Hepatic gene transfer of the catalytic subunit of the apolipoprotein B mRNA editing enzyme results in a reduction of plasma LDL levels in normal and Watanabe heritable hyperlipidemic rabbits

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Abstract Apolipoprotein (apo) B exists in two forms, the full length protein apoB-100 and the carboxyterminal-truncated apoB-48 that is synthesized in the intestine due to editing of the apoB mRNA which generates a premature stop codon. To determine whether gene transfer of the catalytic subunit of the apoB mRNA editing enzyme APOBEC-1 (apoB mRNA editing enzyme catalytic polypeptide 1) into the liver of rabbits reconstitutes hepatic apoB mRNA editing and how this affects the plasma levels of apoB-containing lipoproteins, we constructed an APOBEC-1 recombinant adenovirus (Ad APOBEC-1). After injection of Ad APOBEC-1 into normal New Zealand White (NZW) or Watanabe heritable hyperlipidemic (WHHL) rabbits, up to 50% of the hepatic apoB mRNA was edited and freshly isolated hepatocytes secreted predominantly apoB-48-containing lipoproteins. VLDL isolated from Ad APOBEC-1-treated NZW and WHHL rabbits contained both apoB-100 and apoB-48, whereas that from control rabbits infected with a β-galactosidase recombinant adenovirus (Ad LacZ) contained exclusively apoB-100. VLDL from WHHL rabbits treated with Ad APOBEC-1 had the same particle size, lipid composition, and content of apolipoprotein E as VLDL from Ad LacZ-infected control animals. An increase of VLDL was observed in NZW and WHHL rabbits after infection with Ad APOBEC-1 as well as Ad LacZ. After injection of Ad APOBEC-1, LDL became undetectable in the plasma of NZW rabbits and was reduced by an average of 65% in the plasma of WHHL rabbits compared to Ad LacZ-infected controls. LDL from Ad APOBEC-1-infected WHHL rabbits contained only apoB-100. VLDL isolated from Ad APOBEC-1-infected WHHL rabbits were rapidly cleared from the circulation after injection into NZW rabbits. These results provide further evidence that the switch in the hepatic synthesis from exclusively apoB-100 to partly apoB-48 can result in a reduction of LDL formation that requires the full-length apoB-100.—**Greeve, J., V. K. Jona, N. Roy Chowdhury, M. S. Horwitz, and J. Roy Chowdhury.** Hepatic gene transfer of the catalytic subunit of the apolipoprotein B mRNA editing enzyme results in a reduction of plasma LDL levels in normal and Watanabe heritable hyperlipidemic rabbits. *J. Lipid Res.* 1996. **37:** 2001–2017.

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Apolipoprotein (apo) B exists in two forms, the carboxyterminal-truncated apoB-48 and the full-length protein apoB-100 that represents the core component of the atherogenic LDL and Lp[a] (1–4). The synthesis of apoB-48 results from a posttranscriptional base change in the apoB mRNA from C to U, called mRNA editing (5, 6). During the intranuclear processing of the intestinal apoB pre-mRNA, the apoB mRNA editing enzyme, which is composed of several subunits, catalyzes the site-specific deamination of cytidine 6666 to uridine to generate the premature stop codon UAA whereby only the aminoterminal 48% of the apoB mRNA is translated into apoB-48 (5–11). The catalytic subunit of the apoB mRNA editing enzyme APOBEC-1 (apoB mRNA editing enzyme catalytic polypeptide 1) is an RNA-specific cytidine deaminase with a novel RNA binding motif (12–17). Besides APOBEC-1, the other presumptive components of the apoB mRNA editing enzyme have not been conclusively characterized (18, 19).

In all mammalian species, the intestinal apoB mRNA is completely edited and the intestine secretes chylomi-

Abbreviations: APOBEC-1, apolipoprotein B mRNA editing enzyme catalytic polypeptide 1; apoB, apolipoprotein B; LDL, low density lipoproteins; VLDL, very low density lipoproteins; HDL, high density lipoproteins; Lp[a], lipoprotein[a]; mRNA, messenger RNA; MOI, multiplicity of infection; NZW, New Zealand White; WHHL, Watanabe heritable hyperlipidemic; PCR, polymerase chain reaction; RT-PCR, reverse transcription-coupled polymerase chain reaction.

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crons that contain exclusively apoB-48 (20). In many mammalian species, including humans and rabbits, the hepatic apoB mRNA is not edited and the VLDL produced by their livers contain only apoB-100 (20). In dog, horse, rat, and mouse, however, the hepatic apoB mRNA is also substantially edited and the hepatic secretion of VLDL that contain both apoB-100 and apoB-48 results in low plasma levels of LDL (20). Findings in a woman with homozygous familial hypobetalipoproteinemia indicate a significance of apoB-48-containing VLDL also for humans (21). Due to a premature stop codon in the apoB gene, only the truncated apoB-50 can be translated from her apoB mRNA (21). Her VLDL, which contain exclusively apoB-50, cannot be converted into LDL that are, consequently, absent in her plasma (21).

Editing of apoB mRNA can be reconstituted in vitro by complementing cytosolic fractions from human, baboon, or rabbit liver with recombinant APOBEC-1, suggesting that the livers of these species contain the other components of the apoB mRNA editing enzyme (14, 15, 22). Indeed, in APOBEC-1 transgenic rabbits, the apoB mRNA was found to be edited in both liver and intestine resulting in a reduction of plasma LDL levels (23). This study was designed to investigate *i)* how effectively somatic gene transfer of APOBEC-1 can induce apoB mRNA editing in rabbit liver; and *ii)* how this influences the plasma levels of apoB-containing lipoproteins. Expression of APOBEC-1 in rabbit liver by recombinant adenoviruses efficiently reconstituted apoB mRNA editing and resulted in substantially reduced plasma LDL levels in New Zealand White (NZW) and Watanabe heritable hyperlipidemic (WHHL) rabbits. These results further support the hypothesis that hepatic apoB mRNA editing is a fundamental genetic mechanism to limit the generation of atherogenic apoB-100-containing lipoproteins.

EXPERIMENTAL PROCEDURES

Rabbits

Two- to 6-month-old male NZW rabbits (1–2 kg, purchased from Charles River, Cambridge, MA) and WHHL rabbits (1–2.5 kg, obtained from Camm Research, Wayne, NJ), were maintained on standard rabbit chow and were allowed to acclimatize for at least a week before being subjected to experiments. All animal experiments were performed according to protocols approved by the institutional review board of Albert Einstein College of Medicine for animal experimentation according to NIH guidelines.

Plasmids

pJM17 was kindly provided by Dr. F. L. Graham, McMaster University, Hamilton, Canada and pAd BglII was obtained through the courtesy of Dr. M. Imperiale, University of Michigan, Ann Arbor, MI.

Cloning of a full-length cDNA for APOBEC-1 from rat intestine

Total RNA from rat small intestine was prepared by CsCl density gradient ultracentrifugation (20). RNA (10 μg) was reverse transcribed with 60 pmol oligonucleotide APOBEC-1 V (TCC CAG AAG TCA TTT CAA CCC TGT GGC CCA, rat, antisense nt +9 in the 3′ untranslated region to nt 680 in the coding region). Rat APOBEC-1 cDNA was amplified by 30 cycles of polymerase chain reaction (PCR) using 60 pmol oligonucleotide APOBEC-1 V and APOBEC-1 I (GAG GAA GGA GTC CAG AGA CAC AGA GAG C, sense, rat, nt -31 to -4 in the 5′ untranslated region) and cloned into the expression vector pSVL (Pharmacia). Five recombinant clones obtained by two separate PCR amplifications were completely sequenced. The nucleotide sequences of all five cloned cDNAs were identical to that published by Teng, Burand, and Davidson (12) except for a single base substitution: the G at position 178 was replaced by an A, changing the amino acid residue at position 60 from lysine (AAA) to glutamic acid (GAA). As this sequence variation was found in all clones obtained from the two separate PCRs, it was considered a normal polymorphism.

Construction of an APOBEC-1 recombinant adenovirus and a β-galactosidase recombinant control-adenovirus

pAdBglII was digested with BglII and ligated to a BglII-BamHI fragment that is composed of the BglII-HindIII fragment of pRC-CMV (Invitrogen), containing the promoter and enhancer sequences for the immediate early genes of CMV, and the HindIII-Bam HI fragment of pSVK3, containing the SV40 small T-antigen splice site and SV 40 early polyadenylation site. The resulting plasmid, pAdBgl II-CMV-SV40, was digested with HindIII and ligated to the HindIII fragment of pSVL APOBEC-1 containing the entire coding region for APOBEC-1. The resulting plasmid, pAdBgl II CMV-APOBEC-1-SV40, contains the left end sequences of Ad5 from nt 1–357, the CMV-promoter, the cDNA for APOBEC-1, the SV40 small T-antigen splice site and polyadenylation signal, followed by sequences of Ad5 from nt 3328 to 5788 in the plasmid backbone of pAT153, a pBR322 derivative. The APOBEC-1 recombinant adenovirus, termed Ad APOBEC-1, was generated by cotransfection of pAdBg II-CMV-APOBEC-1-SV40 and pJM17 into 293 cells as described (24–28). Ad

by cotransfection of pAdBg II-CMV-APOBEC-1-SV40 and pJM17 into 293 cells as described (24–28). Ad APOBEC-1 was plaque-purified and the integration of the APOBEC-1 minigene was confirmed by Southern blotting using viral DNA prepared by a modified Hirt procedure (27). An HindIII fragment spanning the entire coding region of *E. coli* β-galactosidase linked to a nuclear localization signal was inserted into pAdBglII-CMV-SV40 and a β-galactosidase recombinant adenovirus, named Ad LacZ, was generated as described above.

Large scale virus preparation

The 293 cells (8×10^8), grown in suspension culture at a cell density of 5×10^5/ml, were infected with the supernatant of 293 cells (2×10^7) that had been completely lysed by Ad APOBEC-1. Recombinant adenovirus was purified from cell lysate by two consecutive CsCl density gradient ultracentrifugations and stored in 30% glycerol at -20°C (28). The virus preparation was dialyzed for 12 h at 4°C against phosphate-buffered saline containing 10% glycerol prior to use. Viral particle number was determined by OD260 of the purified virus as described (28). The virus preparations were also titered by limiting dilution plaque assay on 293 cells. The ratio of viral particles/plaque forming units (pfu) was generally between 20–40:1.

