Disruption of Autosomal Recessive Hypercholesterolemia Gene Shows Different Phenotype In Vitro and In Vivo

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Abstract—We previously characterized the patients with autosomal recessive hypercholesterolemia (ARH) as having severe hypercholesterolemia and retarded plasma low-density lipoprotein (LDL) clearance despite normal LDL receptor (LDLR) function in their cultured fibroblasts, and we identified a mutation in the ARH locus in these patients. ARH protein is an adaptor protein of the LDLR and reportedly modulates its internalization. We developed ARH knockout mice (ARH−/−) to study the function of this protein. Plasma total cholesterol level was higher in ARH−/− mice than that in wild-type mice (ARH+/+), being attributed to a 6-fold increase of LDL, whereas plasma lipoprotein was normal in the heterozygotes (ARH+/−). Clearance of 125I-LDL from plasma was retarded in ARH−/− mice, as much as that found in LDLR−/− mice. Fluorescence activity of the intravenously injected 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-LDL was recovered in the cytosol of the hepatocytes of ARH−/− mice, but not in those of ARH+/− or LDLR−/− mice. Also, less radioactivity was recovered in the liver of ARH−/− or LDLR−/− mice when [3H]cholesteryl oleyl ether (CE)-labeled LDL was injected. In contrast, uptakes of [3H]CE-labeled LDL, 125I-LDL, and DiI-LDL were all normal or slightly subnormal when the ARH−/− hepatocytes were cultured. We thus concluded that the function of the hepatic LDLR is impaired in the ARH−/− mice in vivo, despite its normal function in vitro. These findings were consistent with the observations with the ARH homozygous patients and suggested that certain cellular environmental factors modulate the requirement of ARH for the LDLR function. (Circ Res. 2004;95:945-952.)

Key Words: autosomal recessive hypercholesterolemia ■ knockout mouse ■ low-density lipoprotein receptor ■ primary cultured hepatocytes ■ OmniBank

Hereditary hypercholesterolemia was first characterized by Khachadurian and Kuthman in 1973 as severe hypercholesterolemia with cutaneous and tendon xanthomas and premature atherosclerosis. They proposed two categories, autosomal dominant and recessive.1 Hypercholesterolemia with autosomal dominant inheritance was termed familial hypercholesterolemia. Studies of familial hypercholesterolemia led to the discovery of low-density lipoprotein receptor (LDLR) and identification of its genetic dysfunction as the cause of this disease. The LDLR is now known to play a key role in the internalization of LDL into the cell and in the regulation of plasma LDL concentrations.2,3 However, hypercholesterolemia with autosomal recessive inheritance had never been fully characterized until we first reported this disease.4-5 In these articles, we described siblings with severe hypercholesterolemia, exhibiting huge xanthomas and premature atherosclerosis despite normal LDLR activity in their cultured fibroblasts.

In 2001, Garcia et al6 mapped the ARH locus to chromosome 1p35 using six families of autosomal recessive hypercholesterolemia (ARH). They identified six mutations of the gene encoding a putative LDLR adaptor protein in these ARH families. We showed that an insertion mutation in the gene of the Japanese siblings described causes an early stop codon.7 ARH protein has an N-terminal phosphotyrosine-binding (PTB) domain.6 The PTB domain is found in several adaptor proteins, such as Disabled-2 and numb, and binds to an NPXY sequence in the cytoplasmic tails of cell surface receptors to modulate their internalization. Recently, the PTB domain of ARH protein was shown by the pull-down technique to bind to the FDNPVY sequence of LDLR.8 ARH protein was also reported to interact with clathrin and is thought to function as an adaptor protein that couples LDLR to the endocytic machinery.8 What is unique about the patients with ARH is the apparent inconsistency of the LDLR functions between in vitro and in vivo.
vivo. In ARH patients, clearance of $^{125}$I-LDL from plasma is delayed to the same extent as that found among homozygous familial hypercholesterolemia, whereas LDL binding, internalization, and degradation are normal or subnormal in their cultured fibroblasts. However, a defect in LDLR internalization was observed in Epstein-Barr virus lymphocytes from ARH patients. LDLR activity in these mutant cells could be restored by retrovirus-mediated expression of normal ARH. The results indicated that lymphocytes require ARH for normal functioning of the LDLR even in vitro, whereas fibroblasts express the normal LDLR functions without ARH, at least in vitro. Because ARH patients have delayed clearance of LDL, the LDLR requires ARH for its functions, at least in the liver in vivo. Jones et al reported that ARH-deficient mice have delayed catabolism of LDL, higher LDL cholesterol levels, and greater immunodetectable LDLR on the sinusoidal surface of hepatocytes.

