Hepatic Apo E Expression Is Required for Remnant Lipoprotein Clearance in the Absence of the Low Density Lipoprotein Receptor

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Abstract

According to the secretion-capture model of remnant lipoprotein clearance, apo E secreted by hepatocytes into the space of Disse serves to enrich the remnants with a ligand for receptor-mediated lipoprotein endocytosis. Current evidence supports a two-receptor model of lipoprotein removal, in which apo E–containing remnants bind either the low density lipoprotein receptor (LDLR) or the LDLR-related protein (LRP). Recently, we demonstrated that reconstitution of apo E(−/−) mice with apo E(+/+) marrow results in normalization of plasma lipoprotein levels, indicating that hepatic expression of apo E is not required for remnant clearance and calling into question the relevance of the secretion-capture mechanism. To dissect the relative contributions of LDLR and LRP to the cellular catabolism of remnant lipoproteins by the hepatocyte, bone marrow transplantation (BMT) was used to reconstitute macrophage expression of apo E in mice that were null for expression of both apo E and the LDLR. Reconstitution of macrophage apo E in apo E(−/−)/LDLR(−/−) mice had no effect on serum lipid and lipoprotein concentrations, although it produced plasma apo E levels up to 16-fold higher than in C57BL/6 controls. Immunocytochemistry of hepatic sections revealed abundant staining for apo E in the space of Disse, but no evidence of receptor-mediated endocytosis of remnant lipoproteins. Transient expression of human LDLR in the livers of apo E(+/+)→apo E(−/−)/LDLR(−/−) mice by adenoviral gene transfer resulted in normalization of serum lipid levels and in the clearance of apo E–containing lipoproteins from the space of Disse. We conclude that whereas the LDLR efficiently clears remnant lipoproteins irrespective of the site of origin of apo E, endocytosis by the chylomicron remnant receptor (LRP) is absolutely dependent on hepatic expression of apo E. These data demonstrate in vivo the physiologic relevance of the apo E secretion-capture mechanism in the liver. (*J. Clin. Invest.* 1998. 101:1726–1736.) Key words: macrophages • bone marrow transplantation • gene delivery • dyslipidemia • mouse models

Introduction

The triglyceride-rich lipoproteins, chylomicrons and VLDL, are processed by lipoprotein lipase in the peripheral circulation to form cholesterol-enriched remnant lipoproteins, which are rapidly cleared from the circulation by the liver (1). Genetic deficiency of apo E in humans (2) and in mice (3, 4) results in the accumulation of cholesterol-rich remnant lipoproteins in the circulation, demonstrating that apo E is essential for remnant lipoprotein clearance by the liver. Current evidence supports a two-receptor model in which both the LDL receptor (LDLR)[1] or the LDLR-related protein (LRP) can mediate the endocytosis of chylomicron remnants by binding apo E, but the relative contribution of each of these receptors under physiological conditions has been difficult to discern. The existence of a separate chylomicron remnant receptor was suggested by observations that humans (5), rabbits (6), and mice (7) lacking the LDLR do not significantly accumulate chylomicron remnants in plasma. On the other hand, antibodies to the LDLR partially inhibit chylomicron remnant removal in mice (8), indicating an important physiologic role for the LDLR in remnant clearance. The LRP binds specifically to apo E, thus suggesting a role for the LRP in chylomicron remnant clearance (9). Adenoviral overexpression of the receptor-associated protein (RAP), a 39-kD protein that inhibits binding of LRP to all its known ligands, does not result in significant remnant accumulation in LDLR(+/+) mice but causes massive accumulation of remnant lipoproteins in LDLR(−/−) mice, providing evidence in vivo that LDLR and LRP both contribute to remnant lipoprotein clearance (10). Furthermore, Herz and co-workers have found recently that inactivation of LRP in the liver of LDLR-deficient mice through conditional gene targeting results in remnant lipoprotein accumulation in LDLR(−/−) mice but not in LDLR(+/+) mice (10a). Therefore, neither the LDLR nor the LRP appears to be absolutely essential for chylomicron remnant clearance, but in the absence of both receptors chylomicron remnant clearance is inhibited.

Investigations of the molecular mechanisms of hepatic clearance of chylomicron remnants support a sequential process involving the interactions of a number of complex macromolecules, including apoproteins, proteoglycans, enzymes, and lipoprotein receptors (11). The proposed steps include sequestration of remnant lipoproteins in the hepatic sinusoids, enzymatic processing, and receptor-mediated endocytosis (12). Apo E has been proposed to play a role in the sequestration process through its ability to bind both heparan sulfate proteoglycans (HSPG) and remnant lipoproteins (1, 13, 14). Once

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1. *Abbreviations used in this paper:* BMT, bone marrow transplantation; HSPG, heparan sulfate proteoglycans; LDLR, low density lipoprotein receptor; LRP, LDLR-related protein; RAP, receptor-associated protein.

sequestered in the space of Disse, the remnant lipoproteins are processed by enzymes, such as hepatic lipase (15), resulting in further exposure of the apo E ligand for receptor-mediated endocytosis (16). Apo E–containing β-VLDL bind to the LDLR with high affinity, but require further enrichment with exogenous apo E for high-affinity binding to the LRP (17).

Significant amounts of apo E are secreted by hepatocytes in a lipoprotein-free form into the space of Disse, where it has been proposed to associate with incoming remnant lipoproteins, facilitating their clearance by the LDLR or LRP (18), a process called secretion-recapture (19, 20) or secretion-capture (14). Local hepatic expression of apo E has been proposed to promote receptor-mediated endocytosis by the LRP (20) or by both the LDLR and the LRP (14, 18). Shimano et al. reported that after the injection of human chylomicrons into transgenic mice overexpressing rat apo E in the liver, the density of apo E staining by immunocytochemistry at the basolateral surface of the hepatocyte decreased and there was an increase in vesicular staining in the cytoplasm, suggesting that apo E on the cell surface was used for the endocytosis of chylomicron remnants (21). However, definitive proof of the existence of the secretion-capture mechanism and demonstration of its physiologic relevance in vivo have been lacking.

The majority of apo E in plasma is hepatic in origin (22), but apo E is also synthesized by a variety of other cells, including macrophages (23). By allowing the selective replacement of bone marrow–derived cells, including macrophages, bone marrow transplantation (BMT) provides an approach to compare the role of hepatic and extrahepatic apo E in remnant lipoprotein clearance. After transplantation of apo E(−/−) mice with wild-type marrow, extrahepatic apo E associates with plasma lipoproteins and promotes their clearance by the liver, resulting in normalization of plasma cholesterol levels (24). Plasma levels of extrahepatic apo E that were only 10% of normal were sufficient to promote the clearance of the accumulating remnant lipoproteins in apo E(+/+)→apo E(−/−) mice. These in vivo data demonstrate that in the presence of both LDLR and the LRP, local expression of apo E by the hepatocyte is not required for remnant lipoprotein clearance. The efficiency of extrahepatic apo E in clearing remnant lipoproteins suggests that apo E secretion by the liver is redundant and calls into question the physiologic relevance of the secretion-capture mechanism.

To examine the role of hepatic apo E expression in the secretion-capture mechanism and to further dissect the relative contributions of LDLR and LRP to remnant lipoprotein clearance, BMT was used to reconstitute macrophage expression of apo E in mice homozygous for the disruption of both apo E and LDLR genes (25). The current studies demonstrate that in the absence of hepatic expression of both apo E and the LDLR, extrahepatic apo E expressed by macrophages is not sufficient to promote the clearance of remnant lipoproteins. Therefore, in the absence of LDLR expression, hepatic expression of apo E is absolutely required for LRP-mediated endocytosis of remnant lipoproteins, demonstrating in vivo the physiologic relevance of the secretion-capture model for remnant lipoprotein clearance.