Infection of HuH-7 cells with Ad APOBEC-1 and Ad LacZ

Human hepatoma HuH-7 cells (2×10^6 in 60 mm dishes) were infected with Ad APOBEC-1 or Ad LacZ at ratios of viral particles/cell of 1, 10, 25, 50, and 100 by addition of purified recombinant virus into the tissue culture medium. Two days later, the cells were analyzed for editing of apoB mRNA and the secretion of apoB protein as described below.

Infection of NZW and WHHL rabbits with Ad APOBEC-1 and Ad LacZ

Young adult, age- and sex-matched NZW rabbits were infected with Ad APOBEC-1 (n = 4) or Ad LacZ (n = 3) by infusion of 1×10^{13} viral particles/kg body weight into the marginal ear vein or a mesenteric vein tributary exposed by laparotomy as described (29). Five days after infection, wedge liver biopsies were obtained from all animals. Portions of each liver biopsy were either frozen in liquid nitrogen for analysis of apoB mRNA editing, fixed in formalin for histological examination, or frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) in a methyl–butane bath for histochemical staining of β-galactosidase activity (30). Two rabbits were killed 6 days after infection with Ad APOBEC-1 or Ad LacZ, respectively, and one rabbit at day 15 after infection with Ad APOBEC-1. Liver, pancreas, spleen, intestine, testis, kidney, lung, heart, skeletal muscle, and brain from each animal were frozen in liquid nitrogen for analysis of apoB mRNA editing and expression of rat APOBEC-1. Two additional rabbits infected with Ad APOBEC-1 and two rabbits infected with Ad LacZ underwent surgical wedge liver biopsy for assessment of apoB mRNA editing or β-galactosidase expression. For the subsequent study in WHHL rabbits, the viral dose was lowered to 5×10^{12} viral particles/kg body weight infused into the marginal ear vein. In this series of experiments, one animal from the Ad APOBEC-1-treated WHHL rabbits was killed on each of the days 3, 6, 9, and 15 after infection and the extent of apoB mRNA editing in liver was analyzed. Blood samples were drawn from each WHHL rabbit 1 week before the infection, on the day of infection (day 0), and at days 3, 6, 9, 15, and 28 after infection. The plasma samples of all NZW and WHHL rabbits infected with Ad APOBEC-1 or Ad LacZ were analyzed for total plasma cholesterol and triglycerides, VLDL-cholesterol, LDL-cholesterol, and HDL-cholesterol. The activity of alanine-aminotransferase (ALT) was measured using a commercially available kit (GPT optimized Alanine-Aminotransferase EC 2.6.12 UV test, Sigma).

Analysis of apoB mRNA editing

Total RNA was prepared by CsCl density gradient ultracentrifugation as described (20), digested with 5 units of RNase-free DNaseI, and further purified by phenol–chloroform extraction followed by ethanol precipitation. ApoB mRNA was reverse transcribed using 5 units avian myeloblastosis virus reverse transcriptase (Pharmacia) and 30 pmol oligonucleotide apoB6 (CAA GAA TTT TTA ATT TTT CCA TGA TT, antisense, rabbit, nt 6765–6740) and subsequently amplified by PCR (25 cycles, 1 min at 95°C, 2 min at 52°C, 2.5 min at 72°C) using 30 pmol each of the antisense primer apoB6 and the sense primer apoB4 (TGC CAA AAT CAA CTT GAA TGA AAA AC, sense, rat, nt 6614–6639). A control PCR without reverse transcriptase was performed for each sample. The PCR products were purified on micro-spin columns (Pharmacia) and analyzed for editing using a primer extension assay (7, 20). Briefly, radiolabeled oligonucleotide apoB3 (A ATC ATG TTA ATC ATA ACT ATC TTT AAT ATA CTG A, antisense, human, nt 6708–6674) was annealed to denatured apoB PCR products and extended in the presence of 1 mM ddGTP using avian myeloblastosis virus reverse transcriptase (Pharmacia). This results in a 43-nt extension product for unedited and a 54-nt extension product for edited apoB cDNA, respectively, which were sepa-

rated by electrophoresis on a 8% polyacrylamide–7 M urea sequencing gel. ApoB mRNA editing was quantitated using RadiophosphorImager SF that was shown to be as accurate as liquid scintillation counting of the excised extension products (7, 20).

Cloning and sequencing of apoB PCR products

Gel-purified apoB RT-PCR products from Ad APOBEC-1-infected rabbit liver were cloned into pT7T3 (20) and the complete nucleotide sequence of several clones was determined using oligonucleotide apoB4 and Sequenase 2.0 (United States Biochemical Corporation, Cleveland, OH).

RNA analysis for expression of rat APOBEC-1

Total RNA (30 μg), isolated by CsCl density gradient ultracentrifugation (20) from the organs of one NZW rabbit each at days 6 and 15 after infection with Ad APOBEC-1 and from one NZW rabbit at day 6 after infection with Ad LacZ, was electrophoresed on 0.8% agarose, 6% formaldehyde gels and transferred to nylon membranes (Genescreen®, NEN) by capillary blotting. Filters were hybridized with a ^{32}P-labeled full-length cDNA probe of rat APOBEC-1 for 12 h at 62°C, washed in 0.1 × SSC, 0.1% SDS at 72°C, and exposed to autoradiography at -80°C for 12 h. For analysis by RT-PCR, 5 μg of total RNA, digested with 5 units of RNase-free DNaseI and purified by phenol–chloroform extraction and ethanol precipitation, was reverse transcribed using 5 units avian myeloblastosis virus reverse transcriptase (Pharmacia) and oligo APOBEC-1 III (CAG ATG GGG GTA CCT TGG CCA ATG AGC, sense, rat, nt 564 to 548). The subsequent PCR amplification was for 20 cycles (1 min at 95°C, 2 min at 52°C, and 2 min at 72°C) using oligo APOBEC-1 I and III. A separate control without reverse transcriptase was performed for each sample. After digestion with 1 unit RNaseA for 10 min, 9 μl of the PCR reactions was separated on a 1.5% agarose gel and stained with ethidium bromide.

Analysis of secreted apoB protein

Primary hepatocytes were isolated from two Ad APOBEC-1-infected and from two Ad LacZ-infected rabbits 5 days after infection as described (20). Four hours after isolation, 2 × 10^6 hepatocytes were labeled for 6 h with 50 μCi [^{35}S]methionine/ml in methionine-free Minimal Essential Medium (MEM) containing 0.1% dialyzed fetal calf serum (20). ApoB protein was immunoprecipitated from cell supernatant using a polyclonal goat anti-human apoB antibody (20), separated by electrophoresis on a linear 4–15% gradient SDS-polyacrylamide gel, and visualized by autoradiography (20).

Lipoprotein analysis

Total cholesterol and total triglycerides were measured in rabbit plasma using commercially available colorimetric assays (Monotest Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim). Plasma lipoproteins were separated by buoyant density gradient ultracentrifugation as described (20). Briefly, 3 ml plasma from NZW rabbits or 1 ml plasma from WHHL rabbits was adjusted to a density of 1.21 g/ml by addition of KBr, overlaid stepwise with 3 ml each of KBr solutions of the density d = 1.063 g/ml and d = 1.019 g/ml, and centrifuged in a SW 40 rotor (Beckmann) for 24 h at 40,000 rpm at 4°C. Fractions of the gradients were collected from the bottom of the tube. The cholesterol in each fraction was determined using Monotest Cholesterol, Boehringer Mannheim. The density of each fraction was determined with a densitometer (20). HDL-cholesterol was defined as the density range from d = 1.08–1.2 g/ml, LDL-cholesterol as the density range from d = 1.02–1.05 g/ml, and VLDL-cholesterol as the density range d = 1.006–1.019 g/ml, which possibly also contains some intermediate density lipoproteins (IDL). The amount of cholesterol in these three density ranges was integrated and expressed as mg/dl plasma as a measure for plasma concentrations of HDL, LDL, and VLDL, respectively. In rabbit plasma, no cholesterol was detected in the density range d = 1.05–1.08 g/ml which was, therefore, excluded from the calculations. For further biochemical characterization of apoB-containing lipoproteins from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits, VLDL and LDL were isolated from plasma of fasted animals by preparative sequential ultracentrifugation at densities of d = 1.019 g/ml (VLDL and IDL) and d = 1.05 g/ml (LDL) and recentrifuged at their respective densities. The protein content of each lipoprotein fraction was determined using the Bradford method (Bio-Rad). Concentrations of total cholesterol, total triglycerides, free cholesterol, and phospholipids in the lipoprotein fractions were determined using commercially available colorimetric assays (Monotest Cholesterol, Triglyceride GPO-PAP, free Cholesterol CHOD-PAP and phospholipids MPR2, Boehringer Mannheim, Germany). For analysis of the apoprotein content, VLDL and LDL were delipidated in 20 volumes of ethanol–ether 3:1 at -20°C for 8 h. The protein precipitates were resolved by electrophoresis on 4–15% linear gradient SDS-polyacrylamide gels. Gels were stained with Coomassie brilliant blue or silver. For analysis of apoB in LDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits, gels were electroblotted onto nitrocellulose and apoB was detected using a goat anti-human apoB antibody (Boehringer Mannheim) followed by incubation with protein A coupled to horseradish peroxidase (Bio-Rad). Horseradish peroxidase activity was detected using 4-chloro-1-

naphthol as described (31). For determination of the diameters of the VLDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits, purified VLDL was extensively dialyzed against ammonium acetate buffer, pH 7.4, and stained with 2% phosphotungstate, pH 7.4, as described (32). Electron microscopy of VLDL was performed as described (32). The diameters of 1000 particles of VLDL from both Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits were measured.

