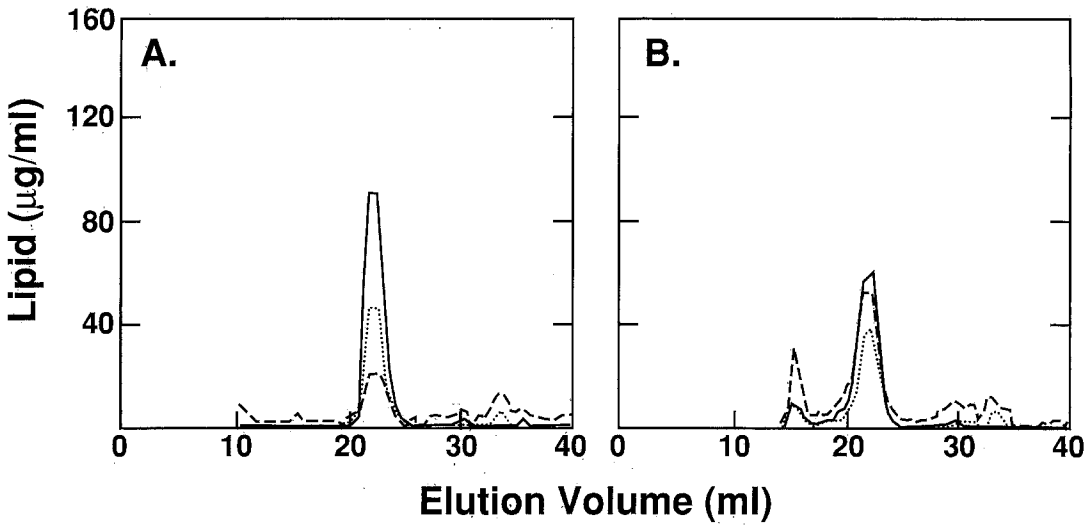

Fig 4. hLDLR-rAdV treatment–induced changes in lipoprotein particle lipid composition as evaluated by Superose 6B FPLC gel filtration. Representative plasma FPLC profiles from a representative rabbit following hLDLR-rAdV (5 to 7 × 10^9 PFU/kg) treatment are shown. Elution volumes for peak VLDL, IDL/LDL, HDL, and nascent HDL fractions are 15.5, 22.0, 30.0, and 33.5 mL, respectively. Plasma was analyzed before treatment (A) and 9 days after treatment (B). (——) Cholesterol, (----) triglycerides, (·····) phospholipids.

significantly affected by hLDLR-rAdV treatment, consistent with the results shown in Fig 6. The nascent HDL pools contained apo A-I particles with two distinct electrophoretic mobilities, pre-β and near-origin. The size and electrophoretic properties of the pre-β species were characteristic of nascent HDL. Densitometric scans of the immunoblot shown in Fig 7 revealed that pre-β–migrating apo A-I increased by 220% following hLDLR-rAdV treatment. The identity of the near-origin species was unclear; it was also visible in Fig 6 and probably represented nonspecific aggregation of apo A-I protein. The slight increase in the electrophoretic mobilities of lipoprotein species compared with those shown in Fig 6 resulted from sample manipulations associated with gel filtration.

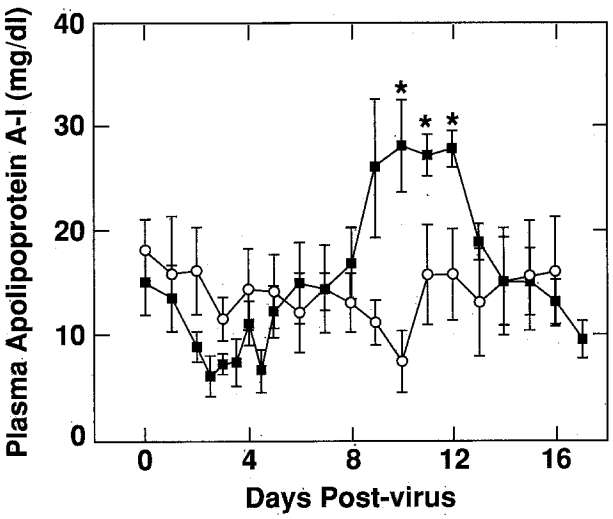

Fig 5. Plasma apo A-I response to rAdV treatment. Plasma apo A-I levels in rabbits treated at doses of 5 to 7 × 10^9 PFU/kg with either Luc-rAdV (○, n = 3) or hLDLR-rAdV (■, n = 6) on day 0 were determined daily for 16 or 17 days. Data are the mean ± SEM (*$P < .05$).