In the present study, we characterized ARH-deficient mice to study the functions of ARH. ARH$^{−/−}$ mice showed a higher level of plasma LDL cholesterol than wild-type mice, whereas ARH$^{+/−}$ mice did not show hypercholesterolemia, being consistent with clinical manifestation of the human ARH patients. The clearance of $^{125}$I-LDL was delayed, and hepatic uptake of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI)-LDL and of [3H]cholesteryl oleyl ether-labeled LDL (3H-CE-LDL) was decreased in ARH$^{−/−}$ mice. However, primary cultured hepatocytes of ARH$^{−/−}$ mice had normal functions to internalize 3H-CE-LDL, 125I-LDL, and DiI-LDL. Thus, the results indicate that the cellular environment modulates the regulation of LDLR function by ARH protein.

Materials and Methods

General Procedure

Plasma lipoproteins were analyzed by high-performance liquid chromatography using molecular sieve columns (Skylight Biotech, Inc). Cholesterol and triglycerides were measured using enzyme assay kits (Wako, Tokyo, Japan). Na$^{125}$I (37 GBq/mL) and [1α,2α(m)-$^{3}$H]cholesterol oleyl ether labeled LDL (3H-CE-LDL) were purchased from Amersham (Buckinghamshire, UK). LDL was isolated by sequential ultracentrifugation in a density range of 1.019<density<1.064 from pooled plasma of apolipoprotein E-deficient mice (Jackson Laboratory, Bar Harbor, Me) or normal human volunteers after overnight fasting. Human lipoprotein-deficient serum (LPDS) (density:0.925 g/mL) was prepared by sequential ultracentrifugation.

Generation of Knockout Mouse

To generate ARH knockout mice, mutations were created by insertional mutagenesis using the gene trap vectors developed by Lexicon Genetics Incorporated (Woodlands, Tex), based on retroviral-based gene trap technology previously reported. OmniBank Sequence Tag 149604 has an insertion mutation in the third intron of the mouse ARH gene (Figure 1A). The line was obtained from Lexicon Genetics Incorporated. All experiments were performed with the F2-generation or F3-generation descendants, which were backcrossed with the C57Bl/6. LDLR knockout mice (125I-LDL$^{−/−}$) were generated as previously described, which were backcrossed to C57Bl/6 mice, and were used for the study.

Southern Blot Analysis

Southern blot analysis was performed after digestion of the DNA prepared from liver with Apol. $^{32}$P-labeled polymerase chain reaction products (239 bp) amplified from portions of exon 3 and intron 3 of the mouse ARH gene with primers 5′-ATCATCCTGACCGACACCT-3′ and 5′-GGCACACATAACCGACCTA-3′ or neo gene fragment (850 bp) derived from pBS64neo (Lexicon Genetics Inc) as probes according to the standard procedure.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from the liver of wild-type, heterozygous, and homozygous mice using the acid guanidium thiocyanate-phenol-chloroform method, as described.