Methods

Animal procedures. Animals used were at the fourth backcross, or higher, into C57BL/6 background, and were originally purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were maintained in microisolator cages on a rodent chow diet (No. 5010; Purina Mills, Inc., St. Louis, MO) containing 4.5% fat. All mice were given autoclaved water acidified to pH 2.0. 1 wk before and 2 wk after BMT, 100 mg/liter neomycin (Sigma, St. Louis, MO), and 10 mg/liter polymyxin B sulfate (Sigma) were added to the acidified water. Beginning 2 d after transplantation, mice were given gentamicin (80 μg in 1 ml saline; Sigma) and granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA) at the dose of 3×10^6 U in 100 μl saline daily for 5 d by subcutaneous injection. Animal care and experimental procedures were performed according to the regulations of the Animal Care Committee of Vanderbilt University.

Bone marrow collection and transplantation. Bone marrow was collected from donor mice by flushing femurs and tibias using RPMI 1640 with 2% FBS and 5 U/ml heparin (Sigma) added as described (26). Briefly, recipient mice were irradiated 4 h before BMT with 900 rads from a cesium gamma source. Cells were counted, resuspended in RPMI 1640, and injected into the tail vein of recipient mice (5×10^6 cells in 300 μl).

Serum cholesterol and triglyceride analysis. Blood samples were collected by retroorbital venous plexus puncture using heparinized tubes and serum was separated by centrifugation and preserved using 1 mM PMSF (Sigma). All blood collections were performed after a 4-h fast. Serum cholesterol levels were determined using Sigma kit No. 352 adapted for a microtiter plate assay (27). Briefly, 100 μl of a 1:100 dilution of serum was mixed with 100 μl of reagent and incubated at 37°C for 20 min in a microtiter plate. The 490 nm absorbance was read on a microplate reader (Molecular Devices, Menlo Park, CA). Serum triglyceride levels were determined using Sigma kit No. 339 similarly adapted for microtiter plate assay (absorbance was read at 540 nm).

Lipoprotein separation. Serum from mice was subjected to FPLC analysis using a Superose 6 column from Pharmacia (Piscataway, NJ) on an HPLC system (model 600; Waters, Milford, MA). A 100-μl aliquot of serum was injected onto the column and separated with a buffer containing 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA, pH 7.5, at a flow rate of 0.5 ml/min. 40 0.5-ml fractions were collected and tubes 11–40 were analyzed for cholesterol and apo E content. Fractions 14–17 contain VLDL and chylomicrons; fractions 18–24 contain LDL and IDL, fractions 25–29 contain HDL, and fractions 30–40 contain non–lipoprotein-associated proteins.

Ultracentrifugation. Preparation of VLDL ($d < 1.019$), IDL/LDL ($d = 1.019$–1.040), and HDL ($d = 1.040$–1.121) was performed by ultracentrifugation on a table-top centrifuge (TL120; Beckman Instruments, Inc., Fullerton, CA). These lipoprotein pools were desalted by centrifugation over microcon-10 columns (Amicon, Beverly, MA) and the total protein in each fraction was determined according to a modification of the Lowry procedure (28).

Lipoprotein turnover studies. VLDL and LDL were prepared from C57BL/6 and LDLR(−/−) mice, respectively, by ultracentrifugation as described above. The lipoprotein preparations had only trace amounts of albumin. After dialysis, the lipoproteins were iodinated according to a modification of the McFarlane procedure (29, 30). The study was performed with both VLDL and LDL in four mice from each of the following three groups: C57BL/6, LDLR(+/−), and apo E(+/+)/LDLR(−/−)→apo E(−/−)/LDLR(+/−). Jugular vein injections were performed as described (26). Blood was collected from animals immediately after injection (< 10 s) as well as at 1 and 3 h after injection. Data are reported as the percentage of injected counts (as determined by immediate blood collection) assuming that the mouse plasma volume is 3.5% of body weight (31).

Apo E Western blot analyses. Serum samples were tested for the presence of mouse apo E by Western blot analysis. Samples were separated by electrophoresis on 12% SDS polyacrylamide gels. After electrophoresis, proteins were transferred from the gel to nitrocellulose membranes (Gelman, Ann Arbor, MI). The primary antibody was a polyclonal rabbit anti–mouse apo E (BioDesign International, Kennebunk, ME). The secondary antibody was a horseradish peroxi-

dase–conjugated goat anti–rabbit IgG (Amersham, Arlington Heights, IL). Signal was detected using the ECL kit by Amersham. Film was typically exposed for 1 min.

Adenovirus preparation and injections. The adenoviral vector (AdLDLR) containing the human LDLR cDNA was kindly provided to us by Dr. J. Herz (University of Texas, Southwestern Medical Center, Dallas, TX). The virus was constructed using the Ad5 viral backbone and the cDNA is driven by the cytomegalovirus promoter. This virus has been demonstrated to restore LDLR activity in LDLR(−/−) mice (7) and to accelerate cholesterol clearance in normal mice (32). Virus was prepared by collection of infected human embryonic kidney 293 cells (Microbix Biosystems Inc., Ontario, Canada), lysis of cells to release virus, and purification of virus from lysate by cesium chloride gradient centrifugation. The virus was dialyzed against a 10% glycerol solution and frozen at −70°C in multiple aliquots. In this study, two apo E(+/+)/LDLR(−/−)→apo E(−/−)/LDLR(−/−) mice (8 mo after BMT) were given 5×10^8 pfu of AdLDLR via tail vein injection. Blood samples were collected from mice before injection, 4 d after injection, and at the time of killing (7 d after injection). Livers were collected after flushing the carcass with PBS at physiologic pressure. They were then fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analyses.

Immunocytochemical analyses. To collect liver tissues, mice were anesthetized by exposure to methoxyflurane vapors, the abdominal and thoracic cavities were exposed, and the animals were exsanguinated through heart puncture. After clipping the inferior vena cava below the level of the renal arteries, the carcasses were flushed with 20 ml of cold saline injected through the left ventricle at physiologic pressure causing the liver to turn pale. Liver slices ($\sim 1.0 \times 0.7 \times 0.3$ cm) were fixed by immersion in a fresh 4% paraformaldehyde-PBS solution for 2–5 d at 4°C and embedded in paraffin. Serial 5-μm-thick paraffin sections were cut on a microtome (1130/Bio-cut; Reichert-Jung; Leica Instruments, Nussloch, Germany), mounted on Superfrost-plus slides (Fisher Scientific, Pittsburgh, PA), and the presence of apo E, apo B, and LDLR antigens was assessed. The sections were pretreated for 30 min with proteinase K, 50 μg/ml (Promega, Madison, WI) at 37°C to enhance epitope exposure, and the sections were blocked for 20 min in 10% normal goat serum. Each set of sections was incubated overnight at 4°C with either rabbit antiserum against mouse apo E (BioDesign), rabbit antiserum to rat apo B cross-reacting with mouse apo B (a gift of Dr. L. Swift, Vanderbilt University), or with rabbit antiserum to bovine LDLR (AbRb455, a gift of Dr. T. Innerarity, Gladstone Institutes, San Francisco, CA; and Ab638, a gift of Dr. J. Herz). After washing, the sections were treated with biotinylated goat anti–rabbit IgG (PharMingen, San Diego, CA) and incubated with avidin–biotin complex labeled with alkaline phosphatase (Vector Laboratories Inc., Burlingame, CA). The reaction product was visualized with Fast Red TR/Naphthol AS-NX substrate (Sigma). All photomicrographs were taken on an Axioskop microscope (Zeiss, Thornwood, NY) using a ×40 Plan-NEOFLUAR objective resulting in a magnification of 100.