To confirm apo A-I distribution among size-fractionated lipoprotein particles, plasma samples both before and 9 days after hLDLR-rAdV treatment were chromatographed by Superose 6B gel filtration. Individual column fractions were evaluated by SDS–polyacrylamide gel electrophoresis and apo A-I immunoblot analysis. Results from a representative rabbit (no. 200142) are shown in Fig 8. In all animals tested, treatment with hLDLR-rAdV increased total plasma apo A-I by promoting the accumulation of apo A-I specifically in lipoprotein particles the size of HDL and nascent HDL but not in VLDL, IDL, or LDL. (A trace of apo A-I was detected in the IDL/LDL size range [peak, 21.0 to 22.5 ml], but the level was not affected by hLDLR-rAdV treatment.) The most intense apo A-I staining was observed at elution volumes corresponding to mature HDL (peak, 29.5 to 31.0 mL), but the greatest percent increase following hLDLR-rAdV treatment was observed in nascent HDL (>31.0 mL). Densitometric scanning of the immunoblot shown in Fig 8 revealed increases in posttreatment apo A-I of 30%, 50%, 100%, 520%, 950%, and 240% at elution volumes of 29, 30, 31, 32, 33, and 34 mL, respectively.

DISCUSSION

The hyperlipidemic LDLR-defective rabbit is a well-studied model of human FH.[21-23] Although cardiovascular disease pathology in this animal model is remarkably similar to human atherosclerosis,[21] the model does demonstrate some unique features. In contrast to rodents and humans, rabbits have low levels of hepatic lipase.[70,71] This probably explains the relative abundance of IDL in LDLR-defective rabbits compared with FH patients.[25] Apo A-II, an apoprotein present in human and rodent HDL, is undetectable in rabbit HDL.[72] In addition, rabbits have abundant plasma cholesteryl ester transfer protein (CETP) activity,[73-75] nearly threefold that of human plasma and 20-fold that of rodent plasma.[73] Furthermore, several investigators have noted a twofold to threefold elevation in

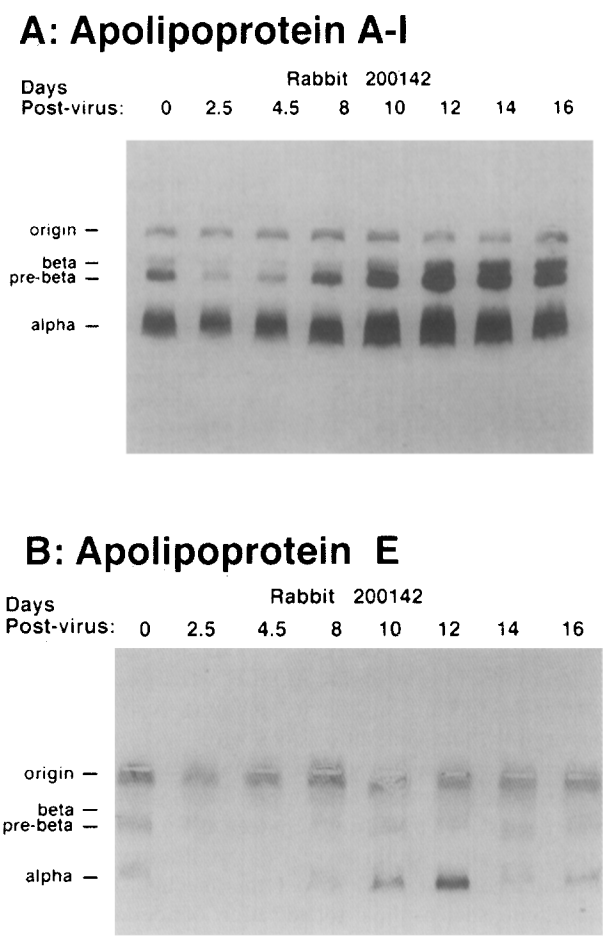

Fig 6. Effects of hLDLR-rAdV treatment on the distribution of apo A-I and apo E among plasma lipoproteins isolated from a representative animal. Plasma samples were obtained before hLDLR-rAdV treatment and at various times following treatment (5 to 7 × 10^9 PFU/kg). Whole-plasma samples were fractionated by agarose gel electrophoresis, and lipoproteins were transferred to Immobilon-P. Membranes were immunostained for apo A-I (A) or apo E (B). Electrophoretic mobilities of β-, pre-β–, and α-lipoproteins from whole plasma are indicated.