$^{125}$I-LDL Turnover Study

Mouse LDL was iodinated with $^{125}$I by the iodine monochloride method to give a specific activity of $^{125}$I-LDL >200 cpm/ng.
protein. Female mice of the genotypes of wild-type (ARH\textsuperscript{+/+}), ARH\textsuperscript{−/+}, ARH\textsuperscript{−/−}, and LDLR\textsuperscript{−/+}, 12 to 15 weeks of age, were fasted for 13 hours. Three mice in each genotype received an intravenous bolus via the external jugular vein of \textsuperscript{125}I-labeled mouse LDL (5 \( \mu \)g of protein). Blood was collected from the tail vein with heparinized Pasteur pipette at the indicated timings. The plasma \textsuperscript{125}I-labeled apoB was measured by isopropanol precipitation, followed by gamma counting as previously described.\textsuperscript{17}

**In Vivo Hepatic Uptake of Intravenously Injected DiI-LDL**

Twelve-week-old female mice of the genotypes of wild-type, ARH\textsuperscript{+/+}, ARH\textsuperscript{−/+}, and LDLR\textsuperscript{−/+} were fasted for 13 hours. Each mouse received an intravenous bolus via the external jugular vein of 50 \( \mu \)g of human DiI-LDL (Molecular Probes Inc, Eugene, Ore). To detect nonspecific incorporation of DiI-LDL, 2.5 mg of unlabeled human LDL was injected 2 minutes before \textsuperscript{125}I-labeled LDL, 5 mg of unlabeled LDL was injected 2 minutes before 50 \( \mu \)g of DiI-LDL was injected. Two minutes after DiI-LDL injection, blood was collected for determination of cholesterol and lipoproteins. After 4 hours, the right atrium was punctured, 10 mL of phosphate-buffered saline (PBS) was injected via the left ventricle, and subsequently 10 mL of PBS containing 4\% paraformaldehyde. The tissues were immersed in PBS containing 4\% paraformaldehyde and incubated at 4°C for 12 hours. The liver samples were frozen in liquid nitrogen and subjected to microscopic analysis. To stain Kupffer cells, BM8 (rat antibody against mouse pan-macrophage) (BMA Biomedicals AG, Augst, Switzerland) was used as a primary antibody and fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (DAKO Cytomation, Glostrup, Denmark) was used as a secondary antibody. The samples were observed by confocal laser scanning microscopy (LSM5 PASCAL; Zeiss, Co, Tokyo, Japan).

**Labeling of Human LDL With \textsuperscript{3}H-CE**

LDL was labeled with \textsuperscript{3}H-CE according to the previously described method,\textsuperscript{21} with a minor modifications.

**In Vivo Hepatic Uptake of Intravenously Injected \textsuperscript{3}H-CE-LDL**

\textsuperscript{3}H-CE-LDL (0.8 \( \mu \)Ci) was injected via the external jugular vein of wild-type (ARH\textsuperscript{+/+}), ARH\textsuperscript{−/+}, and LDLR\textsuperscript{−/+} female mice, 12 to 15 weeks of age. Blood was collected from the tail vein with heparinized Pasteur pipette at 2 minutes and 4 hours after the injection. The blood was dispersed in chloroform/methanol, 2/1 (v/v). Immediately after the second blood collection, 2 mg of human LDL was injected into the blood by the method of Folch.\textsuperscript{22} Radioactivity in each sample was measured by isopropanol precipitation, followed by gamma analysis. To stain Kupffer cells, BM8 (rat antibody against mouse pan-macrophage) (BMA Biomedicals AG, Augst, Switzerland) was used as a primary antibody and fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (DAKO Cytomation, Glostrup, Denmark) was used as a secondary antibody. The samples were observed by confocal laser scanning microscopy (LSM5 PASCAL; Zeiss, Co, Tokyo, Japan).

**Preparation and Culture of Mouse Hepatocytes**

Mice (14 to 16 weeks of age) of the indicated genotype were used for the study. The livers were perfused via the portal vein and hepatocytes were obtained by the method of Seglen.\textsuperscript{23} After 24-hour incubation in Waymouth MB 752/1 medium containing 10% fetal calf serum in six-well plates, the cells were subjected to the study.