In situ hybridization. RNA probes for mouse LDLR were generated as follows: a fragment of 167 bp (2100–2267 bp) was cut from the mouse LDLR cDNA (courtesy of Dr. Ishibashi, University of Tokyo, Japan) and cloned into pBluescript II SK plasmid (Promega). Antisense and sense RNA probes were prepared after linearization of the plasmid with XbaI and SalI enzymes, and were labeled using an RNA transcription kit (Stratagene, La Jolla, CA). The ^{35}S-labeled 104-bp nucleotide antisense or sense riboprobes for mouse apo E were prepared as described previously (24). In situ hybridization was performed essentially as described (33). Briefly, the serial paraffin sections were treated for 15 min with proteinase K and incubated overnight at 54°C with the RNA probes. The sections were then treated with RNase A, coated with autoradiographic emulsion (Kodak NTB-2) and exposed for 3 wk. After development, the slides were counterstained with hematoxylin.

Table I. Serum Cholesterol and Triglyceride Values from apo E(−/−) Mice with 1, 2, or No LDLR Alleles after Transplantation with apo E(+/+)/LDLR(−/−) Marrow

		Pre-BMT		Post-BMT	
Recipient mouse	*n*	Cholesterol	Triglycerides	Cholesterol	Triglycerides
LDLR(−/−)	15	646±145	144±58	705±186	186±78
LDLR(+/−)	6	705±157	239±87	247±67	192±85
LDLR(+/+)	6	762±315	80±56	147±33	111±54

Total serum cholesterol and triglyceride levels of apo E(−/−) mice with 0, 1, or 2 functional LDLR alleles [LDLR(−/−), LDLR(+/−), and LDLR(+/+), respectively] transplanted with apoE(+/+)/LDLR(−/−) marrow at 12 wk after transplantation. Values are in mg/dl (mean±SD); *n* is the number of animals in each group at each time point.

Results

To examine the role of hepatic expression of apo E in the clearance of remnant lipoproteins by the liver, BMT was used to reconstitute macrophage expression of apo E into mice that were null for expression of both apo E and the LDLR. Previous studies have demonstrated that extrahepatic apo E is sufficient to promote the clearance of remnant lipoproteins, when both the LRP and the LDLR are present (24, 34, 35). Thus, the current studies were designed to examine whether extrahepatic apo E can promote the clearance of lipoproteins in the absence of the LDLR, when the LRP is the main receptor for endocytosis of remnant lipoproteins.

Female recipient mice that were homozygous for targeted disruption of the apo E gene and either homozygous, heterozygous, or wild-type for the targeted disruption of the LDLR were lethally irradiated (9 Gy) and rescued with 5×10^6 bone marrow cells from female apo E(+/+)/LDLR(−/−) donor mice by tail vein injection. To examine the effect of reconstituting macrophage apo E in mice lacking both apo E and LDLR genes, serum cholesterol and triglyceride levels were determined in each group at baseline and 12 wk after BMT (Table I). No significant differences in serum cholesterol or triglyceride levels from baseline were detected in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice 12 wk after BMT. In a subgroup of six mice followed for 5 mo after BMT, serum cholesterol and triglycerides remained essentially unchanged from baseline (cholesterol 786±214 mg/dl, difference from baseline, $P = 0.76$; triglycerides 190±77 mg/dl, difference from baseline, $P = 0.16$). This contrasts with the expected cholesterol-lowering effect of transplantation of apo E(+/+) marrow in mice lacking only the apo E gene (24). In the current study, the mean serum cholesterol level in the apo E(+/+)→apo E(−/−)/LDLR(+/+) control mice dropped by 88% in 4 wk and was normalized for the period of observation. Interestingly, transplantation of apo E(+/+) mouse marrow into apo E(−/−)/LDLR(+/−) mice produced a significant cholesterol-lowering effect (65% reduction) but failed to induce normalization of lipid levels.

Gel filtration chromatography was used to examine the changes in the lipoprotein distribution in the mice after BMT. In the absence of hepatic expression of apo E and the LDLR, reconstitution of macrophage apo E does not promote signifi-

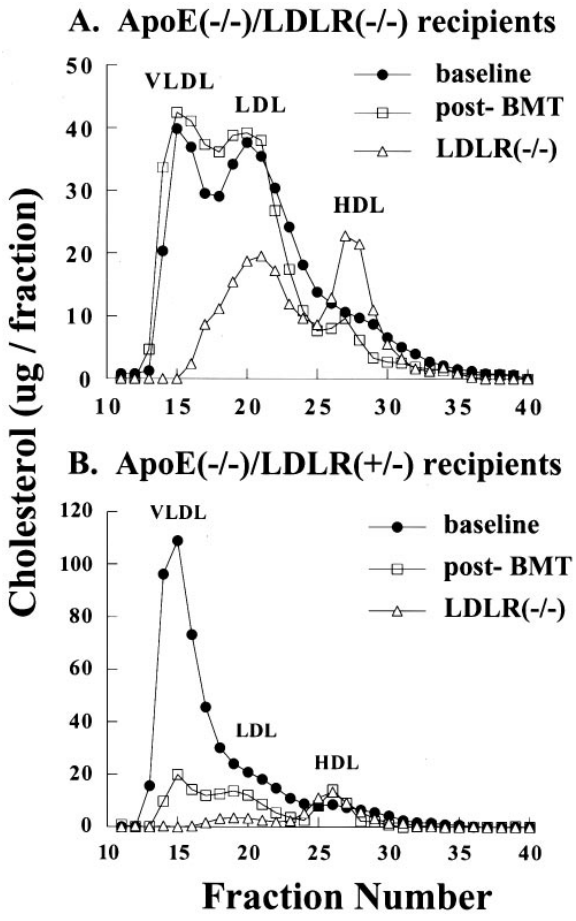

Figure 1. Lipoprotein distribution in apo E(−/−)/LDLR(−/−) or apo E(−/−)/LDLR(+/−) mice before and after transplantation. apo E(−/−) mice having either one or no copies of the LDLR were transplanted with apo E(+/+)/LDLR(−/−) marrow. Lipoprotein distribution was determined by FPLC followed by cholesterol analysis of each fraction. Fractions 14–17 contain VLDL; fractions 18–24 contain IDL/LDL; and fractions 25–29 contain HDL. Fractions 29–35 contain the major plasma proteins. *A* shows the lipoprotein profile of an apo E(−/−)/LDLR(−/−) mouse before transplantation (*filled circles*), compared with its profile after transplantation (*open boxes*), as well as to the profile of an apo E(+/+)/LDLR(−/−) mouse (*open triangles*). *B* shows the average lipoprotein distribution from four apo E(−/−)/LDLR(+/−) mice before transplantation (*filled circles*), compared with their profile after transplantation (*open boxes*), as well as to the profile of three apo E(+/+)/LDLR(+/−) mice (*open triangles*, average of three mice).

cant changes in the lipoprotein profile (Fig. 1 *A*). Fig. 1 *B* shows the effect of reconstitution of macrophage apo E in apo E(−/−) mice heterozygous for the LDLR gene disruption. After transplantation there was a dramatic reduction in the levels of VLDL/IDL, but LDL levels were still higher than normal and higher than in LDLR(+/−) and LDLR(−/−) mice. Turnover studies in these apo E(+/+)→apo E(−/−)/LDLR(+/−) mice revealed that the clearance of ^{125}I-labeled LDL (d = 1.019–1.040 g/ml) was normal in comparison to LDLR(+/−) control mice, whereas the clearance of ^{125}I-labeled remnant lipoproteins ($d < 1.019$ g/ml) was impaired dramatically (Fig. 2).