CEPT activity and mass[76] in hypercholesterolemic rabbits, including Watanabe rabbits,[77] relative to normocholesterolemic controls. This high level of CETP activity is thought to contribute to the low levels of HDL cholesteryl ester seen in hypercholesterolemia.[78]

We have used adenoviral-mediated gene therapy as a tool to further dissect the effects of LDLR function on lipoproteins, with particular attention to HDL. Since previous studies have demonstrated that the human receptor functions efficiently in rabbits[41-44] and as part of an ongoing development of gene therapies applicable to humans, we have studied the consequences of human LDLR cDNA delivery to LDLR-deficient rabbits. Hepatic gene expression has been achieved without evidence of toxicity or changes in tests of liver function. Our studies confirm the results of previous investigators who showed that adenoviral-mediated LDLR expression transiently enhanced IDL and LDL particle clearance and reduced plasma levels of total, IDL, and LDL cholesterol.[44,65,66] We now extend these observations with the demonstration of a complex biphasic response involving changes in plasma cholesterol and triglyceride levels and alterations in lipoproteins containing apo A-I and apo E. Not only was IDL/LDL cholesterol decreased, but cholesterol in VLDL and triglyceride in VLDL and IDL/LDL were increased. Early in the response, when plasma cholesterol levels were reduced, α- and pre-β–migrating apo E and pre-β–migrating apo A-I were decreased. Late in the response, when plasma cholesterol had returned to baseline, significant increases in total plasma apo A-I levels and enrichment in pre-β–migrating apo A-I were observed. The early-phase reduction in apo E–containing lipoproteins was consistent with the known activity of the LDLR and probably represented receptor-mediated clearance of apo B-100– and apo E–containing IDL[79,80] and apo E–containing α-HDL.[39,81] The parallel early-phase reduction in pre-β–migrating apo A-I could have been either a primary or secondary response to increased LDLR activity.

The late-phase increase in apo A-I represented a more

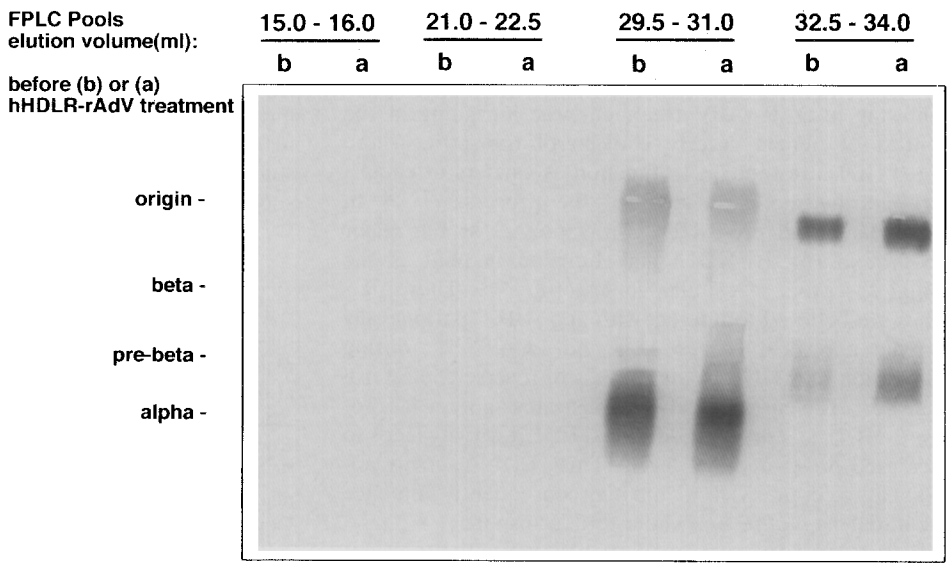

Fig 7. Effects of hLDLR-rAdV treatment on distribution of apo A-I among size-fractionated lipoproteins analyzed by agarose gel electrophoresis. Rabbit plasma obtained before (b) and 10 days after (a) hLDLR-rAdV treatment was fractionated by Superose 6B gel filtration. Elution volumes of lipoprotein pools: VLDL, 15.0 to 16.0 mL; IDL/LDL, 21.0 to 22.5 mL; HDL, 29.5 to 31.0 mL; nascent HDL, 32.5 to 34.0 mL. Electrophoretic mobilities of β-, pre-β–, and α-lipoprotein samples following gel filtration are indicated.