**In Vitro Uptake of \textsuperscript{3}H-CE-LDL Into Primary Cultured Hepatocytes**

The cells were incubated in Waymouth MB 752/1 medium containing 10% LPDS for 48 hours, and then incubated in DMEM containing 2\% bovine serum albumin (without free fatty acid) and \textsuperscript{3}H-CE-LDL (5 to 100 \( \mu \)g/mL) for an additional 3 hours. Then, the cells were washed twice with 150 mmol/L NaCl, 50 mmol/L of Tris-HCl (pH 7.4) containing 2 mg/mL of bovine serum albumin, and once with the same buffer without bovine serum albumin. The cells were incubated with LDL-releasing buffer (50 mmol/L NaCl, 10 mmol/L Hepes (pH 7.4), containing 10 mg/mL heparin) at 4°C for 1 hour. After removal of the heparin-releasable fraction, 1 mL of hexane/isopropyl alcohol (3/2) was added to the cells to extract lipids in the cells.\textsuperscript{24} After delipidation, the cells were dissolved in 1 N NaOH and protein concentration was measured.

**\textsuperscript{125}I-LDL Binding, Incorporation, and Degradation Assays**

Waymouth MB 752/1 medium containing 10\% LPDS was added to the cells and incubated for another 24 hours. Binding, internalization, and degradation of \textsuperscript{125}I-LDL were analyzed according to the method previously described.\textsuperscript{25}

**Results**

**Generation of ARH Knockout Mouse**

OmniBank Sequence Tag 149604 (Lexicon Genetics Inc) corresponded to the insertion mutation in the third intron of ARH gene in mouse chromosome 4 (Figure 1A). The mutated allele encodes a marker fusion transcript and OmniBank Sequence Tag fusion transcript instead of ARH mRNA. The offspring heterozygous animals were mated to produce ARH\textsuperscript{+/−} and ARH\textsuperscript{−/−} mice. Southern blot analysis was performed to genotype the mice using a probe comprising portions of mouse ARH gene containing both exon 3 and intron 3 (Figure 1B) after digestion of DNA with ApaI. ApaI site is not present in the insertion fragment used for development of the knockout mice. Southern blot analysis with the exon probe showed a 3405-bp band for the wild-type allele and was observed in both the wild-type and ARH\textsuperscript{+/−} mice, whereas an 8581-bp band, the disrupted allele which was produced by the insertion of 5176-bp fragment (Figure 1A), was observed in ARH\textsuperscript{−/−} and ARH\textsuperscript{+/−} mice after digestion of DNA with ApaI. Southern blot analysis with neo gene probe demonstrated that only one position and one copy insertion event occurred in a mouse genome because ARH\textsuperscript{−/−} and ARH\textsuperscript{+/−} exhibited an 8581 bp.

To confirm that the mutated allele does not express the mRNA, total RNA was isolated from the livers of the animals of each genotype and analyzed by reverse-transcription polymerase chain reaction (Figure 1C). A 944-bp band, the ARH transcript that was expressed in wild-type and ARH\textsuperscript{+/−} mice, was not detectable in ARH\textsuperscript{−/−} mice. The results thus confirmed that the insertion mutation by gene trap vector disrupted the ARH gene expression.
**TABLE 1. Lipid and Lipoprotein Profiles of ARH Knockout Mice (mean±SEM)**