In the secretion-capture model of remnant lipoprotein clearance, enrichment of remnant lipoproteins with apo E is required for LRP-mediated endocytosis. This enrichment has been proposed to come from either hepatic apo E expression or possibly from the transfer of apo E from other lipoproteins (20). Therefore, the amount of apo E in plasma and on the plasma lipoproteins may be a crucial determinant of remnant lipoprotein clearance. The levels of apo E in plasma of apo E(+/+)→apo E(−/−)/LDLR(−/−) mice were examined by immunoblot analysis using a polyclonal antibody to mouse apo E (Fig. 3). By 1 mo after BMT, apo E levels in plasma of transplanted mice were already higher than the levels in normal C57BL/6 mouse plasma, and progressively increased with time. Computerized scanning densitometry was performed to determine the relative amount of apo E in plasma at serial time points in the apo E(+/+)→apo E(−/−)/LDLR(−/−)

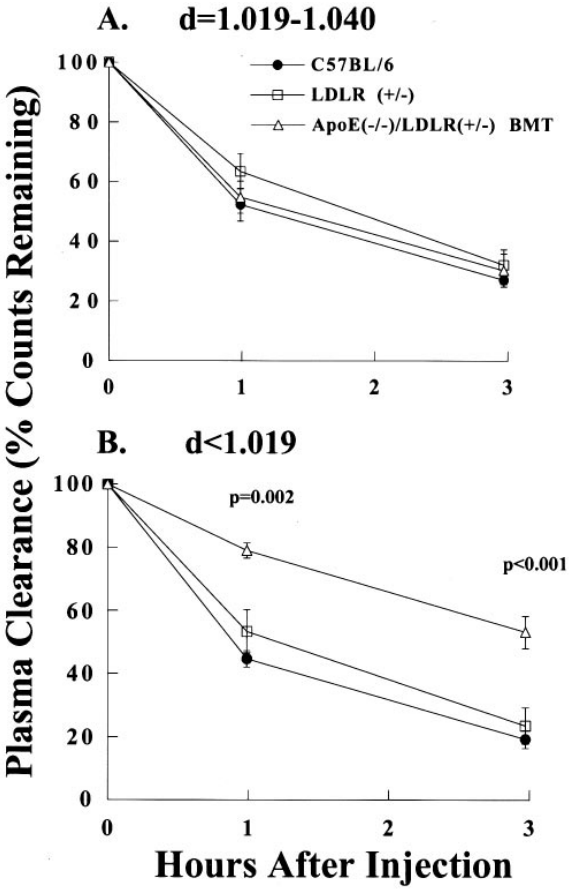

Figure 2. Turnover of ^{125}I-labeled VLDL and LDL in apo E(+/+) →apo E(−/−)/LDLR(+/−) mice. The turnover of both VLDL and LDL was measured in C57BL/6 (*filled circles*), LDLR(+/−) (*open boxes*), and apo E(+/+)→apo E(−/−)/LDLR(+/−) BMT mice (*open triangles*) (four mice in each group). VLDL and LDL were collected and iodinated as described in Methods. The injected dose was 5 μg of lipoprotein protein per mouse (specific activity was 1,558 cpm/ng for the VLDL experiment and 670 cpm/ng for the LDL experiment). Blood was collected at the specified time points and plasma γ-activity was determined using an automated gamma counter (Micromedic Systems, Horsham, PA). *A* shows the turnover of LDL (d = 1.019–1.040) and *B* shows the turnover of VLDL ($d <$ 1.019). Plasma clearance is given as a percentage of counts at the immediate collection (< 10 s after injection).

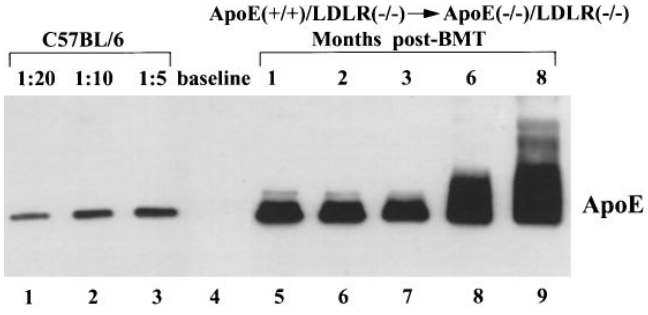

Figure 3. Immunoblot analysis for apo E in serum of an apo E(−/−)/LDLR(−/−) mouse transplanted with apo E(+/+)/LDLR(−/−) marrow. Immunoblot analysis on serum from transplanted mice was performed as described in Methods. Lanes *1–3* contain 3 μl of the indicated dilution of C57BL/6 serum. Lane *4* contains 3 μl of serum from an apo E(−/−)/LDLR(−/−) mouse before transplantation. Lanes *5–9* contain a 1:10 dilution of serum from an apo E(+/+)→apo E(−/−)/LDLR(−/−) mouse at 1, 2, 3, 6, and 8 mo after BMT, respectively.

mice. The plasma levels of apo E in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice at 4 wk after BMT were approximately four times the levels seen in normal C57BL/6 mice. By 6 mo after BMT, the plasma levels of apo E had reached levels greater than nine times above normal. These same mice showed no significant decrease in serum cholesterol or triglyceride levels at this time (845±207 and 247±83 mg/dl, respectively), and no significant changes from baseline were seen in the lipoprotein profile (data not shown). The increasing plasma levels of apo E with time indicate that, in the absence of the LDLR, the remnant lipoproteins are not being cleared from plasma, despite containing progressively more extrahepatic apo E.

The amount of apo E per remnant lipoprotein particle may be an important determinant of receptor mediated endocytosis. Therefore, the $d < 1.019$ g/ml lipoprotein particles were isolated by ultracentrifugation from the serum of apo E(+/+)→apo E(−/−)/LDLR(−/−) mice at 1 and 6 mo after BMT, and the amount of apo E per milligram of VLDL protein was examined by immunoblot analysis (Fig. 4 *A*). The apo E reconstituted on the $d < 1.019$ g/ml (VLDL-IDL) lipoprotein particles of apo E(+/+)→apo E(−/−)/LDLR(−/−) mice 4 wk after BMT was equivalent to or greater than that on normal mouse VLDL/IDL (Fig. 4 *A*). Furthermore, the distribution of apo E among the various lipoprotein fractions was examined in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice 6 mo after BMT. All of the apo E was bound to lipoproteins, and > 95% was present in the VLDL-IDL density fractions (Fig. 4 *B*). Because the level of apo E in plasma 6 mo after BMT was ninefold higher than the level in normal C57BL/6 mice, the VLDL-IDL fraction in plasma of apo E(+/+)→apo E(−/−)/LDLR(−/−) mice should be enriched in apo E compared with the VLDL-IDL in C57BL/6 mice. In fact, the amount of apo E per milligram of VLDL-IDL ($d < 1.019$) protein was twofold greater in apo E(+/+)→apo E(−/−)/LDLR(−/−) mice than in control C57BL/6 mice (Fig. 4 *B*).