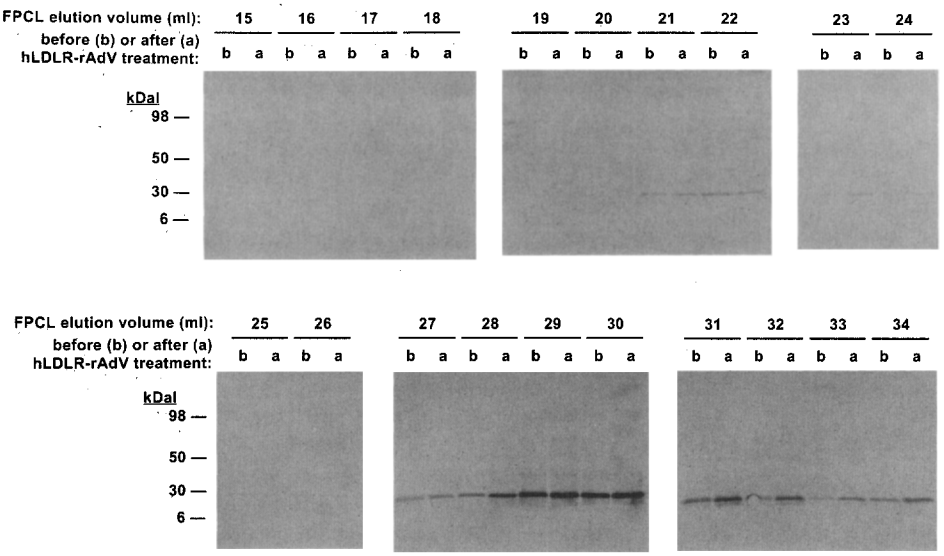

Fig 8. Effects of hLDLR-rAdV treatment on distribution of apo A-I among size-fractionated lipoproteins analyzed by SDS–polyacrylamide gel electrophoresis. Rabbit plasma obtained before (b) and 9 days after (a) hLDLR-rAdV treatment was fractionated by Superose 6B FPLC gel filtration. Individual column fractions were evaluated for apo A-I content by SDS–polyacrylamide gel electrophoresis and immunoblot analysis. Mobilities of protein molecular-weight standards are indicated.

complex process, since it occurred after total plasma cholesterol had returned to baseline and was therefore a secondary event. This response was particularly interesting in that it was not necessarily predicted by the known activities of the LDLR and it illuminated another level of interaction between LDL and HDL. Kinetic studies have previously shown that untreated LDLR-deficient rabbits[27] and humans[13] both exhibit decreased apo A-I production and increased apo A-I clearance, resulting in the observed low steady-state levels of apo A-I. Our findings suggest that transient reconstitution of LDLR function following hLDLR-rAdV treatment leads to transient correction of one or both of these kinetic defects and to an increase in plasma apo A-I levels. Several mechanisms are potentially involved in this process, including (1) increased hepatic VLDL particle production secondary to increased hepatic LDL uptake,[82] leading to VLDL, IDL, and LDL particle triglyceride enrichment; (2) CETP activity (abundant in the hyperlipidemic rabbit[76,77]) promoting redistribution of cholesteryl ester and triglycerides between VLDL, LDL, and HDL; (3) decreased rates of apo A-I particle clearance due to conformational changes associated with particle triglyceride enrichment; and (4) increased net apo A-I synthesis.

The late-phase increase in pre-β–migrating apo A-I following hLDLR-rAdV treatment was intriguing in the context of human reverse cholesterol transport, where pre-β-HDL are thought to function as carriers of cholesterol during the early steps of the process.[31,32,34-36] In particular, small pre-β-HDL with apo A-I as the major apoprotein (pre-β1-HDL) may function in man as the initial acceptors of cellular cholesterol.[31,83-85] These particles are believed to interact with apo A-II,[86] peripherally derived apo E,[37,38,87] apo D,[32] and LCAT[32,88,89] during conversion to α-HDL in the process of reverse cholesterol transport. The current study demonstrates another facet of pre-β-HDL, ie, adenoviral-directed LDLR activity leads to increased pre-β–apo A-I levels. Thus, LDLR activity appears to effect pre-β-HDL and may consequently influence the initial steps of reverse cholesterol transport.

Several mechanisms probably contribute to the transient quality of hLDLR-rAdV responses observed in this study. The human LDLR is likely to be moderately immunogenic in the rabbit, but the loss of hLDLR activity occurs earlier than expected for a primary immune response. More importantly, first-generation adenovirus vectors as used in this study are known to be immunogenic. MHC class I–restricted cytotoxic T cells directed against viral antigens have been shown to destroy hepatocytes[90,91] and respiratory epithelial cells[92] within several days following adenoviral-mediated gene transfer in vivo. Other mechanisms may also contribute, such as the cytotoxic effect of adenoviral capsid proteins independent of the immune response and progressive loss of CMV promoter activity with time. The loss of transgene expression with time in the rabbit has been observed by others[44] and occurred by days 6 to 13 posttreatment, similar to our observed return to baseline at 9 to 10 days posttreatment.