Turn-over studies with VLDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits in NZW rabbits

VLDL from one Ad APOBEC-1- and one Ad LacZ-infected WHHL rabbit were radiolabeled with ^{125}I to a specific activity of 10^6 cpm/mg protein using iodine monochloride as described (31). Ninety five percent of the radioactivity was precipitated in 10% trichloracetic acid. Radioiodinated VLDL (5×10^5 cpm) from the Ad

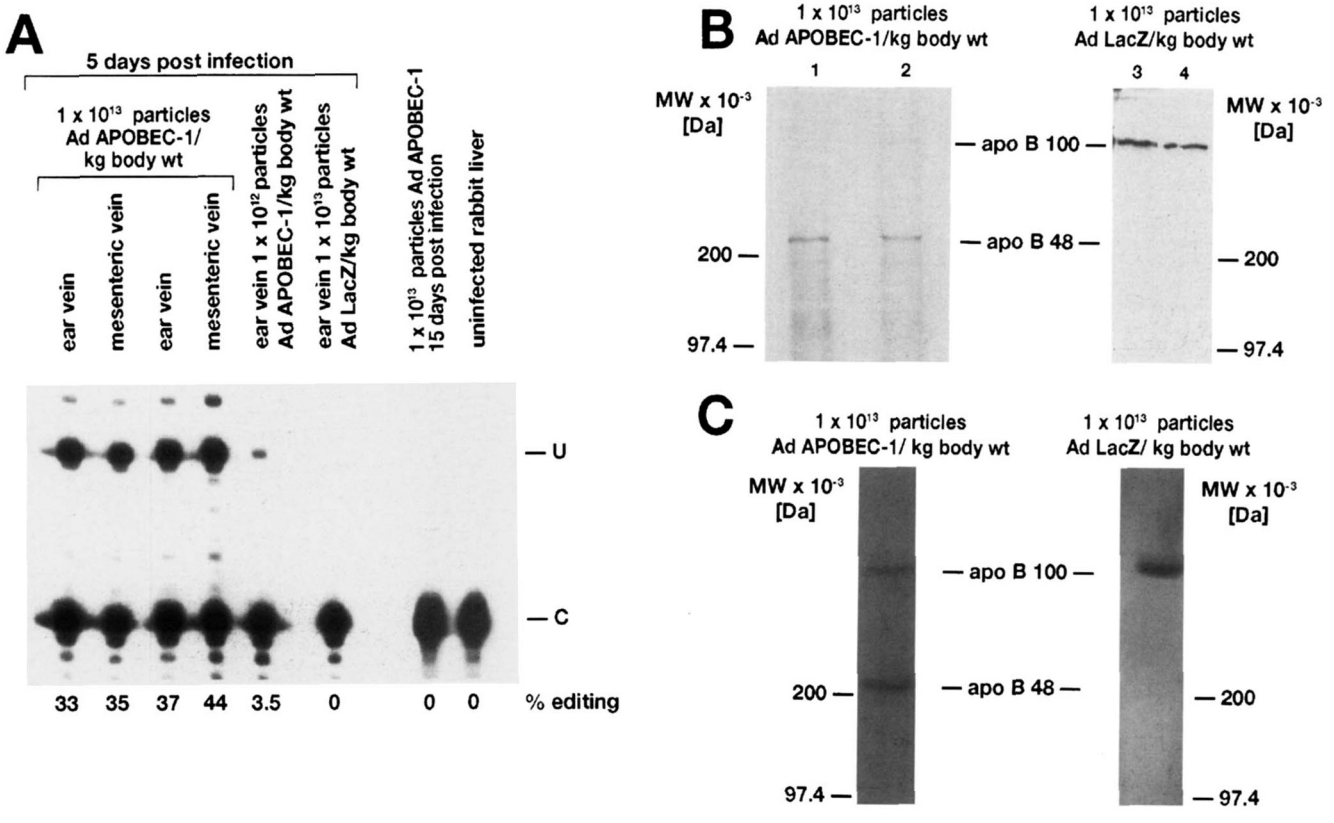

Fig. 1. A: Primer extension analysis of apoB mRNA editing in the liver of rabbits infected with Ad APOBEC-1. NZW rabbits were infected with 1×10^{13} viral particles of Ad APOBEC-1 per kg body weight by injection into a peripheral vein or a mesenteric vein tributary, with 1×10^{12} viral particles of Ad APOBEC-1 per kg body weight and with 1×10^{13} viral particles of Ad LacZ per kg body weight by injection into a peripheral vein. Five and 15 days post infection the apoB mRNA was amplified from total hepatic RNA by RT-PCR. The apoB cDNA was denatured, annealed with ^{32}P-labeled oligonucleotide apoB3, and extended with reverse transcriptase in the presence of ddGTP. This results in extension products of 43 nucleotides for unedited (C) and 54 nucleotides for edited (U) apoB cDNA which were separated by electrophoresis on a 8% polyacrylamide sequencing gel and visualized by autoradiography. The positions for the extension products of unedited (C) and edited (U) apoB cDNA are indicated. The extension products for the apoB RT-PCR products from uninfected rabbit liver are shown as a control. The extension products for unedited (C) and edited (U) apoB cDNA were quantitated using a RadiophosphorImager SF. The proportion of edited cDNA is shown as % editing. B: Immunoprecipitation of apoB from the supernatant of primary hepatocytes isolated from rabbits infected with Ad APOBEC-1 and Ad LacZ. Primary hepatocytes were isolated from the liver of rabbits 5 days after infection with Ad APOBEC-1 (n = 2; each 1×10^{13} viral particles per kg body weight) by infusion into a mesenteric vein tributary (lane 1) or a peripheral vein (lane 2), or with Ad LacZ (n = 2; each 1×10^{13} viral particles per kg body weight) by infusion into a peripheral vein (lane 3 and 4). After incubation of the hepatocytes in medium containing [^{35}S]methionine, apoB secreted into the supernatant was immunoprecipitated using a polyclonal goat anti-human apoB antibody and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Representative autoradiograms are shown. The position of the protein molecular weight markers for 200 kDa and 97.4 kDa are indicated by arrows. C: ApoB in VLDL from plasma of fasted rabbits infected with Ad APOBEC-1 or Ad LacZ. VLDL were isolated from plasma of a fasted rabbit infected with Ad APOBEC-1 (1×10^{13} viral particles per kg body weight) and a fasted rabbit infected with Ad LacZ (1×10^{13} viral particles per kg body weight) by sequential ultracentrifugation at a density of d = 1.019 g/ml. Apoproteins were delipidated in 20 volumes ethanol–ether 3:1 and separated by electrophoresis on a 4% SDS-polyacrylamide gel.

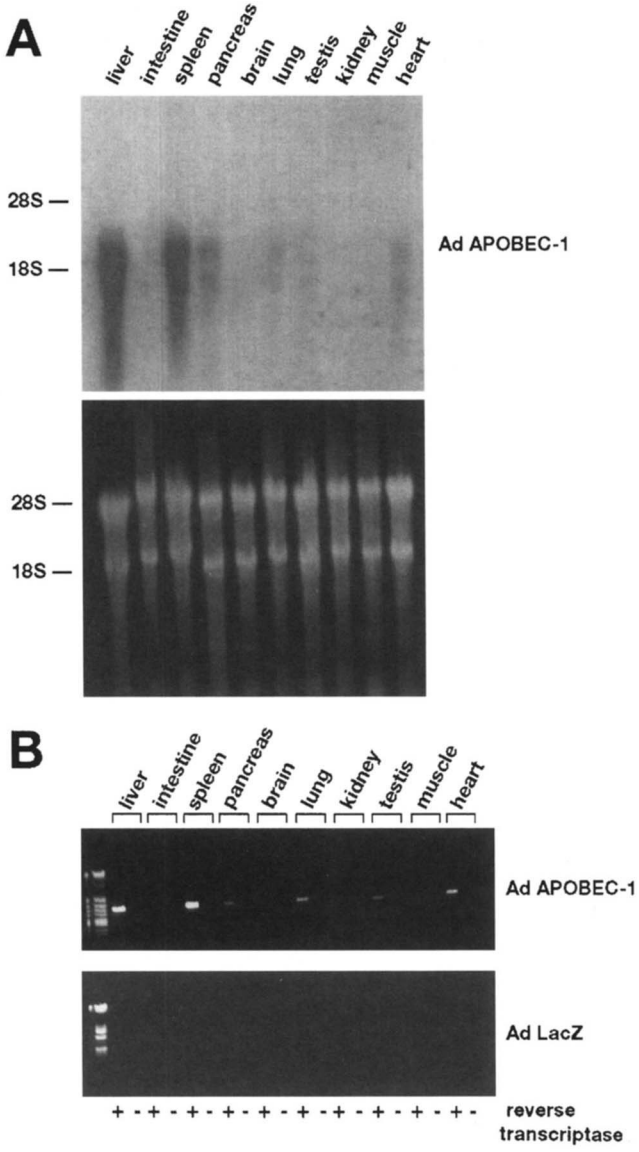

Fig. 2. A: Northern blot analysis for expression of rat APOBEC-1 in a rabbit infected with Ad APOBEC-1. Five days after intravenous infection of a NZW rabbit with Ad APOBEC-1 (1×10^{13} viral particles per kg body weight) total RNA was prepared from ten organs and separated by electrophoresis on a 0.8% agarose, 6% formaldehyde gel, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled full-length cDNA for rat APOBEC-1. A representative autoradiogram is shown above, the ethidium bromide-stained gel is shown below. The positions of 28 S ribosomal RNA and 18 S ribosomal RNA are indicated by arrows. Two transcript sizes for rat APOBEC-1 of 1.0 and 1.8 kb, respectively, corresponding to spliced and unspliced APOBEC-1 transcripts from the APOBEC-1 minigene in Ad APOBEC-1, are found in liver, spleen, pancreas, lung, testis, and heart. B: RT-PCR analysis for expression of rat APOBEC-1 in a rabbit infected with Ad APOBEC-1. Five days after infection of one rabbit with Ad APOBEC-1 (1×10^{13} viral particles per kg body weight) and one rabbit with Ad LacZ (1×10^{13} viral particles per kg body weight), rat APOBEC-1 mRNA was amplified from ten organs by RT-PCR using rat APOBEC-1 specific primers. The RT-PCR products and their respective controls lacking reverse transcriptase during cDNA synthesis were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The photograph of the representative gel for the rabbit infected with Ad APOBEC-1 and for the control rabbit infected with Ad LacZ are shown above and below, respectively.

APOBEC-1- and the Ad LacZ-infected WHHL rabbits were resolved by electrophoresis on 4–15% linear gradient SDS-polyacrylamide gels and analyzed by autoradiography. Subsequently, the radioiodinated VLDL (3×10^7 cpm) were injected into the marginal ear vein of NZW rabbits. Over a period of 2 h, blood samples were drawn from the ear artery and the radioactivity remaining in the blood was measured.

Statistical analysis

For the experiments in the WHHL rabbits, repeated measurement analysis of variants was performed for total cholesterol, triglycerides, VLDL-cholesterol, LDL-cholesterol, and serum alanine-aminotransferase (ALT) both between the two groups using the observations at days 3 and 6 and also within the two groups comparing the observations at days 3 and 6 with the observations at days -7, 0, 15, and 28.