High levels of apo E in plasma have been reported to impair the clearance of remnant lipoproteins after adenoviral-mediated apo E gene transfer in apo E–deficient mice, and it was proposed that high plasma levels of apo E may block receptor-mediated endocytosis of remnant lipoproteins (36). Our previous studies have demonstrated that in the presence of functional LDLR, low plasma levels of extrahepatic apo E efficiently promote lipoprotein clearance (24). To examine whether the LDLR can promote lipoprotein clearance in the presence of extremely high plasma levels of extrahepatic apo E, we used an adenovirus encoding the human LDLR (Ad-CMV-LDLR) (7) to introduce expression of the LDLR into two apo E(+/+)→apo E(−/−)/LDLR(−/−) mice 8 mo after BMT, when plasma levels of apo E were 16 times higher than normal (see Fig. 3). 4 d after injection of 5×10^8 pfu of Ad-CMV-LDLR, a significant reduction in smaller VLDL and LDL was observed (Fig. 5). By 7 d after injection, the levels of the larger VLDL were dramatically reduced and the lipoprotein profile was almost normalized. This effect was reflected in a significant reduction in total serum cholesterol from 841 mg/dl at baseline to 380 mg/dl at 4 d and to 148 mg/dl at 7 d after injection. Fig. 6 shows the effect of the adenoviral injection on plasma apo E levels in the post-BMT mice. Lanes *2* and *3* in Fig. 6 show the high level accumulation of apo E in plasma at baseline. Lanes *4* and *5* in Fig. 6 show significant reduction in plasma apo E levels 4 d after the injection. Apo E levels were further reduced by 7 d after injection (Fig. 6, lanes *6* and *7*). 7 d after injection of the AdCMV-LDLR, high level expression of the LDLR mRNA was detected in the majority of hepatocytes by in situ hybridization (Fig. 7 *A*), and computerized analysis

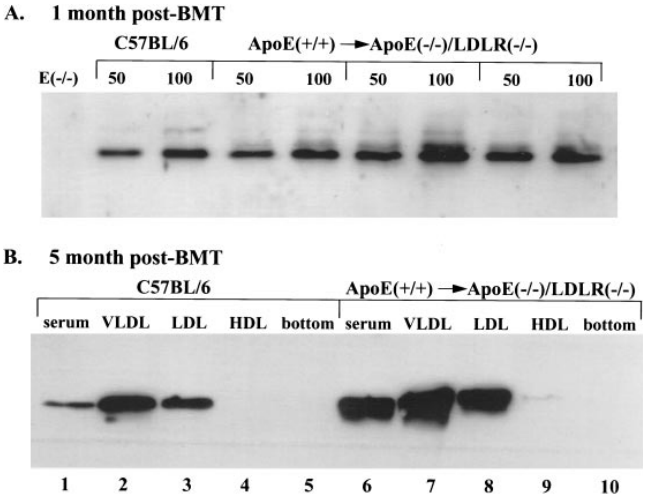

Figure 4. Immunoblot analysis for apo E on VLDL of apo E(−/−)/LDLR(−/−) mice transplanted with apo E(+/+)/LDLR(−/−) marrow at 1 or 5 mo after BMT. Immunoblot analysis for apo E was performed on VLDL/IDL ($d < 1.019$), LDL ($d = 1.019$–1.040), and HDL ($d = 1.040$–1.121) isolated from apo E(+/+)/LDLR(−/−)→apo E(−/−)/LDLR(−/−) mice by ultracentrifugation. (*A*) Lane *1* contains serum from an apo E(−/−) mouse. Lanes *2* and *3* contain 50 and 100 ng of VLDL from a C57BL/6 mouse; lanes *4* and *5*, *6* and *7*, and *8* and *9* contain 50 and 100 ng, respectively, of VLDL from three separate apo E(−/−)/LDLR(−/−) mice transplanted with apo E(+/+)/LDLR(−/−) marrow. (*B*) Lane *1* contains a 1:10 dilution of C57BL/6 serum; lanes *2–5* contain 3 μg of VLDL, IDL/LDL, HDL, and bottom fraction from the same mouse; lane *6* contains a 1:10 dilution of serum from an apo E(+/+)/LDLR(−/−)→apo E(−/−)/LDLR(−/−) mouse; lanes *7–10* contain 3 μg of VLDL, IDL/LDL, HDL, and bottom fraction from the same mouse.

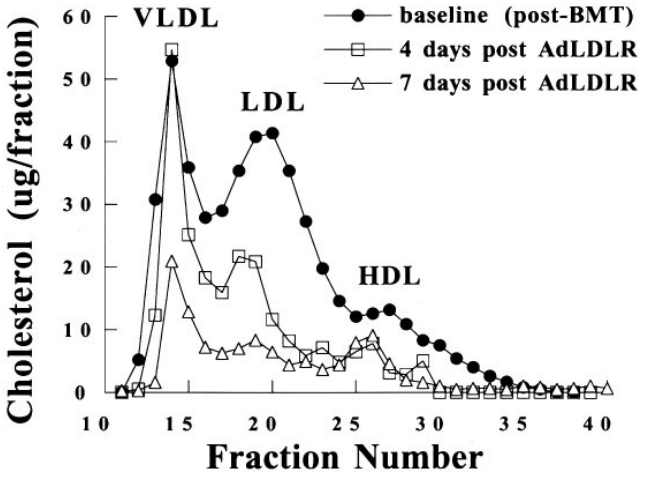

Figure 5. Lipoprotein distribution in apo E(−/−)/LDLR(−/−) mice transplanted with apo E(+/+)/LDLR(−/−) marrow 4 and 7 d after injection with an LDLR-containing adenovirus. apo E(−/−)/LDLR(−/−) BMT mice were given 5×10^8 infectious AdLDLR viral particles by tail vein injection. The lipoprotein distribution was analyzed from serum of mice before adenoviral injection, 4 d after infection, and 7 d after infection. Distribution of lipoproteins among fractions is similar to that described in the legend to Fig. 1.

of hepatic sections stained with an antiserum to the LDLR revealed that 84% of the hepatocytes were expressing the LDLR (Fig. 7 *C*).

The distribution of apo E mRNA expression in the liver was examined by in situ hybridization (Fig. 8). The expression of apo E in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice was abundant in Kupffer cells but was not detected in hepatocytes. Immunocytochemistry was used to examine the distribution of both apo E and apo B in liver sections in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice before and after injection of the AdCMV-LDLR (Fig. 9). In normal C57BL/6 mice, the abundant staining for apo E was distributed according to a dual pattern (Fig. 9 *A*). Intracellularly, apo E was present in a punctate staining pattern representing both apo E synthesized by the hepatocyte and apo E on lipoproteins after internalization. In addition, abundant staining for apo E was seen in the region of the cell surface and the space of Disse. The staining pattern for apo B in C57BL/6 mice was virtually identical to that of apo E (Fig. 9 *E*). As expected, no staining for apo E was detected in control sections from apo E(−/−) mice (Fig. 9 *B*). However, there was abundant staining for apo B in apo E(−/−) mice both intracellularly and in the region of the cell surface and the space of Disse (Fig. 9 *F*). In contrast, in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice intense immunoreactivity for apo E was present on the cell surface and in the space Disse, but the pattern of punctate staining was not detected within the cytoplasm of the hepatocytes, an indication that no uptake of apo E–containing lipoproteins was occurring (Fig. 9 *C*). On the other hand, apo B immunoreactivity was abundant both in the region of the cell surface and the space of Disse and in the cytoplasm of the hepatocytes in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice (Fig. 9 *G*). After the injection of the AdCMV-LDLR in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice, there was virtually no staining for apo E and apo B in the space of Disse, and an intense pattern of staining for both proteins was detected in the cytoplasm (Fig. 9, *D* and *H*), indicating that the acute expression of the LDLR gene induced internalization of the apo E–containing lipoproteins that were present in the space Disse. The vacuolated appearance of cells in Fig. 9, *D* and *H*, is likely related to the sudden intake of large amounts of lipoproteins.