Using adenoviral-mediated hLDLR gene transfer to the hyperlipidemic LDLR-deficient rabbit, we have explored relationships between LDL and HDL, including effects on plasma lipid concentrations, lipoprotein particle composition, and apo A-I and Apo E lipoprotein distribution. Although gene-transfer technology has great potential for the development of new therapeutic agents, the consequences of manipulating gene expression within complex metabolic systems in vivo are essentially unexplored. This study provides an important example of a metabolic system subjected to analysis in vivo using a gene-transfer paradigm.

ACKNOWLEDGMENT

The authors wish to thank Dr Frank Graham for providing the plasmids pJM17 and pXCX2, and for his pioneering studies with recombinant adenoviruses that have made this study possible. We also are indebted to Dr David Usher for providing the monoclonal mouse antirabbit apo A-I (clone 2C3) antibody; to Rosemary Ronan for providing the purified rabbit apo A-I protein; to Barbara Winterrowd, Geretta Wood, Cornelio Duarte, and Kurt Schumacher for technical assistance; and to Drs Robert Hoyt and Douglas Powell for veterinary and surgical care of our rabbit colony.

REFERENCES

1. Gofman JW, Lindgren F, Elliot H, et al: The role of lipids and lipoproteins in atherosclerosis. Science 111:166-171, 186, 1950
2. Barr DP, Russ EM, Eder HA: Protein-lipid relationship in human plasma. II. In atherosclerosis and related conditions. Am J Med 11:480-493, 1951
3. Gofman JW, Young W, Tandy R: Ischemic heart disease, atherosclerosis, and longevity. Circulation 34:679-697, 1966
4. Miller GJ, Miller NE: Plasma high-density lipoprotein concentration and development of ischaemic heart-disease. Lancet 1:16-19, 1975
5. Breckenridge WC, Roberts A, Kuksis A: Lipoprotein levels in genetically selected mice with increased susceptibility to atherosclerosis. Arteriosclerosis 5:256-264, 1985
6. Gordon DJ, Knoke J, Probstfield JL, et al: High-density lipoprotein cholesterol and coronary heart disease in hypercholesterolemic men: The Lipid Research Clinics Coronary Primary Prevention Trial. Circulation 74:1217-1225, 1986
7. Gordon T, Castelli WP, Hjortland MC, et al: High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 62:707-714, 1977
8. Gordon DJ, Rifkind BM: High-density lipoprotein—The clinical implications of recent studies. N Engl J Med 321:1311-1316, 1989
9. Goldstein JL, Hobbs HH, Brown MS: Familial hypercholesterolemia, in Scriver CR, Beaudet AL, Sly WS, et al (eds): The Metabolic and Molecular Bases of Inherited Disease, vol 7. New York, NY, McGraw-Hill, 1995, pp 1981-2030
10. Brewer HB Jr, Gree RE, Hoeg JM: Apolipoproteins, lipoprotein, and atherosclerosis, in Braunwald E (ed): Heart Disease: A Textbook of Cardiovascular Medicine, vol 3. New York, NY, Saunders, 1989, pp 121-144
11. Streja D, Steiner G, Kwiterovich PJ Jr: Plasma high-density lipoproteins and ischemic heart disease: Studies in a large kindred with familial hypercholesterolemia. Ann Intern Med 89:871-880, 1978
12. Sprecher DL, Schaefer EJ, Kent KM, et al: Cardiovascular features of homozygous familial hypercholesterolemia: Analysis of 16 patients. Am J Cardiol 54:20-30, 1984
13. Schaefer JR, Rader DJ, Ikewaki K, et al: In vivo metabolism of apolipoprotein A-I in a patient with homozygous familial hypercholesterolemia. Arterioscler Thromb 12:843-848, 1992
14. Hoeg JM, Maher MB, Zech LA, et al: Effectiveness of mevinolin on plasma lipoprotein concentrations in type II hyperlipoproteinemia. Am J Cardiol 57:933-939, 1986
15. Bilheimer DW, Goldstein JL, Grundy SM, et al: Liver transplantation to provide low-density-lipoprotein receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. N Engl J Med 311:1658-1664, 1984
16. Figuera D, Ardaiz J, Martin-Judez V, et al: Combined transplantation of heart and liver from two different donors in a patient with familial type IIa hypercholesterolemia. J Heart Transplant 5:327-329, 1986