RESULTS

Adenovirus-mediated gene transfer of rat APOBEC-1 in NZW rabbits

The cDNA of APOBEC-1 was recombined into an E1a-defective adenovirus, producing the recombinant virus Ad APOBEC-1. HuH-7 cells were infected with Ad APOBEC-1 at increasing ratios of viral particles/cell (1 to 100), equivalent to MOIs (multiplicity of infection) of 0.05 to 5. Three days after infection, 5%, 38%, 40%, 43%, and 45% of the apoB mRNA was edited at MOIs of 0.05, 0.5, 1.25, 2.5, and 5, respectively. At MOIs of 0.5 to 5,

apoB-48 became the predominant apoB form secreted by HuH-7 cells. Infection of HuH-7 cells with a β-galactosidase recombinant adenovirus (Ad LacZ) at a ratio of 25 viral particles/cell, equivalent to an MOI of 1, resulted in positive staining for β-galactosidase activity in nearly every cell.

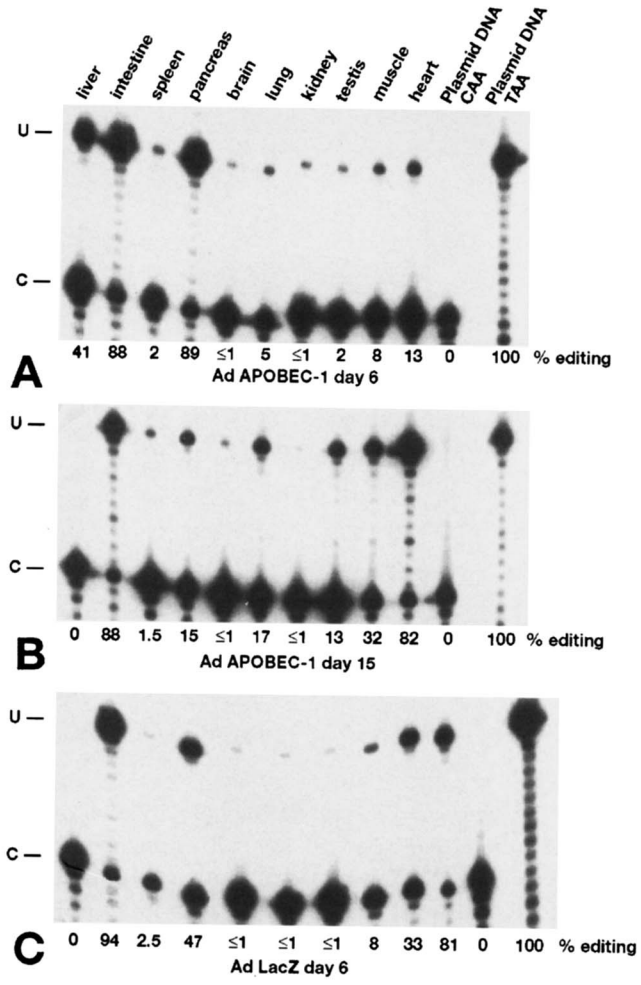

Fig. 3. Primer extension analysis for apoB mRNA editing in organs of rabbits infected with Ad APOBEC-1 or Ad LacZ. Six days (panel A) and 15 days (panel B) after infection of rabbits with Ad APOBEC-1 (1×10^{13} viral particles per kg body weight) and 6 days after infection of a rabbit with Ad LacZ (1×10^{13} viral particles per kg body weight) (panel C), apoB mRNA was amplified from total RNA of ten organs by RT-PCR, annealed with ^{32}P-labeled oligonucleotide apoB3 and extended with reverse transcriptase in the presence of ddGTP. The extension products of 43 nucleotides for unedited (C) and 54 nucleotides for edited (U) apoB cDNA were separated by electrophoresis on a 8% polyacrylamide sequencing gel and visualized by autoradiography. The positions for the extension products of unedited (C) and edited (U) apoB cDNA are indicated. The primer extension analysis of plasmid DNA encoding unedited apoB cDNA sequence (CAA) or edited apoB cDNA sequence (TAA) are shown as controls. The extension products for unedited (C) and edited (U) apoB cDNA were quantitated using RadiophosphorImager SF. The proportion of edited cDNA is given as % editing.

Five days after injection of 1×10^{13} viral particles of Ad APOBEC-1 per kg body weight into the ear vein of two NZW rabbits, 33% and 37% of the hepatic apoB mRNA were edited (**Fig. 1A**). Injection of the same viral dose into mesenteric vein tributaries of two other NZW rabbits resulted in editing of 35% and 44% of the hepatic apoB mRNA (Fig. 1A). Only 3.5% of the hepatic apoB mRNA was edited after intravenous infection with 1×10^{12} viral particles of Ad APOBEC-1 per kg body weight (Fig. 1A). Editing of apoB mRNA was undetectable in the liver of rabbits infected with Ad LacZ (1×10^{13} viral particles/kg body weight), of uninfected rabbits or 15 days after infection with Ad APOBEC-1 (Fig. 1A).

A third extension product, terminating at C6651, was generated in the primer extension assay using hepatic apoB cDNA from rabbits infected with high doses of Ad APOBEC-1 (Fig. 1A). This indicated editing of the apoB mRNA at C6655 in addition to C6666 as previously reported for apoB mRNA from dog liver and intestine (20). To assess the fidelity of apoB mRNA editing reconstituted in rabbit liver by expression of rat APOBEC-1, the apoB PCR products from the liver of a NZW rabbit infected with Ad APOBEC-1 were cloned and sequenced. Six clones contained a T instead of a C at nucleotide position 6666 and one of these edited clones also contained a T at nucleotide position 6655 instead of the C residue present in the genomic sequence at this position. No other sequence heterogeneity was found in 15 apoB cDNA clones between nucleotides 6640–6700.

Predominantly apoB-48 was immunoprecipitated from the supernatant of primary hepatocytes isolated from two Ad APOBEC-1-infected rabbits after labeling the freshly isolated cells with [^{35}S]methionine, whereas exclusively apoB-100 was immunoprecipitated from the supernatant of primary hepatocytes from two Ad LacZ-infected rabbits (Fig. 1B). The apoproteins of VLDL isolated by density gradient ultracentrifugation from plasma of fasted rabbits were analyzed by SDS-polyacrylamide gel electrophoresis. VLDL of Ad APOBEC-1-infected rabbits contained two high molecular weight proteins of 500 and 250 kDa, respectively, corresponding to apoB-100 and apoB-48; of these, the apoB-48 band was more intense (Fig. 1C). In VLDL from rabbits infected with Ad LacZ, apoB-100 was the only high molecular weight protein (Fig. 1C).

Liver biopsy specimens from rabbits infected by intravenous infusion of 1×10^{13} viral particles Ad LacZ/kg body weight demonstrated β-galactosidase activity in 80–90% of hepatocytes, whereas no β-galactosidase activity was detectable in the liver of uninfected or Ad APOBEC-1-infected rabbits. Histological examination of these liver biopsies, performed 5 days after infection with either Ad APOBEC-1 or Ad LacZ, showed a normal liver architecture with microvesicular fat in some hepa-

TABLE 1. Total cholesterol, VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides in NZW rabbits infected with Ad APOBEC-1 or Ad LacZ

	Day 0	Day 5	Day 15	Day 42
Ad APOBEC-1[a] (n = 4)				
Total cholesterol (mg/dl)	39 ± 2.5	102 ± 22	103 ± 21	41, 39
VLDL-cholesterol (mg/dl)	17 ± 3.2	91 ± 24	75 ± 17	10, 7
LDL-cholesterol (mg/dl)	4.3 ± 0.8	not detectable	2.3 ± 0.62	4, 7
HDL-cholesterol (mg/dl)	13 ± 2.9	7.0 ± 2.5	23 ± 4.5	18, 29
Triglycerides (mg/dl)	60 ± 12	196 ± 50	151 ± 17	112, 98
Ad APOBEC-1[b] (n = 2)				
Total cholesterol (mg/dl)	37, 42	44, 48	45, 49	49, 53
VLDL-cholesterol (mg/dl)	9, 17	26, 27	21, 23	17, 21
LDL-cholesterol (mg/dl)	4.0, 6.0	4.2, 5.0	4.5, 6.5	5.1, 6.0
HDL-cholesterol (mg/dl)	18, 22	13, 17	19, 22	22, 27
Triglycerides (mg/dl)	63, 82	59, 65	64, 73	52, 57
Ad LacZ[a] (n = 3)				
Total cholesterol (mg/dl)	45 ± 4.9	98 ± 13	77, 82	48, 52
VLDL-cholesterol (mg/dl)	12 ± 4.5	87 ± 16	61, 65	10, 13
LDL-cholesterol (mg/dl)	6.0 ± 2.2	5.7 ± 0.67	3.0, 4.5	4.9, 6.0
HDL-cholesterol (mg/dl)	29 ± 6.6	8 ± 3.7	15, 22	28, 33
Triglycerides (mg/dl)	72 ± 29	174 ± 12.0	136, 152	108, 123

NZW rabbits were infected with 1×10^{13} viral particles of Ad APOBEC-1 (n = 4) or Ad LacZ (n = 3) per kg body weight or 1×10^{12} viral particles of Ad APOBEC-1 (n = 2) per kg body weight. Total cholesterol, VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides were determined before infection and 5, 15, and 42 days after infection. The mean ± SD are given. Where only two values are available, both are given.

[a] 10^{13} viral particles/kg body weight.

[b] 10^{12} viral particles/kg body weight.

tocytes. Fifteen days after infection, the liver histologies were entirely normal.