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol, mmol/L</th>
<th>Triglyceride, mmol/L</th>
<th>HDL Cholesterol, mmol/L</th>
<th>LDL Cholesterol, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=5)</td>
<td>2.66±0.14</td>
<td>1.04±0.26</td>
<td>2.49±0.12</td>
<td>0.17±0.05</td>
</tr>
<tr>
<td>Female (n=8)</td>
<td>2.57±0.22</td>
<td>0.78±0.07</td>
<td>2.20±0.19</td>
<td>0.30±0.04</td>
</tr>
<tr>
<td>Heterozygote</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=8)</td>
<td>2.92±0.14</td>
<td>1.00±0.13</td>
<td>2.78±0.11</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>Female (n=10)</td>
<td>2.82±0.19</td>
<td>0.76±0.06</td>
<td>2.40±0.14</td>
<td>0.35±0.08</td>
</tr>
<tr>
<td>Homozygote</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=5)</td>
<td>4.53±0.35†</td>
<td>1.20±0.10</td>
<td>3.10±0.52</td>
<td>1.01±0.21†</td>
</tr>
<tr>
<td>Female (n=8)</td>
<td>4.64±0.68†</td>
<td>1.09±0.19</td>
<td>2.96±0.30*</td>
<td>1.46±0.44†</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01 vs wild type.

**Lipid and Lipoprotein Profiles of ARH Knockout Mice**

Table 1 shows total plasma cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol levels of mice from litters derived from the mating of ARH−/− mice fed a normal chow. Plasma total cholesterol levels were 1.8-fold higher in ARH−/− mice than those in ARH+/+. The elevation of total cholesterol was attributed to the six-fold increase of LDL cholesterol. There was no significant difference in the plasma cholesterol levels between ARH−/− and ARH+/+ mice. Historical analysis of the liver showed that ARH−/− mice at 12 weeks of age did not have fatty livers, which were observed in our ARH patients.5

**125I-LDL Turnover Study**

To investigate the LDL metabolism in ARH deficiency, 125I-LDL turnover study was performed in ARH−/−, ARH+/−, ARH+/+ (wild-type), and LDLR−/− mice. The clearance of 125I-LDL from circulation is demonstrated in a semi-logarithmic plot in Figure 2. It was substantially retarded in ARH−/− and LDLR−/− mice compared with wild-type mice. The clearance rate of 125I-LDL in ARH−/− mice was similar to that in wild-type mice. The half-lives of the plasma 125I-LDL were 11.7 hours in ARH−/− and 6.0 hours in LDLR−/− mice, and both were longer than those in ARH+/+ and wild-type mice (2.8 hours).

**Microscopic Findings in the Liver After Injection of DiI-LDL**

To study whether the delayed clearance of LDL in ARH−/− mice is attributable to impaired catabolism of LDL in the liver, the liver specimen was examined after injection of DiI-LDL in wild-type, LDLR−/−, and ARH−/− mice (Figure 3). Kupffer cells were identified by fluorescent isothiocyanate (Figure 3B, 3D, 3F, 3H, 3J, and 3L). Fluorescence activity was detected in the cytosol of the hepatocytes and Kupffer cells in wild-type mice, indicating that both types of cells effectively take-up DiI-LDL (Figure 3A). Pre-injection of 2.5 mg LDH raised serum total cholesterol from 2.18±0.66 (mean±SD) mmol/L to 14.67±0.68 mmol/L, and LDL cholesterol from 0.21±0.07 mmol/L to 11.42±1.09 mmol/L in wild-type mice (Table 2). In this condition, the incorporation of DiI-LDL into hepatocytes was decreased by the excess amount of LDL in plasma (Figure 3C), whereas its incorporation into Kupffer cells was not affected. In contrast, hepatocytes of ARH−/− mice were stained less extensively than those of wild-type mice by DiI-LDL fluorescence (Figure 3I), indicating that the uptake of DiI-LDL by the hepatocytes of ARH−/− mice was smaller than that of wild-type mice, and the presence of excess amount of LDL in plasma did not influence this result (Figure 3K). The fluorescence in the hepatocytes of LDLR−/− mice was also less intense than that in ARH−/− (Figure 3E).