17. Hoeg JM, Starzl TE, Brewer HB Jr: Liver transplantation for treatment of cardiovascular disease: Comparison with medication and plasma exchange in homozygous familial hypercholesterolemia. Am J Cardiol 59:705-707, 1987
18. Postiglione A, Thompson GR: Experience with plasma-exchange in homozygous familial hypercholesterolaemia. Prog Clin Biol Res 188:213-220, 1985
19. Frick MH, Elo O, Haapa K, et al: Helsinki Heart Study: Primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety in treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 317:1237-1245, 1987
20. The Lovastatin Study Group II: Therapeutic response to lovastatin (Mevinolin) in nonfamilial hypercholesterolemia. A multicenter study. JAMA 256:2829-2834, 1986
21. Watanabe Y: Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL-rabbit): Incidence and development of atherosclerosis and xanthoma. Atherosclerosis 36:261-268, 1980
22. Tanzawa K, Shimad Y, Kuroda M, et al: WHHL-rabbit: A low density lipoprotein receptor–deficient animal model for familial hypercholesterolemia. FEBS Lett 18:81-84, 1980
23. Buja LM, Kita T, Goldstein JL, et al: Cellular pathology of progressive atherosclerosis in the WHHL rabbit: An animal model of familial hypercholesterolemia. Arteriosclerosis 3:87-101, 1983
24. Yamamoto T, Bishop RW, Brown MS, et al: Deletion in cysteine-rich region of LDL receptor impedes transport to cell surface in WHHL rabbit. Science 232:1230-1237, 1986
25. Havel RJ, Kita T, Kotite L, et al: Concentration and composition of lipoproteins in blood plasma of the WHHL rabbit. An animal model of human familial hypercholesterolemia. Arteriosclerosis 2:467-474, 1982
26. Goldstein JL, Kita T, Brown MS: Defective lipoprotein receptors and atherosclerosis. Lessons from an animal counterpart of familial hypercholesterolemia. N Engl J Med 309:288-296, 1983
27. Saku K, Yamamoto K, Sakai T, et al: Kinetics of HDL-apo A-I in the WHHL rabbit, an animal model of familial hypercholesterolemia. Atherosclerosis 79:225-230, 1989
28. Mezdour H, Nomura S, Yamamura T, et al: Concentration and distribution of apolipoproteins A-I and E in normolipidemic, WHHL and diet-induced hyperlipidemic rabbit sera. Biochim Biophys Acta 1127:116-123, 1992
29. Hoeg JM, Remaley AT: Reverse cholesterol transport, in Goldbourt U, de Faire U, Berg K (eds): Genetic Factors in Coronary Heart Disease. Dordrecht, The Netherlands, Kluwer Academic, 1994, pp 351-369
30. Kunitake ST, La Sala KJ, Kane JP: Apolipoprotein A-I–containing lipoproteins with pre-beta electrophoretic mobility. J Lipid Res 26:549-555, 1985
31. Castro GR, Fielding CJ: Early incorporation of cell-derived cholesterol into pre-beta–migrating high-density lipoprotein. Biochemistry 27:25-29, 1988
32. Francone OL, Gurakar A, Fielding C: Distribution and functions of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in plasma lipoproteins. Evidence for a functional unit containing these activities together with apolipoproteins A-I and D that catalyzes the esterification and transfer of cell-derived cholesterol. J Biol Chem 264:7066-7072, 1989
33. Kunitake ST, Mendel CM, Hennessy LK: Interconversion between apolipoprotein A-I–containing lipoproteins of pre-beta and alpha electrophoretic mobilities. J Lipid Res 33:1807-1816, 1992
34. Fielding CJ, Fielding PE: Evidence for a lipoprotein carrier in human plasma catalyzing sterol efflux from cultured fibroblasts and its relationship to lecithin:cholesterol acyltransferase. Proc Natl Acad Sci USA 78:3911-3914, 1981
35. Barbaras R, Puchois P, Fruchart JC, et al: Cholesterol efflux from cultured adipose cells is mediated by LpAI particles but not by LpAI:AII particles. Biochem Biophys Res Commun 142:63-69, 1987
36. Barkia A, Barbaras R, Ghalim N, et al: Effect of different APO A-I containing lipoprotein particles on reverse cholesterol transport in fat cells. Horm Metab Res Suppl 19:10-12, 1988
37. Basu SK, Ho YK, Brown MS, et al: Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes. J Biol Chem 257:9788-9795, 1982
38. Basu SK, Goldstein JL, Brown MS: Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. Science 219:871-873, 1983