Total RNA, prepared from liver, intestine, spleen, pancreas, brain, lung, testis, kidney, muscle, and heart of Ad APOBEC-1- and Ad LacZ-infected rabbits, was analyzed for the expression of rat APOBEC-1. Six days after infection with Ad APOBEC-1, a very high concentration of rat APOBEC-1 mRNA was found in liver and spleen, while a comparably low level of expression was detected in pancreas, lung, testis, and heart (**Fig. 2A**). No hybridization signal was generated for RNA from brain, kidney, muscle, and, notably, intestine (Fig. 2A). The absence of cross-hybridization of the rat APOBEC-1 cDNA probe with the endogenous rabbit APOBEC-1 mRNA in the intestine demonstrated the specificity of the detection for the transgene. In all organs expressing rat APOBEC-1, two distinct mRNAs with sizes of 1 kb and 1.8 kb were detected. These two bands most probably represent spliced and unspliced transcripts, respectively, from the APOBEC-1 minigene in Ad APOBEC-1 that contains the SV40 small T-antigen splice site. Fifteen days after infection with Ad APOBEC-1, rat APOBEC-1 mRNA was not detectable in any of the organs studied. No specific hybridization signal was generated using RNA from organs of a rabbit that had been infected with Ad LacZ. At day 6 after infection with Ad APOBEC-1, rat APOBEC-1 mRNA concentrations, as determined by radiophosphorimaging, were equal in spleen and liver. Therefore, based on the weights of spleen (5 g) and liver (180 g), > 95% of rat APOBEC-1 expression was found in rabbit liver after intravenous injection of Ad APOBEC-1. To further confirm the expression pattern of rat APOBEC-1 in Ad APOBEC-1- and Ad LacZ-infected rabbits, RT-PCR was performed using rat APOBEC-1 specific primers. An RT-PCR product specific for rat APOBEC-1 mRNA was generated using total RNA from liver, spleen, pancreas, lung, testis, and heart of Ad APOBEC-1-infected rabbits, while no product was generated using RNA from brain, kidney, muscle, and intestine (Fig. 2B). In addition, no PCR product was obtained using RNA from any organ of a rabbit infected with Ad LacZ (Fig. 2B).

In all ten organs studied, the apoB mRNA was amplified by RT-PCR and analyzed for editing by primer

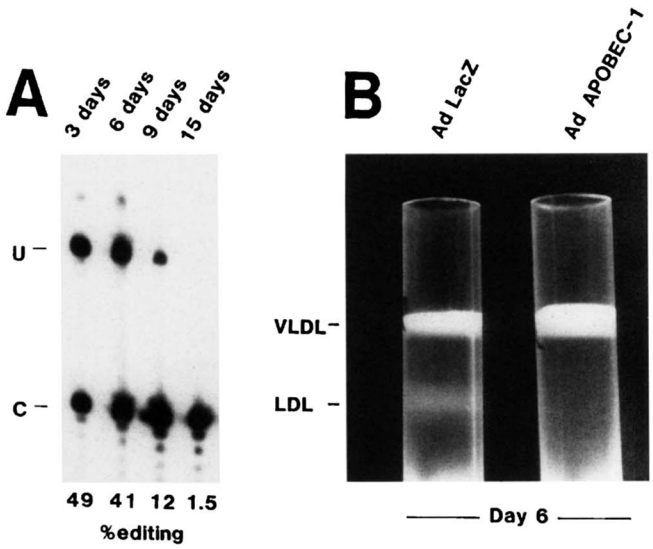

Fig. 4. A: Primer extension analysis for apoB mRNA editing in the liver of WHHL rabbits infected with Ad APOBEC-1. Three, 6, 9, and 15 days after infection of WHHL rabbits with Ad APOBEC-1 (5×10^{12} viral particles per kg body weight) the apoB mRNA was amplified from total hepatic RNA, annealed to ^{32}P-labeled oligonucleotide apoB3 and extended with reverse transcriptase in the presence of ddGTP. The extension products of 43 nucleotides for unedited (C) and 54 nucleotides for edited (U) apoB cDNA were separated by electrophoresis on a 8% polyacrylamide sequencing gel and visualized by autoradiography. A representative autoradiogram is shown. The positions for the extension products of unedited (C) and edited (U) apoB cDNA are indicated. The extension products for unedited (C) and edited (U) apoB cDNA were quantitated using RadiophosphorImager SF and the proportion of edited cDNA is given as % editing. B: Native density gradients after ultracentrifugation of plasma of WHHL rabbits infected for 6 days with Ad APOBEC-1 or Ad LacZ. Six days after infection with Ad APOBEC-1 (n = 2) and Ad LacZ (n = 1) (each 5×10^{12} viral particles of per kg body weight) the plasma of WHHL rabbits was separated by KBr density gradient ultracentrifugation. A photograph of the native density gradients immediately after ultracentrifugation is shown. The bands for VLDL and LDL are indicated by arrows.

extension. Six days after infection with Ad APOBEC-1, high levels of editing were found in liver (41%), intestine (88%), and pancreas (89%) (**Fig. 3A**). Intermediate levels of editing were found in lung, muscle, and heart (5%, 8%, and 13%, respectively) and low levels (1–2%) were found in all other organs including spleen, in which rat APOBEC-1 was highly expressed (Figs. 2 and 3A). The lung continued to exhibit a moderate degree of apoB mRNA editing (17%) 15 days after infection with Ad APOBEC-1, but only < 1% of the apoB mRNA was edited in lung after infection with Ad LacZ (Fig. 3B and C). In all other organs, however, by day 15 after infection with Ad APOBEC-1 the degree of apoB mRNA editing (Fig. 3B) had returned to the levels of editing found in rabbits at day 6 after infection with Ad LacZ (Fig. 3C) or in uninfected rabbits (data not shown). In uninfected rabbits, in Ad LacZ-treated control rabbits, and in Ad APOBEC-1 treated rabbits 15 days after infection, the liver was the only organ in which the extent of apoB mRNA editing was clearly below the detection limit (0.5%) of the primer extension analysis.

The plasma lipoprotein concentrations of Ad APOBEC-1- and of Ad LacZ-infected NZW rabbits were determined by buoyant density gradient ultracentrifugation. The combined results are shown in **Table 1.** Five days after infection with Ad APOBEC-1 at a viral dose of 1×10^{13} viral particles per kg body weight, LDL was undetectable, whereas in the Ad LacZ-treated rabbits the plasma LDL concentrations were unchanged (4–6 mg/dl). However, in both Ad APOBEC-1- and Ad LacZ-infected NZW rabbits, there was a 5- to 6-fold increase of plasma VLDL concentrations. As a result, there was a 2- to 2.5-fold increase of total plasma cholesterol levels in both the Ad APOBEC-1- and the Ad LacZ-treated groups. In addition, HDL concentrations were reduced by 40–50% in both groups. These changes of the plasma

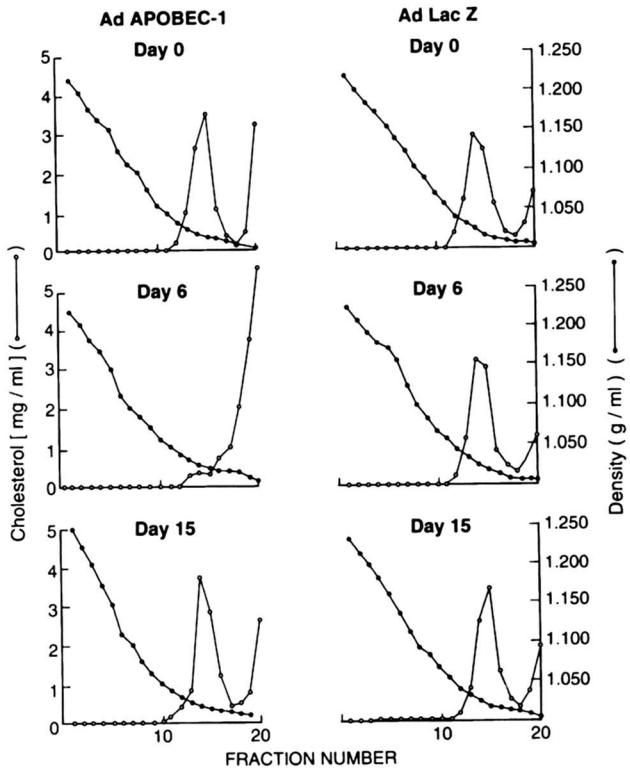

Fig. 5. Density gradient ultracentrifugation analysis of plasma of one WHHL rabbit infected with Ad APOBEC-1 and one WHHL rabbit infected with Ad LacZ. Before infection and 6 and 15 days after infection of rabbits with Ad APOBEC-1 or Ad LacZ (each 5×10^{12} viral particles per kg body weight) the rabbit plasma was subjected to density gradient ultracentrifugation. The cholesterol concentration and the density of each fraction was measured. Representative individual gradients of one Ad APOBEC-1-infected WHHL rabbit and one Ad LacZ-infected control WHHL rabbit are shown. LDL are banding in the fractions 12–16 and VLDL are banding at the top of the gradient in the fractions 19 and 20. No discrete HDL peak (expected in fractions 7–10) was observed in the plasma of WHHL rabbits.