Discussion

The current studies demonstrate that after the reconstitution of macrophage expression of apo E in apo E(−/−)/LDLR(−/−) mice, apo E accumulates on plasma lipoproteins and in the space of Disse, yet there is no receptor-mediated endocytosis of remnant lipoproteins. The establishment of transient high level expression of the human LDLR in the livers of apo E(+/+)→apo E(−/−)/LDLR(−/−) mice by adenoviral gene transfer results in normalization of the plasma lipids and lipoprotein profiles and in the clearance of the apo E–containing lipoproteins from the space of Disse. Combining the observations from these in vivo experiments, we conclude that the LDLR is capable of efficient clearance of apo E containing remnant lipoproteins even in the absence of hepatic expression of apo E. Conversely, receptor-mediated endocytosis by the chylomicron remnant receptor (LRP) is absolutely dependent on hepatic expression of apo E.

The relative contributions of the LDLR and the LRP to remnant lipoprotein clearance under physiologic conditions has been a subject of intense investigation and debate. Reconstitution of macrophage apo E in apo E(−/−)/LDLR(+/+) mice results in normalization of serum lipids and lipoprotein profiles (reference 24 and Table I). Thus, in the presence of hepatic expression of the LDLR and the LRP, local expression of apo E by the hepatocyte is not required for remnant lipoprotein clearance. One of the goals of this study was to examine the relative contributions of the LDLR and the LRP to the hepatic endocytosis of remnant lipoproteins in this model. Therefore, we examined the capacity of macrophage apo E to promote remnant lipoprotein clearance by the LRP in the ab-

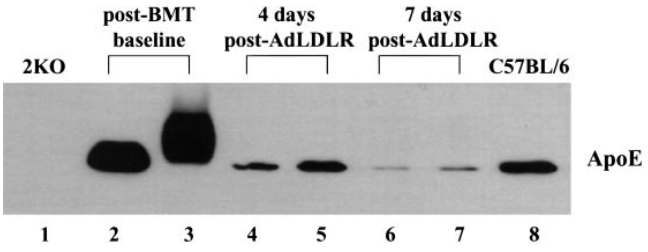

Figure 6. Immunoblot analysis for apo E in serum of an apo E(−/−)/LDLR(−/−) transplant mice after injection with an LDLR-containing adenovirus. The serum from apo E(−/−)/LDLR(−/−) mice before injection, 4 d after injection, and 7 d after injection was analyzed for apo E content. For each sample, 3 μl of 1:10 dilution was subjected to SDS-PAGE, followed by immunoblot analysis as described in Methods. Lane *1* contains serum from an apo E(−/−)/LDLR(−/−) mouse; lanes *2* and *3* are from apo E(−/−)/LDLR(−/−) transplanted mice before AdLDLR injection; lanes *4* and *5* are from these same mice 4 d after AdLDLR injection and lanes *6* and *7* are from these mice 7 d after AdLDLR injection. Lane *8* contains serum from a C57BL/6 mouse.

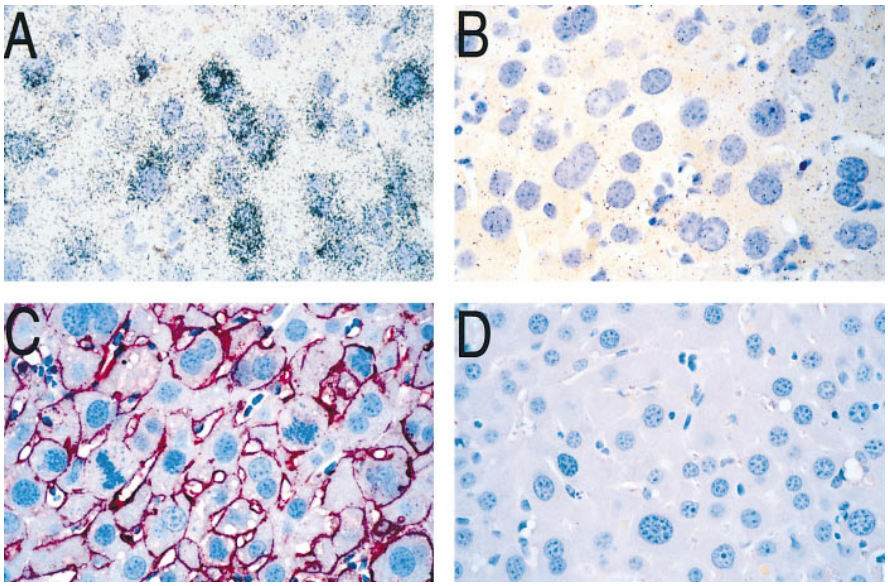

Figure 7. Analysis of LDLR expression in apo E(+/+)→apo E(−/−)/LDLR(−/−) mice after infection with an LDLR-containing adenovirus. 5-μm paraffin-embedded liver sections obtained from the apo E (+/+)→apo E(−/−)/LDLR(−/−) mice on day 7 after infection with the AdLDLR viral particles were examined for expression of LDLR mRNA by in situ hybridization and LDLR antigen by immunocytochemistry. High level expression of the LDLR was detected in the sections hybridized with a ^{35}S-labeled 167 nucleotide antisense RNA probe to the mouse LDLR (*A*) as compared with control sections probed with the corresponding sense probe (*B*). Immunocytochemistry of the sections with a rabbit antiserum to the bovine LDLR (*C*) shows high levels of LDLR antigen expression as compared with negative control sections in which nonimmune rabbit serum was used in place of the primary antibody (*D*). ×100.

sence of LDLR expression, by reconstituting macrophage apo E expression in apo E(−/−)/LDLR(−/−) mice. Serum lipid levels and lipoprotein profiles did not change significantly in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice (Table I and Fig. 1). These results indicate that the LDLR is crucial for remnant lipoprotein clearance in the apo E(+/+)→apo E(−/−) mice and that, in the absence of hepatic apo E expression, the LDLR efficiently promotes the clearance of remnant lipoproteins containing macrophage apo E.

The efficiency of the LDLR in promoting lipoprotein clearance in this model was examined by transplanting apo E(−/−)/LDLR(+/−) mice with apo E(+/+)/LDLR(+/+) marrow. On a normal chow diet, total plasma cholesterol levels in LDLR(+/−) mice are ∼ 35% higher than the levels in wild-type littermates due to accumulation of cholesterol in the IDL/LDL range (7). Serum cholesterol level decreased by 65% from baseline in the apo E(+/+)→apo E(−/−)/LDLR(+/−) mice (Table I), but remained significantly higher than the cholesterol level in apo E(+/+)→apo E(−/−)/LDLR(+/+) control mice. Analysis of the lipoprotein distribution in the apo E(+/+)→apo E(−/−)/LDLR(+/−) mice revealed a dramatic decrease of cholesterol in the VLDL/IDL as compared with baseline apo E(−/−)/LDLR(+/−) mice, but compared with LDLR(+/−) mice there was a significant accumulation of cholesterol in the VLDL-IDL-LDL range (Fig. 1*B*). Turnover studies indicated delayed clearance of ^{125}I-labeled VLDL/IDL but normal clearance of ^{125}I-labeled LDL cholesterol in these apo E(+/+)→apo E(−/−)/LDLR(+/−) mice (Fig. 3). Thus, in the absence of hepatic expression of apo E, a gene–dosage effect of LDLR expression can be detected in the clearance of macrophage apo E–containing remnant lipoproteins. Although hepatic apo E expression is not required for the clearance of apo E–containing remnant lipoproteins when the full complement of LDLR is available, its function be-