39. Koo C, Innerarity TL, Mahley RW: Obligatory role of cholesterol and apolipoprotein E in the formation of large cholesterol-enriched and receptor-active high density lipoproteins. J Biol Chem 260:11934-11943, 1985

40. Li Q, Kay MA, Finegold M, et al: Assessment of recombinant adenoviral vectors for hepatic gene therapy. Hum Gene Ther 4:403-409, 1993

41. Dichek DA, Bratthauer BL, Beg ZH, et al: A genetic therapy for familial hypercholesterolemia. Trans Assoc Am Physicians 103:73-79, 1990

42. Wilson JM, Chowdhury NR, Grossman M, et al: Temporary amelioration of hyperlipidemia in low density lipoprotein receptor–deficient rabbits transplanted with genetically modified hepatocytes. Proc Natl Acad Sci USA 87:8437-8441, 1990

43. Chowdhury JR, Grossman M, Gupta S, et al: Long-term improvement of hypercholesterolemia after ex vivo gene therapy in LDLR-deficient rabbits. Science 254:1802-1805, 1991

44. Kozarsky KF, McKinley DR, Austin LL, et al: In vivo correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses. J Biol Chem 269:13695-13702, 1994

45. Donnelly TM, Kelsey SF, Levine DM, et al: Control of variance in experimental studies of hyperlipidemia using the WHHL rabbit. J Lipid Res 2:1087-1098, 1992

46. Yamamoto T, Davis CG, Brown MS, et al: The human LDL receptor: A cysteine-rich protein with multiple Alu sequences in its mRNA. Cell 39:27-38, 1984

47. de Wet JR, Wood KV, DeLuca M, et al: Firefly luciferase gene: Structure and expression in mammalian cells. Mol Cell Biol 7:725-737, 1987

48. Nordeen SK: Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 6:454-457, 1988

49. McGrory WJ, Bautista DS, Graham FL: A simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. Virology 163:614-617, 1988

50. McKinnon RD, Bacchetti S, Graham FL: Tn5 mutagenesis of the transforming genes of human adenovirus type 5. Gene 19:33-42, 1982

51. Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745-2752, 1987

52. Graham FL, Smiley J, Russel WC, et al: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36:59-72, 1977

53. Harrison T, Graham FL, Williams J: Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology 77:319-329, 1977

54. Green M, Pina M: Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. Virology 20:199-207, 1963

55. Chirgwin JM, Przbyla AE, MacDonald RJ, et al: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299, 1979

56. Patterson AP, Tennyson GE, Hoeg JM, et al: Ontogenetic regulation of apolipoprotein B mRNA editing during human and rat development in vivo. Arterioscler Thromb 12:468-473, 1992

57. Allain CA, Poon LS, Chan CSG, et al: Enzymatic determination of total serum cholesterol. Clin Chem 20:470-475, 1974

58. McGowan MW, Artiss JD, Strandbergh DR, et al: A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin Chem 29:538-542, 1983

59. Finley PR, Schifman RB, Williams RJ, et al: Cholesterol in high-density lipoprotein: Use of Mg^{2+}/dextran sulfate in its enzymic measurement. Clin Chem 24:931-933, 1978

60. Warnick GR, Benderson J, Albers JJ, et al: Dextran sulfate-Mg^{2+} precipitation procedure for quantitation of high-density-lipoprotein cholesterol. Clin Chem 28:1379-1388, 1982

61. Jiao S, Cole TG, Kitchens RT, et al: Genetic heterogeneity of lipoproteins in inbred strains of mice: Analysis by gel-permeation chromatography. Metabolism 39:155-160, 1990

62. Pitas RE, Innerarity TL, Weinstein JN, et al: Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. Arteriosclerosis 1:177-185, 1981

63. Hsu IC, Lipsky MM, Cole KE, et al: Isolation and culture of hepatocytes from human liver of immediate autopsy. In Vitro Cell Dev Biol 21:154-160, 1985

64. Edge SB, Hoeg JM, Triche T, et al: Cultured human hepatocytes. Evidence for metabolism of low density lipoproteins by a pathway independent of the classical low density lipoprotein receptor. J Biol Chem 261:3800-3806, 1986

65. Herz J, Gerard RD: Adenovirus-mediated in vivo gene transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. Proc Natl Acad Sci USA 90:2812-2816, 1993

66. Ishibashi S, Brown MS, Goldstein JL, et al: Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 92:883-893, 1993

67. Smith TAG, Mehaffey MG, Kayda DB, et al: Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. Nature Genet 5:397-402, 1993