**In Vivo Hepatic Uptake of Intravenously Injected 3H-CE-LDL**

Hepatic uptake of LDL was also investigated by injecting 3H-CE-LDL. Four hours after the injection, the tritium count in the blood decreased by 44% in wild-type mice, whereas it did not decrease in either LDLR−/− or ARH−/− mice (Figure 4A). The liver of wild-type mice incorporated 27.4±8.0% (mean±SD) of the injected 3H-CE-LDL, whereas 9.0±1.0% was incorporated in LDLR−/− mice and 11.8±0.5% in ARH−/− mice (Figure 4B). The amount of 3H-CE-LDL taken-up by the liver in ARH−/− was significantly smaller than that in wild-type mice but larger than that in LDLR−/− mice (P<0.05).

**Incorporation of LDL in Primary Cultured Hepatocytes**

In an attempt to reproduce the in vivo observations in vitro, incorporation of 3H-CE-LDL was examined in primary cul-
tured hepatocytes. Interestingly, the hepatocytes of ARH$^{-/-}$ mice internalized $^{3}$H-CE-LDL as much as those of wild-type mice, whereas the hepatocytes of LDLR$^{-/-}$ mice took-up significantly less $^{3}$H-CE-LDL (Figure 5A). The cultured hepatocytes of ARH$^{-/-}$ mice bound larger amounts of $^{125}$I-LDL than those of wild-type and LDLR$^{-/-}$ mice. The hepatocytes of ARH$^{-/-}$ internalized and degraded $^{125}$I-LDL as much as those of wild-type mice, which was significantly more than those of LDLR$^{-/-}$ mice (Figure 5B through 5D).

Dil-LDL Incorporation in Primary Cultured Hepatocytes

To confirm the positive incorporation of LDL by the primary cultured hepatocytes without ARH, the hepatocytes of ARH$^{-/-}$, LDLR$^{-/-}$, and wild-type mice were incubated with Dil-LDL and were observed by a laser confocal microscopy. Fluorescence-positive substances were observed in the cytosol of the hepatocytes of wild-type mice (Figure 6A), and they were suppressed in the presence of excess amounts of LDL (Figure 6B). The cultured hepatocytes of LDLR$^{-/-}$ mice showed very low fluorescence activity (Figure 6C and 6D). In contrast, the hepatocytes of ARH$^{-/-}$ mice showed fluorescence uptake of amounts similar to those observed for the wild-type (Figure 6E), and this was efficiently suppressed by excess amounts of LDL (Figure 6F).

**Discussion**

ARH knockout mice were developed by insertion mutation in the third intron of mouse chromosome 4. ARH$^{-/-}$ mice had a 1.8-fold higher plasma total cholesterol than wild-type litter mates fed normal chow, and this was attributed to a six-fold increase of LDL. Total cholesterol levels in ARH$^{-/-}$ mice (Table 1) were slightly lower than those reported for LDLR$^{-/-}$ mice (5.90±0.23 mmol/L in male, 6.18±0.21 mmol/L in female),$^{17}$ and cholesterol levels in ARH$^{-/-}$ mice (2.92±0.14 mmol/L in male, 2.82±0.19 mmol/L in female) were almost the same as those in ARH$^{-/-}$ mice (2.68±0.14 mmol/L in male, 2.57±0.22 mmol/L in female). The hypercholesterolemia in our ARH$^{-/-}$ mice appears to be milder than that reported by Jones et al.$^{14}$ who have recently reported that total cholesterol levels were moderately higher both in ARH$^{-/-}$ mice (7.96±1.63 mmol/L) and in ARH$^{-/-}$ mice (4.06±0.58 mmol/L) compared with that in ARH$^{+/-}$ mice (3.26±1.00 mmol/L) fed a chow containing 0.2% cholesterol. The difference in the plasma total cholesterol values between our ARH$^{-/-}$ mice and theirs may stem from different cholesterol content of the diet, because the chow we used contained 0.1% cholesterol.