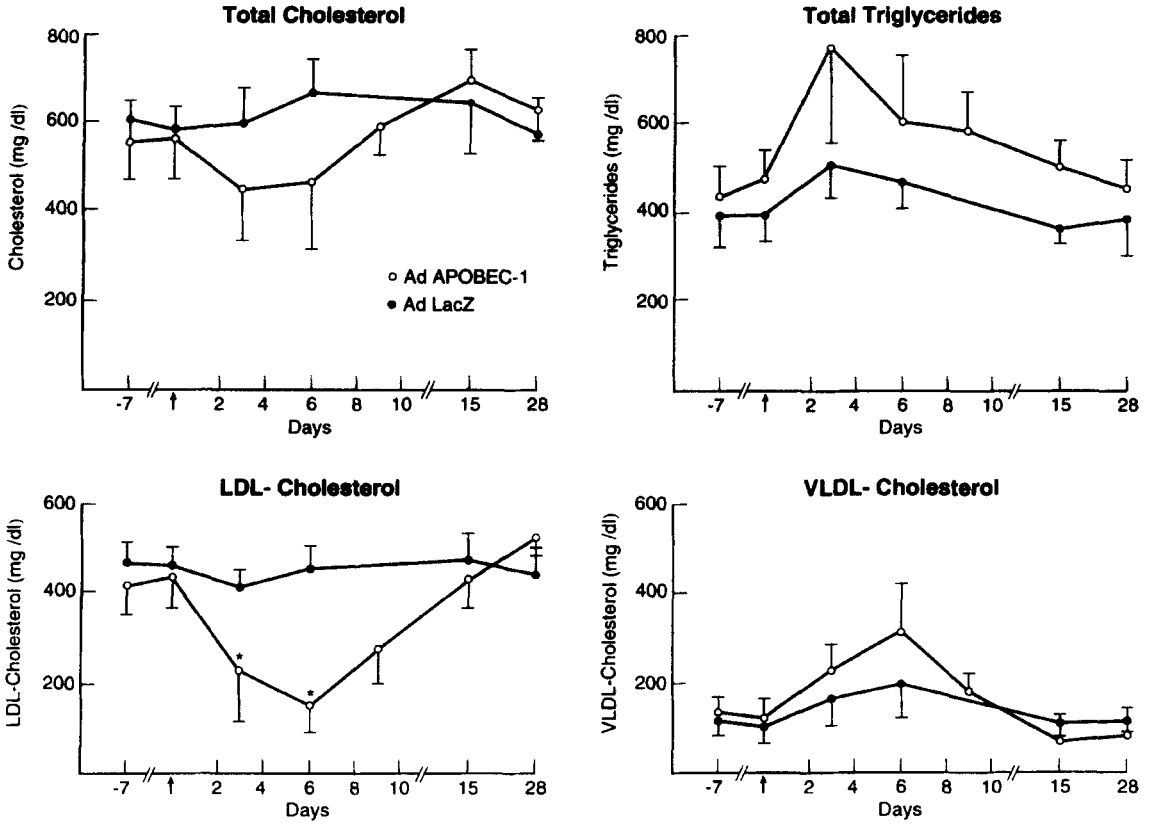

Fig. 6. Total cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol in the plasma of WHHL rabbits before and after infection with Ad APOBEC-1 and Ad LacZ. WHHL rabbits were infected with Ad APOBEC-1 (n = 6) or Ad LacZ (n = 4) (each 5×10^{12} viral particles per kg body weight) by infusion into the marginal ear vein. Seven days before infection, at the day of infection, and at days 3, 6, 9, 15, and 28 after infection, total plasma cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol were determined. Each point represents the mean ± SEM of the two groups. Statistically significant differences in LDL-cholesterol are indicated by an asterisk.

lipoprotein concentrations were less pronounced at day 15 post infection and were completely resolved by day 42. When NZW rabbits were infected with a reduced viral dose of Ad APOBEC-1 (1×10^{12} instead of 1×10^{13} viral particles per kg body weight), only 3–4% of hepatic apoB mRNA was edited (Fig. 1) and plasma lipoprotein concentrations, including plasma LDL levels, did not change compared to the pretreatment values (Table 1).

Adenovirus-mediated gene transfer of rat APOBEC-1 in WHHL rabbits

The low plasma LDL concentrations (4–6 mg/dl) of NZW rabbits impair an assessment of the impact of hepatic apoB mRNA editing on the plasma levels of apoB-100-containing lipoproteins, especially of LDL. Therefore, Ad APOBEC-1 and Ad LacZ were intravenously injected into WHHL rabbits that have 100- to 150-fold higher LDL concentrations than NZW rabbits (33, 34). Comparable to the results in the NZW rabbits, 49%, 41%, 12%, and 1.5%, respectively, of the hepatic apoB mRNA was edited on days 3, 6, 9, and 15 after infection with Ad APOBEC-1 (**Fig. 4A**). Six days after infection with Ad APOBEC-1, concomitant to the generation of hepatic apoB mRNA editing, the visibly prominent LDL band of the density gradients of WHHL rabbit plasma was markedly reduced compared to the LDL band of an Ad LacZ-infected control WHHL rabbit (Fig. 4B).

The quantification of cholesterol in the gradient fractions confirmed the substantial reduction of plasma LDL in WHHL rabbits 6 days after infection with Ad APOBEC-1 (**Fig. 5,** middle panel left). This LDL decrease was accompanied by an increase of VLDL (Fig. 5, middle panel left). Fifteen days after infection with Ad APOBEC-1, the LDL levels returned to pretreatment levels. HDL-cholesterol was not detectable in plasma from untreated, Ad APOBEC-1-treated, or Ad LacZ-treated WHHL rabbits by density gradient ultracentrifugation (Fig. 5). No major alteration of the plasma lipoproteins was observed in a WHHL rabbit that had

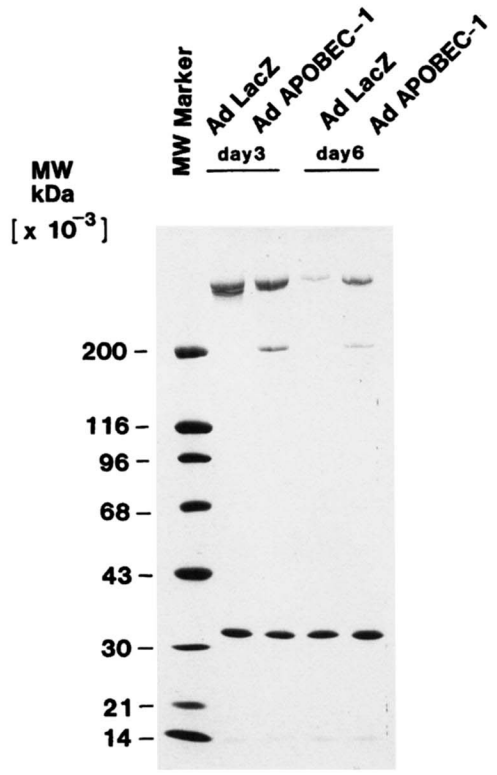

Fig. 7. Apoproteins of VLDL from fasted WHHL rabbits 3 and 6 days after infection with Ad APOBEC-1 or Ad LacZ. VLDL were isolated from plasma of WHHL rabbits 3 and 6 days after infection with Ad APOBEC-1 or Ad LacZ (each 5×10^{12} viral particles per kg body weight) by sequential ultracentrifugation at a density of d = 1.019 g/ml. Twenty-five μg protein was delipidated in 20 volumes of ethanol–ether 3:1 and separated by electrophoresis on a 4–15% linear gradient SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue.

been infected with 5×10^{12} viral particles of Ad LacZ/kg body weight (Fig. 5, right panel).

The combined results of the lipoprotein changes in WHHL rabbits infected with Ad APOBEC-1 or Ad LacZ are shown in **Fig. 6.** Three and 6 days after infection with Ad APOBEC-1, the total serum cholesterol decreased by about 20% and 17%, respectively, from the pretreatment values. After infection with Ad LacZ, however, total cholesterol was unchanged at day 3 and was increased by about 10% at day 6. The LDL-cholesterol was markedly reduced in all six Ad APOBEC-1-infected WHHL rabbits. The maximum reduction of 65% was achieved at day 6. After infection with Ad LacZ, the LDL-cholesterol decreased by 5% at day 3 and increased by 5% at day 6, compared to the pretreatment values. Total plasma triglycerides and VLDL-cholesterol increased in both groups after adenovirus infection. The increase appeared to be greater in the Ad APOBEC-1-infected rabbits than in the Ad LacZ-infected WHHL rabbits, but the difference was not statistically significant. At day 15, the lipoprotein concentrations in the Ad APOBEC-1- and the Ad LacZ-infected WHHL rabbits had returned to pretreatment values. By repeated measurement analysis of variance, the reduction of LDL-cholesterol in Ad APOBEC-1-treated WHHL rabbits was highly significant at days 3 and 6 in comparison to the Ad LacZ-treated WHHL rabbits ($F = 28.26$, $P = 0.0007$). Although there was a trend towards a reduction of total cholesterol in the Ad APOBEC-1-treated compared to the Ad LacZ-treated group, this result was not statistically significant ($F = 4.26$; $P = 0.0728$). No statistically significant difference was found between the two groups in the concentrations of plasma triglycerides and of VLDL-cholesterol comparing the observations at days 3 and 6. Comparing the observations at days -7 and 0 with those at days 15 and 28, no significant difference between or within the two groups was found for any of the four variables. Using repeated measurement analysis of variance within the two groups comparing the observations at days -7, 0, 15, and 28 with those at days 3 and 6, the decrease of LDL-cholesterol in the Ad APOBEC-1-treated rabbits was again highly significant ($F = 41.60$; $P = 0.0001$), but the decrease in total cholesterol again did not reach statistical significance ($F = 3.80$; $P = 0.0669$). The increases in triglycerides and VLDL-cholesterol at days 3 and 6 were significant within both the Ad APOBEC-1-treated ($F = 4.94$; $P = 0.0393$ and $F = 13.39$; $P = 0.0018$, respectively) and the Ad LacZ-treated group ($F = 9.08$; $P = 0.0093$ and $F = 7.63$; $P = 0.0153$, respectively).

To monitor potential hepatic injury in the WHHL rabbits after infection with recombinant adenoviruses, serum alanine-aminotransferase (ALT) activity was measured before infection and at days 3, 6, 9, and 15 after infection. In both the Ad APOBEC-1- and the Ad LacZ-treated groups, serum ALT increased about 3-fold, peaking at day 6 and returning to almost baseline levels by day 15 after infection. Using repeated measurement analysis of variance, no statistically significant difference in the ALT levels was found between the two groups.

The apoproteins of VLDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits were analyzed by SDS-polyacrylamide gel electrophoresis. ApoB-48 was a prominent apoB form in VLDL from fasted WHHL rabbits infected with Ad APOBEC-1, whereas VLDL of fasted WHHL rabbits that had been infected with Ad LacZ contained exclusively apoB-100 (**Fig. 7**). The VLDL from Ad APOBEC-1- and Ad LacZ-treated WHHL rabbits contained equal amounts of apoE (Fig. 7). The content of protein, total cholesterol, triglycerides, free cholesterol, and phospholipids was determined in VLDL and LDL from two Ad APOBEC-1- and two Ad LacZ-infected WHHL rabbits (**Table 2**). No obvious

TABLE 2. Chemical composition of VLDL from WHHL rabbits infected with Ad APOBEC-1 and Ad LacZ

	Total Cholesterol/Protein	Triglycerides/Protein	Free Cholesterol/Protein	Phospholipids/Protein
		mg/mg		
VLDL, untreated	1.66	2.16	0.42	0.16
VLDL, untreated	1.72	1.44	0.49	0.31
Ad APOBEC-1				
VLDL, day 3	1.42	2.62	0.48	0.15
LDL, day 3	1.49	1.62	0.49	0.29
VLDL, day 6	2.26	3.22	0.79	0.16
LDL, day 6	1.36	1.59	0.49	0.26
Ad LacZ				
VLDL, day 3	1.65	2.56	0.46	0.21
LDL, day 3	1.55	1.18	0.41	0.19
VLDL, day 6	3.32	3.57	0.88	0.18
LDL, day 6	1.88	1.77	0.65	0.23

VLDL and LDL were prepared from uninfected WHHL rabbits (n = 2) and from WHHL rabbits 3 and 6 days after infection with Ad APOBEC-1 (n = 2) or Ad LacZ (n = 2) (each 5×10^{12} viral particles per kg body weight) by sequential ultracentrifugation at densities of d = 1.019 g/ml and 1.05 g/ml. For both VLDL and LDL, total cholesterol, triglycerides, free cholesterol, phospholipids, and protein were measured as mg/dl. The ratio of total cholesterol, triglycerides, free cholesterol, and phospholipids per amount of protein (mass/mass) is given as the mean of two independent determinations in two animals each.

differences in the chemical composition of the apoB-containing lipoproteins were found comparing the respective fractions from untreated, Ad APOBEC-1- and Ad LacZ-treated animals (Table 2). For a detailed estimation of the particle diameters, the VLDL were analyzed by electron microscopy (**Fig. 8A and B**). The majority of VLDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits had particle diameters in the range of 25–41 nm, the peak range being from 29–33 nm (Fig. 8C). No differences in particle sizes could be detected comparing VLDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits.