68. Jaffe HA, Danel C, Longenecker G, et al: Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. Nature Genet 1:372-378, 1992

69. Shaked A, Csete ME, Drazan KE, et al: Adenovirus-mediated gene transfer in the transplant setting. II. Successful expression of transferred cDNA in syngeneic liver grafts. Transplantation 57:1508-1511, 1994

70. Clay MA, Hopkins GJ, Ehnholm CP, et al: The rabbit as an animal model of hepatic lipase deficiency. Biochim Biophys Acta 1002:173-181, 1989

71. Warran RJ, Ebert DL, Mitchell A, et al: Rabbit hepatic lipase cDNA sequence: Low activity is associated with low messenger RNA levels. J Lipid Res 32:1333-1339, 1991

72. Borresen AL: High density lipoprotein (HDL) polymorphisms in rabbit. I. A comparative study of rabbit and human serum high density lipoprotein. J Immunogenet 3:73-81, 1976

73. Ha YC, Barter PJ: Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. Comp Biochem Physiol 71B:265-269, 1982

74. Ko KWS, Oikawa K, Ohnishi T, et al: Purification, characterization, and conformational analysis of rabbit plasma lipid transfer protein. Biochemistry 32:6729-6736, 1993

75. Rea TJ, DeMattos RB, Pape ME: Hepatic expression of genes regulating lipid metabolism in rabbits. J Lipid Res 34:1901-1910, 1993

76. Tall A, Granot E, Brocia R, et al: Accelerated transfer of cholesteryl esters in dyslipidemic plasma. Role of cholesteryl ester transfer protein. J Clin Invest 79:1217-1225, 1987

77. Son Y-SC, Zilversmit DB: Increased lipid transfer activities in hyperlipidemic rabbit plasma. Arteriosclerosis 6:345-351, 1986

78. Tall AR: Plasma cholesteryl ester transfer protein. J Lipid Res 34:1255-1274, 1993

79. Goldstein JL, Brown MS: The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem 46:897-930, 1977

80. Kita T, Brown MS, Bilheimer DW, et al: Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. Proc Natl Acad Sci USA 79:5693-5697, 1982

81. Gordon V, Innerarity TL, Mahley RW: Formation of cholesterol- and apoprotein E–enriched high density lipoproteins in vitro. J Biol Chem 258:6202-6212, 1983

82. Tanaka M, Jingami H, Otani H, et al: Regulation of apolipoprotein B production and secretion in response to the change of intracellular cholesteryl ester contents in rabbit hepatocytes. J Biol Chem 268:12713-12718, 1993

83. Francone OL, Fielding CJ: Cholesterol transport: The role of short-lived cholesterol acceptors. Eur Heart J 2:218-224, 1990 (suppl E)

84. Kawano M, Miida T, Fielding CJ, et al: Quantitation of preβ-HDL–dependent and nonspecific components of the total efflux of cellular cholesterol and phospholipid Biochemistry 32:5025-5028, 1993

85. Fielding PE, Kawano M, Catapano AL, et al: Unique epitope of apolipoprotein A-I expressed in pre-β-1 high-density lipoprotein and its role in the catalyzed efflux of cellular cholesterol. Biochemistry 33:6981-6985, 1994

86. Hara H, Yokoyama S: Role of apolipoproteins in cholesterol efflux from macrophages to lipid microemulsion: Proposal of a putative model for the pre-β high-density lipoprotein pathway. Biochem 31:2040-2046, 1992

87. Basu SK, Brown MS, Ho YK, et al: Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. Proc Natl Acad Sci USA 78:7545-7549, 1981

88. Neary R, Bhatnagar D, Durrington P, et al: An investigation of the role of lecithin:cholesterol acyltransferase and triglyceride-rich lipoproteins in the metabolism of pre-beta high density lipoproteins. Atherosclerosis 89:35-48, 1991

89. Miida T, Kawano M, Fielding CJ, et al: Regulation of the concentration of preβ high-density lipoprotein in normal plasma by cell membranes and lecithin-cholesterol acyltransferase activity. Biochem 31:11112-11117, 1992

90. Yang Y, Ertl HCJ, Wilson JM: MHC class I–restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1:433-442, 1994

91. Engelhardt JF, Ye X, Doranz B, et al: Ablation of *E2A* in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc Natl Acad Sci USA 91:6196-6200, 1994

92. Yang Y, Nunes FA, Berencsi K, et al: Inactivation of *E2a* in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. Nature Genet 7:362-369, 1994