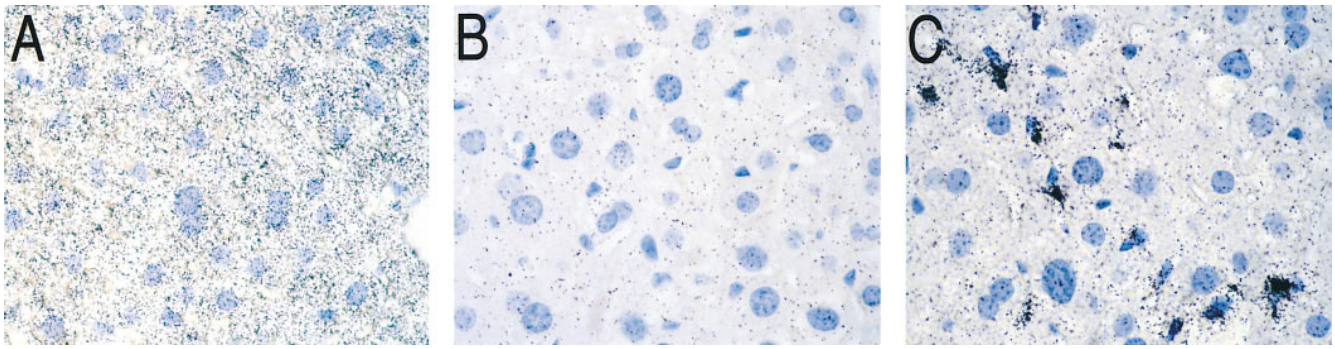

Figure 8. Analysis of expression of apo E mRNA in the livers of apo E(+/+) and apo E(−/−)→apo E(−/−)/LDLR(−/−) mice by in situ hybridization. 5-μm paraffin-embedded liver sections were hybridized with either a ^{35}S-labeled 104-nt antisense RNA probe to the mouse apo E gene or the corresponding sense probe as a negative control. High level expression of apo E mRNA is seen in the hepatocytes of liver sections from wild-type C57BL/6 mice hybridized with the ^{35}S-labeled antisense RNA probe to the mouse apo E gene (*A*) but not in control sections probed with the corresponding sense probe (*B*). Hybridization of the liver sections from the apo E(+/+)→apo E(+/+)/LDLR(−/−) mice with the antisense probe to mouse apo E mRNA shows that apo E expression is confined to the Kupffer cells and is not present in the hepatocytes (*C*). ×100.

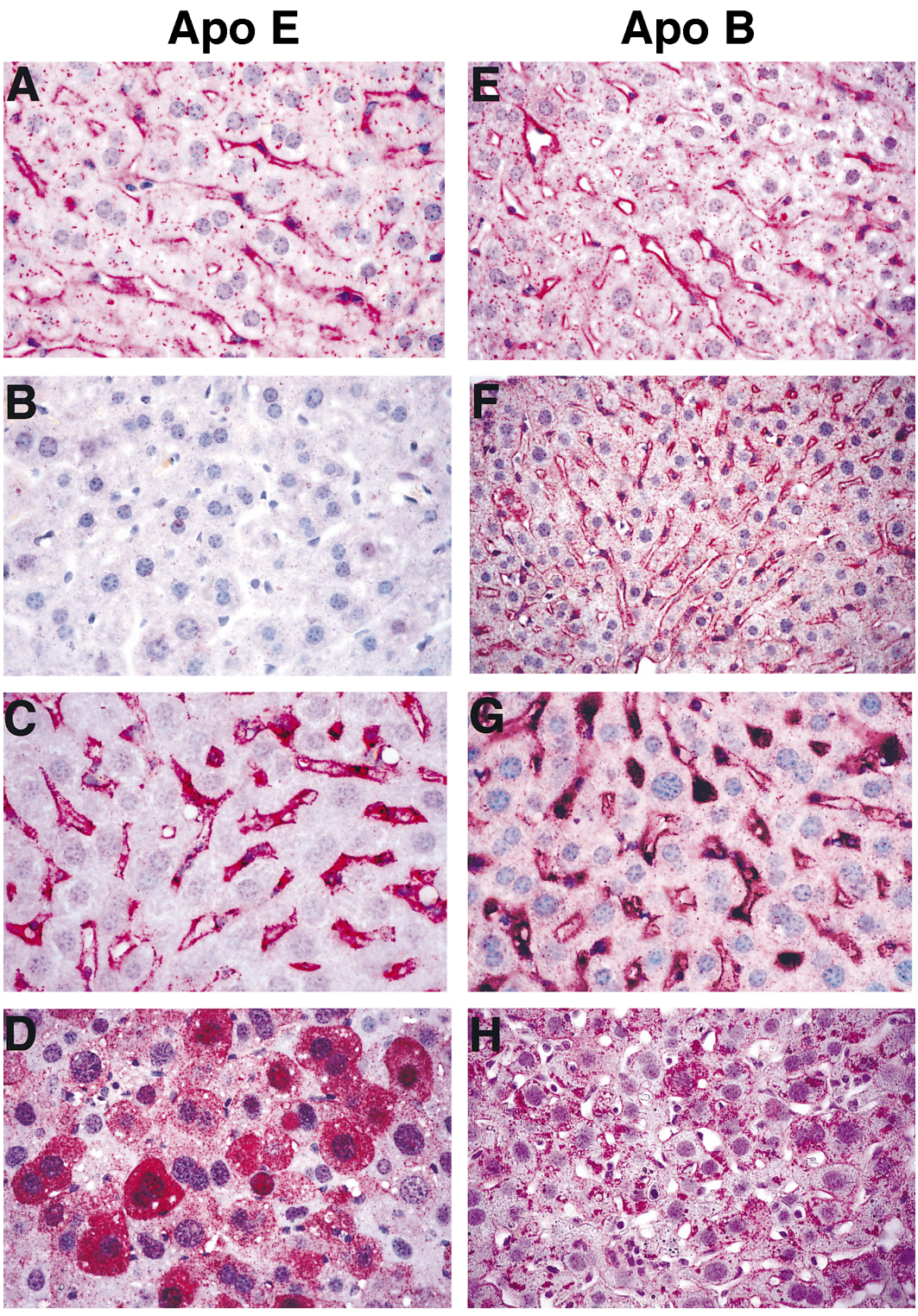

Figure 9. Immunohistochemical analysis for apo E and apo B in liver sections from apo E(+/+)→apo E(−/−)/LDLR(−/−) mice before and after infection with an LDLR-containing adenovirus. Paraffin-embedded liver sections were prepared from the mice and stained with either a rabbit polyclonal antibody to mouse apo E (BioDesign) (*A–D*) and with a rabbit antiserum to rat apo B (*E–H*) and were visualized with Fast Red TR/Napthol AS-NX substrate as described in Methods. *A* and *E* contain liver sections from a C57BL/6 mouse as normal controls, and *B* and *F* contain sections from an apo E(−/−) mouse as a negative control. *C* and *G* contain liver sections from an apo E(+/+)→apo E(−/−)/LDLR(−/−) mouse. *D* and *H* contain sections from an apo E(+/+)→apo E(−/−)/LDLR(−/−) mouse 7 d after infection with AdLDLR (as described in Methods). ×100.

comes obvious when LDLR expression is reduced, and remnant clearance depends mostly on LRP-mediated endocytosis stimulated by hepatic apo E.