The apoproteins of LDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. Exclusively apoB-100 was found in the LDL of both Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits; apoB-48 was not detectable in the remaining LDL of Ad APOBEC-1-infected WHHL rabbits (**Fig. 9**).

To evaluate whether the VLDL increase in Ad APOBEC-1-treated rabbits could be explained by a reduced plasma clearance of the apoB-48-containing VLDL, we isolated VLDL from one WHHL rabbit each 3 days after injection of Ad APOBEC-1 or Ad LacZ and labeled their apoproteins with ^{125}I. About 95% of the radioactivity in the VLDL was precipitated in 10% trichloracetic acid. The protein-associated radioactivity in the two VLDL preparations, assessed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, was distributed between apoE, the apoB forms, and the small C-apoproteins according to their relative amounts (**Fig. 10A**). After reinjection of the radiolabeled VLDL into normal NZW rabbits, the radioactivity in blood was serially measured up to 2 hours and the fraction of the injected dose per ml blood at each time point was calculated (Fig. 10B). Based on a calculated blood volume of 200–260 ml in these NZW rabbits, 80–95% of the injected dose was recovered at the earliest time point (1 min) after injection. VLDL from both the Ad APOBEC-1- and the Ad LacZ-infected WHHL rabbits exhibited a rapid initial removal from the circulation with half of the injected dose disappearing within 5–10 min. This was followed by a relatively slower second phase. The VLDL from the Ad APOBEC-1-infected WHHL rabbit demonstrated a slightly higher plasma clearance at all time points (Fig. 9B). After 24 h, only 0.5% of the radioactivity from the VLDL of the Ad APOBEC-1-infected WHHL rabbit and 2% from the VLDL of the Ad LacZ-infected WHHL rabbit remained in plasma, based on the amount of radioactivity 1 min after injection. Similar results were obtained with two other preparations of VLDL from Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits.

DISCUSSION

We used the rabbit as an animal model to study the effect of adenovirus-mediated gene transfer of APOBEC-1 on hepatic apoB mRNA editing, hepatic apoB secretion, and the concentrations of apoB-containing lipoproteins in plasma. The rabbit liver, similar to human liver, lacks apoB mRNA editing activity (20). In addition, the elevated plasma LDL levels of WHHL rabbits enabled us to evaluate the impact of hepatic apoB mRNA editing on plasma LDL concentrations (33, 34). This investigation demonstrates that infection of

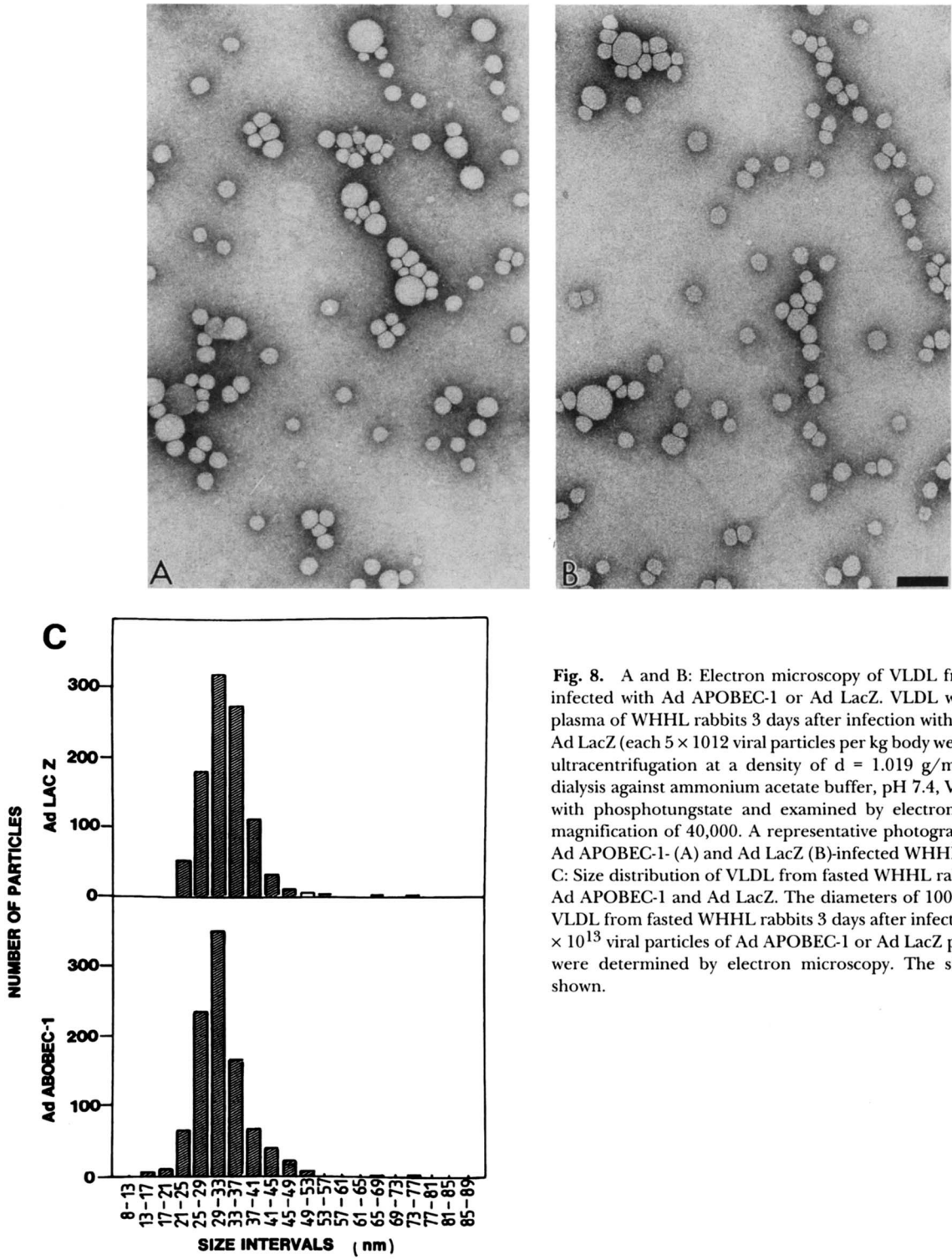

Fig. 8. A and B: Electron microscopy of VLDL from fasted rabbits infected with Ad APOBEC-1 or Ad LacZ. VLDL were isolated from plasma of WHHL rabbits 3 days after infection with Ad APOBEC-1 or Ad LacZ (each 5 × 1012 viral particles per kg body weight) by sequential ultracentrifugation at a density of d = 1.019 g/ml. After extensive dialysis against ammonium acetate buffer, pH 7.4, VLDL were stained with phosphotungstate and examined by electron microscopy at a magnification of 40,000. A representative photograph of VLDL from Ad APOBEC-1- (A) and Ad LacZ (B)-infected WHHL rabbits is shown. C: Size distribution of VLDL from fasted WHHL rabbits infected with Ad APOBEC-1 and Ad LacZ. The diameters of 1000 particles each of VLDL from fasted WHHL rabbits 3 days after infection with either 0.5 × 10^{13} viral particles of Ad APOBEC-1 or Ad LacZ per kg body weight were determined by electron microscopy. The size distribution is shown.

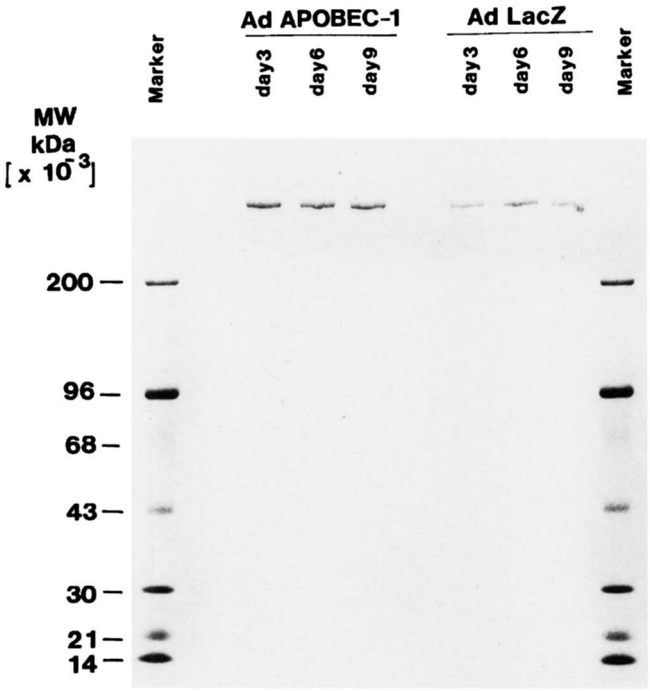

Fig. 9. Immunoblot analysis of LDL from WHHL rabbits infected with Ad APOBEC-1 and Ad LacZ. Three, six, and nine days after infection with Ad APOBEC-1 or Ad LacZ (each 5×10^{12} viral particles per kg body weight) LDL were isolated from plasma of WHHL rabbits by sequential ultracentrifugation at densities of d = 1.019 g/ml and d = 1.050 g/ml. Five µg of protein was delipidated in 20 volumes ethanol–ether 3:1, separated by electrophoresis on a 4–15% linear gradient SDS-polyacrylamide gel, and transferred to a nitrocellulose sheet by electroblotting. ApoB was immunostained using a polyclonal goat anti-human apoB antibody and protein A coupled to peroxidase. Peroxidase activity was detected with 4-chloro-1-naphthol. As molecular weight markers prestained proteins (Rainbow Markers, Amersham) were used. A photograph of the immunoblot including the prestained molecular weight markers is shown.

NZW and WHHL rabbits with Ad APOBEC-1 leads to de novo induction of hepatic apoB mRNA editing and to decreased plasma LDL concentrations.

To assess the overall effect of an adenoviral infection on lipoprotein synthesis, secretion, and metabolism, in every series of experiments equal viral doses of the β-galactosidase recombinant adenovirus Ad LacZ were injected into control animals. A similar VLDL increase and HDL decrease were observed in the Ad APOBEC-1- and Ad LacZ-infected NZW rabbits. This strongly argues for an altered VLDL metabolism after infection of rabbits with recombinant adenoviruses. Despite the identical VLDL increase, only in the Ad APOBEC-1-infected NZW rabbits were the low initial LDL plasma concentrations (4–6 mg/dl) reduced to undetectable levels. Further studies in WHHL rabbits corroborated the specific effect of Ad APOBEC-1 on LDL concentrations. The high LDL plasma levels were clearly reduced only in the Ad APOBEC-1-infected WHHL rabbits. The apoE content, the lipid composition, and the size distribution were very similar in the VLDL of Ad APOBEC-1- and Ad LacZ-infected WHHL rabbits. Thus, a differential metabolism of the apoB-48-containing compared to the apoB-100-containing VLDL remains as the only plausible explanation for the LDL decrease in the Ad APOBEC-1-infected WHHL rabbits. The absence of apoB-48 in the LDL of Ad APOBEC-1-infected WHHL rabbits strengthens this assumption. The full-length protein apoB-100 appears to be required for LDL formation.

A number of recent investigations support these interpretations. In mice, that naturally edit 70–80% of their hepatic apoB mRNA (20), administration of APOBEC-1 recombinant adenoviruses increased the editing of the hepatic apoB mRNA to nearly 100% and reduced the low plasma LDL to undetectable levels (35). Another recent study in mice demonstrated a substantially faster turn-over of apoB-48- relative to apoB-100-containing VLDL (36). In APOBEC-1 transgenic rabbits that express APOBEC-1 in liver, the hepatic apoB mRNA was efficiently edited (23). In our investigation using recombinant adenoviruses for somatic gene transfer of APOBEC-1, editing of apoB mRNA was also efficiently reconstituted in rabbit liver and remained site-specific. Thus, APOBEC-1 is clearly the only component of the apoB mRNA editing enzyme missing in rabbit liver. As in our study, editing of hepatic apoB mRNA in APOBEC-1 transgenic rabbits was accompanied by a substantial reduction of plasma LDL concentrations compared to the non-transgenic litter mates (23). Therefore, the conclusion from our study that induction of apoB mRNA editing in liver with subsequent secretion of apoB-48-containing VLDL results in a reduction of plasma LDL levels in species without natural hepatic apoB mRNA editing finds support from an independent investigation using transgenic animals to address the same biological question.

About 40–50% of the hepatic apoB mRNA was edited in the livers of Ad APOBEC-1-infected NZW rabbits, yet apoB-48 became the predominant apoB form secreted from freshly isolated hepatocytes. Previously, a similar phenomenon was observed in horse liver that naturally edits about 40–50% of hepatic apoB mRNA, but secretes predominantly apoB-48 (20). In rat hepatoma McArdle RH7777 cells, expression of apoB-48 inhibits the synthesis and secretion of apoB-100 by interference with the posttranscriptional regulations of apoB-100 production (37). In addition, alternative polyadenylation has been shown to cause translation of apoB-48 in hepatoma cells without the need for a stop codon by RNA editing (38). Thus, editing of hepatic apoB mRNA does not necessarily have to be extensive to decrease the production of apoB-100-containing VLDL.

The slightly larger VLDL increase in the Ad APOBEC-1- compared with the Ad LacZ-infected WHHL rabbits could be interpreted as a greater toxicity of Ad APOBEC-1. Yet, the increase of serum ALT levels was identical in Ad APOBEC-1- and Ad LacZ-infected animals and in the same range (3-fold) as observed previously using similar doses of LDL-receptor recombinant adenoviruses in WHHL rabbits (39, 40). In NZW rabbits, the infection with Ad APOBEC-1 and Ad LacZ evoked a similar VLDL increase and liver biopsies did not reveal major differences in toxicity between the two viruses. The defect of the LDL-receptor is the more likely cause for the larger VLDL increase in Ad APOBEC-1-infected WHHL rabbits. The LDL-receptor that mediates the rapid hepatic uptake of the apoB-100-containing VLDL remnants is also involved in the clearance of apoB-48-containing chylomicron remnants and therefore most probably of apoB-48-containing VLDL (2, 31, 41, 42). The lack of the full-length protein apoB-100 deprives the Ad APOBEC-1-infected WHHL rabbits of the metabolic route from VLDL to LDL further contributing to the general VLDL increase. The rapid plasma turn-over of apoB-48-containing VLDL in normal NZW rabbits, however, excludes the possibility that editing of apoB mRNA in rabbit liver creates VLDL that per se are inefficiently metabolized due to the lack of apoB-100.

Administration of recombinant adenoviruses into a peripheral ear vein was as effective as injection into a portal vein tributary, as previously reported (39). More than 90% of the expression of rat APOBEC-1 was found in liver and editing of the apoB mRNA was substantially increased only in the liver and to some extent in the pancreas. Therefore, adenovirus-mediated gene transfer of APOBEC-1 to reconstitute apoB mRNA editing is remarkably specific for liver. In contrast to a previous in vitro study (15), our results demonstrate natural apoB mRNA editing and the existence of the putative auxiliary factors in the pancreas and the heart but not in the spleen.

Investigations in subjects with hypobetalipoproteinemia have established that truncated apoB forms lead to hypocholesterolemia because the VLDL that contain truncated apoB forms are rapidly metabolized (43, 44). In addition, a recent study in individuals with heterozygous familial hypobetalipoproteinemia suggests that

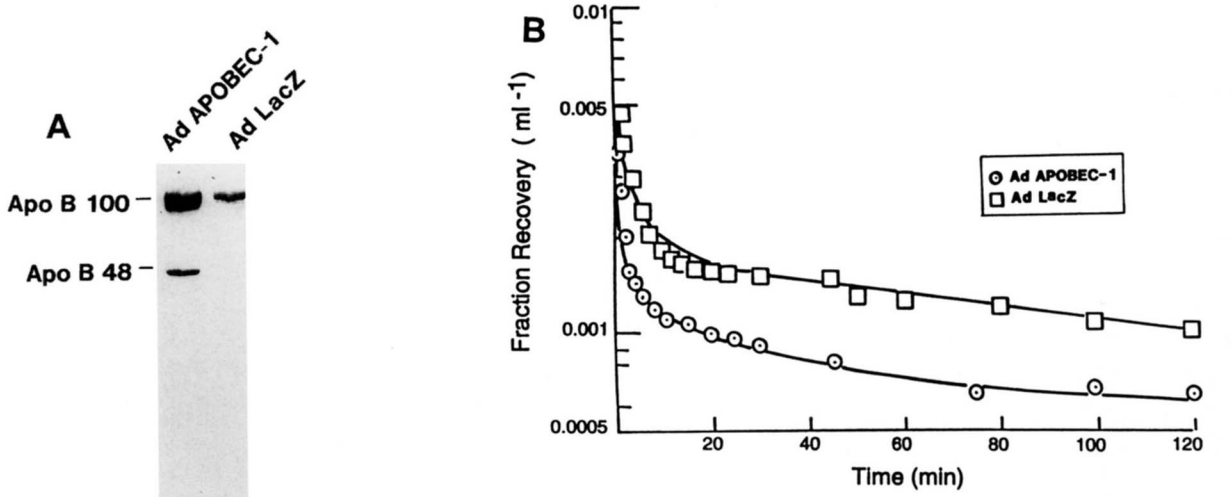

Fig. 10. A: Autoradiogram of ^{125}I-labeled VLDL from WHHL rabbits infected with Ad APOBEC-1 and Ad LacZ. VLDL were isolated from the plasma of WHHL rabbits infected with Ad APOBEC-1 or Ad LacZ (each 5×10^{12} viral particles per kg body weight) by sequential ultracentrifugation at a density of d = 1.019 g/ml. VLDL were labeled with ^{125}I using iodine monochloride. ^{125}I-labeled VLDL (5×10^5 cpm) were separated by electrophoresis on a 4–15% linear gradient SDS-polyacrylamide gel and analyzed by autoradiography. An overnight autoradiogram is shown. B: Disappearance of ^{125}I-labeled VLDL, isolated from WHHL rabbits infected with Ad APOBEC-1 or Ad LacZ, from the plasma of NZW rabbits. VLDL were isolated from the plasma of a fasted WHHL rabbit infected with Ad APOBEC-1 or Ad LacZ (each 5×10^{12} viral particles per kg body weight) by density gradient ultracentrifugation. Purified VLDL were labeled with ^{125}I using iodine monochloride. These two ^{125}I-labeled VLDL preparations (each 3×10^7 cpm) were injected into two normal NZW rabbits. The disappearance of the radioactivity was followed over 2 h. The fraction of the injected dose per 1 ml blood at each time point is given for the two VLDL preparations.

truncated apoB forms decrease the hepatic synthesis and secretion of the remaining apoB-100, possibly by interference with the posttranscriptional regulations of apoB-100 production (45). Lowering of the atherogenic apoB-100-containing lipoproteins by induction of hepatic apoB mRNA editing would require a persistent expression of APOBEC-1 in a large fraction of hepatocytes. The methods presently available for hepatic gene transfer do not achieve these goals (46). Moreover, in APOBEC-1 transgenic mice and rabbits the strong and continuous hepatic expression of APOBEC-1 can lead to hepatocellular carcinoma (23). Therefore, the regulation of APOBEC-1 expression in the liver of species such as dog, horse, rat, and mouse that naturally edit the hepatic apoB mRNA should be further studied to learn how this genetic mechanism with its intrinsic potential to reduce plasma levels of apoB-100-containing lipoproteins could eventually be made available also for humans. Liver-directed gene transfer of APOBEC-1 may be only one possibility. Induction of the endogenous APOBEC-1 gene expression may provide an alternative.

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