Ishibashi et al. have reported previously that intravenous injection of a recombinant replication-defective adenovirus encoding the human LDL receptor driven by the cytomegalovirus promoter into LDLR-deficient mice reduced the elevated IDL/LDL cholesterol to normal in 4 d (7). In this study, we examined the ability of adenovirus-mediated gene transfer of the human LDLR to correct the severe hypercholesterolemia in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice. 7 d after injection of the AdCMV-LDLR, serum cholesterol levels and the lipoprotein profile were normalized. Analysis of hepatic sections by immunocytochemistry revealed intense staining for apo E and apo B in a punctate pattern in the cytoplasm of the infected hepatocytes and almost a total absence of staining for apo B or apo E in the region of the cell surface and the space of Disse (Fig. 9, *D* and *H*). Thus, acute restoration of LDLR

expression by adenoviral gene transfer promotes rapid clearance of remnant lipoproteins containing macrophage-derived apo E, supporting our hypothesis that LDLR-mediated clearance of apo E–containing remnant lipoproteins is not dependent on hepatic expression of apo E.

Apo E has been proposed to play a role in the initial sequestration of remnant lipoproteins by the liver in the space of Disse (1). Apo E binds to both lipoproteins and HSPG, and might help sequester remnant lipoproteins in the space of Disse, bringing them into close proximity to lipoprotein receptors (12). Herz and others showed that the initial sequestration of lipoprotein remnants in the space of Disse is a phenomenon independent from either LDLR or LRP, and postulated that it depends on the presence of apo E (37, 38). In the current study, abundant apo B was detected in the space of Disse in apo E–deficient mice as well as in C57BL/6 mice (Fig. 9, *E* and *F*). The presence of abundant apo B in the sinusoidal space of apo E(−/−) mice may represent both newly secreted lipoprotein particles and particles derived from the circulation. In the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice, apo E–containing remnant lipoproteins are present in the region of the cell surface and the space of Disse, yet no punctate staining for apo E in the cytoplasm of hepatocytes is detected (Fig. 9 *C*). Although these apo E–containing particles may represent remnants sequestered in the space of Disse through binding to either HSPG, apo E, or other molecules anchored to the cell surface, it is also possible that they represent particles entrapped in the sinusoidal space independent of binding processes. That these particles containing extrahepatic apo E are in close proximity to lipoprotein receptors on the hepatocyte cell membrane is demonstrated by the fact that they are rapidly endocytosed when LDLR expression is introduced by adenoviral gene transfer. These data are compatible with the interpretation that, in the absence of LDLR and hepatic apo E expression, close proximity between apo E–containing lipoproteins in the space of Disse and the remnant receptor (LRP) is not followed by particle endocytosis. We speculate that local hepatic expression of apo E is not required for the sequestration of lipoproteins in the space of Disse, and some process other than sequestration of remnant lipoproteins underlies the requirement for hepatic expression of apo E in the receptor-mediated endocytosis by the LRP.

A central tenet of the secretion-capture model of chylomicron remnant clearance is that hepatic expression of apo E serves to enrich the incoming remnant lipoproteins with ligand, activating them for receptor-mediated endocytosis by the LRP (20). As early as 4 wk after BMT, the plasma levels of apo E in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice are significantly higher than those seen in normal C57BL/6 mice (Fig. 3). The amount of apo E in plasma increased with time to levels that were ~ 9 times normal by 6 mo after BMT and 16 times normal by 8 mo (Fig. 4 *B*). Analysis of the distribution of apo E on the plasma lipoproteins 6 mo after BMT revealed that the majority of apo E was located in the VLDL-IDL fraction, indicating that the remnant lipoproteins were significantly enriched with apo E compared with VLDL-IDL from normal mice (Fig. 4 *B*). At 5 mo after BMT, the VLDL-IDL contain twice as much apo E per particle as VLDL-IDL from normal C57BL/6 mice (Fig. 4 *B*). In addition, immunocytochemical studies reveal that the amount of apo E in the space of Disse of the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice is equivalent to or greater than that seen in C57BL/6 mice (Fig. 9, *A* and *C*). Despite the presence of elevated levels of apo E on plasma VLDL-IDL and abundant staining for apo E in the region of the cell surface and the space of Disse, LRP-mediated endocytosis does not occur in the absence of hepatic expression of apo E.

This observation is particularly relevant because it proves that local production of apo E is necessary for LRP-mediated remnant clearance, and suggests that the secretion-capture mechanism involves more than a simple process of apo E transfer to incoming remnants. Although it is possible that hepatic apo E secretion may produce a local concentration of apo E in the space of Disse higher than that achieved from the 16-fold increase in plasma levels of apo E observed in the transplanted double knockout mice, it is difficult to envision a simple quantitative mechanism with such a high threshold and without any measurable dose-related effects below it. Kuchenhoff et al. have reported recently that a β-VLDL subfraction isolated from cholesterol-fed rabbits by gel filtration designated β-$VLDL_{II}$ bound specifically to LRP on mouse peritoneal macrophages and HepG2 cells without addition of apo E (39). Interestingly, of the three different β-VLDL subfractions that were isolated, the β-$VLDL_{II}$ contained the least amount of apo E. These observations suggest that the amount of apo E per particle may not be the most important determinant of binding to the LRP.

Several alternative explanations for the dependence of LRP-mediated endocytosis on hepatocyte expression of apo E can be envisioned. For example, it is possible that the conformation of extrahepatic apo E on the lipoproteins in the apo E(+/+)→apo E(−/−)/LDLR(−/−) mice is not amenable to LRP-mediated endocytosis. The expression of free (non–lipoprotein-bound) apo E by the hepatocyte may be required for the proper positioning of apo E on the remnant lipoproteins to activate binding to the LRP. Alternatively, free hepatic apo E may be required to bind to the LRP before it binds the sequestered remnant lipoproteins. In addition, it is possible that hepatic expression of apo E is required to promote the ability of LRP to function as a chylomicron remnant receptor. Studies by Herz and co-workers suggest that RAP may work as a chaperone protein for LRP (40), preventing premature association of LRP with apo E and thereby preventing aggregation of LRP within the cell (40). Adenoviral overexpression of apo E in fibroblasts results in a decrease in LRP expression in fibroblasts, which can be prevented by coexpression with adenoviral RAP(−/−) (41). Therefore, apo E and RAP both appear to affect the intracellular transport of LRP. Although normal transport of LRP to the cell surface occurs in neurons and fibroblasts in the absence of apo E production, and apo E overexpression disrupts LRP transport in fibroblasts, it is possible that apo E expression is involved in the normal transport of LRP in the liver cell. However, we favor the hypothesis that hepatic apo E affects the conformation of LRP and enhances its ability to bind the incoming remnants. We postulate that hepatic apo E exerts its physiologic action by associating with the LRP on the basolateral cell surface, in this way enhancing the binding of incoming remnants to the LRP. This role of apo E as "receptor extension" is specific for LRP-mediated catabolism of remnants, and is additional to the known function of apo E as a lipoprotein-associated ligand for LDLR-mediated catabolism of apo E–containing lipoproteins.

In conclusion, this paper provides definitive evidence of the existence of a unique pathway of remnant lipoprotein removal

that is dependent on the availability of apo E deriving from hepatocytes, and that is not activated by lipoproteins enriched in extrahepatic apo E. This pathway, partially described by the apo E secretion-capture hypothesis, operates only through the chylomicron remnant receptor (LRP) pathway, not through the LDLR pathway, and consists of a more complex set of events than a simple quantitative enrichment of remnants in apo E in the sinusoidal space. The LRP is a multifunctional receptor that is expressed in a wide variety of tissues and binds a diverse array of ligands. The dependence of the LRP on high-level expression of apo E may serve functionally to target the binding and endocytosis of chylomicron remnants by the LRP only to cells that express high levels of apo E such as hepatocytes and macrophages.

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