The clearance of $^{125}$I-LDL from the circulation was significantly delayed in ARH$^{-/-}$ mice like in LDLR$^{-/-}$ mice (Figure 2), indicating that the in vivo catabolic rate of LDL is decreased in ARH$^{-/-}$ mice. The half-life for the disappearance of $^{125}$I-LDL in ARH$^{-/-}$ mice (11.7 hours) was longer than that observed in wild-type mice (2.8 hours) but not significantly different from that in LDLR$^{-/-}$ (6.0 hours). The LDL clearance in ARH$^{+/-}$ mice was same as that in the wild-type mice, suggesting that their LDL catabolism is normal. These results appear to be consistent with the findings in the ARH patients.$^{5}$

To investigate the in vivo mechanism for the delayed catabolism of LDL directly in ARH$^{-/-}$ mice, hepatic uptake of

**TABLE 2. Cholesterol Levels of Total, VLDL, LDL, and HDL Fractions Before and After Administration of 2.5 mg LDL**

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol, mmol/L</th>
<th>VLDL Cholesterol, mmol/L</th>
<th>LDL Cholesterol, mmol/L</th>
<th>HDL Cholesterol, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Wild-1</td>
<td>2.36</td>
<td>14.30</td>
<td>0.02</td>
<td>1.99</td>
</tr>
<tr>
<td>Wild-2</td>
<td>2.72</td>
<td>14.27</td>
<td>0.05</td>
<td>1.37</td>
</tr>
<tr>
<td>Wild-3</td>
<td>1.45</td>
<td>15.45</td>
<td>0.06</td>
<td>2.10</td>
</tr>
<tr>
<td>Mean</td>
<td>2.18</td>
<td>14.67</td>
<td>0.04</td>
<td>1.82</td>
</tr>
<tr>
<td>SD</td>
<td>0.66</td>
<td>0.68</td>
<td>0.02</td>
<td>0.39</td>
</tr>
</tbody>
</table>

VLDL indicates very-low-density lipoprotein.
DiI-LDL was monitored. The fluorescence was recovered in the cytosols of both hepatocytes and Kupffer cells of wild-type mice (Figure 3A), and the fluorescence in the hepatocytes was suppressed by pretreatment with excess amounts of LDL (Figure 3C), suggesting that DiI-LDL was incorporated into the hepatocytes via the LDLR. Lower levels of DiI-LDL were found in hepatocytes of LDLR<sup>−/−</sup>/H11002/H11002 and ARH<sup>−/−</sup>/H11002/H11002 mice (Figure 3E, I). Because LDL cholesterol levels were higher in ARH<sup>−/−</sup>/H11002/H11002 mice (1.01 ± 0.21 mmol/L in male, 1.46 ± 0.44 mmol/L in female) than that in wild-type mice (0.17 ± 0.05 mmol/L in male, 0.30 ± 0.04 mmol/L in female), injected DiI-LDL may become more diluted in ARH<sup>−/−</sup> by their own LDL. However, hepatic DiI-LDL uptake in ARH<sup>−/−</sup> mice was much lower than the observed uptake in wild-type mice when the excess LDL was given to raise LDL cholesterol (11.42 ± 1.09 mmol/L). This indicates that the lower uptake of DiI-LDL in ARH<sup>−/−</sup> was not attributable to a dilution effect caused by their high LDL levels (Figure 3C, I).

Small numbers of cells appeared highly fluorescence-positive in the liver of mice of all the genotypes including LDLR<sup>−/−</sup> (Figure 3A, E, I). Their fluorescent activity was not
Hepatic uptake of LDL in ARH−/− was also examined by measuring the uptake of 3H-CE-LDL by the liver. The tritium count in the blood decreased significantly in the wild-type mice, whereas it did not change in LDLR−/− and ARH−/− mice (Figure 4A). This is in agreement with the 125I-LDL turnover study (Figure 2). The incorporation of 3H-CE-LDL into the liver of LDLR−/− and ARH−/− mice was significantly lower than that of the wild-type mice (Figure 4B), suggesting again that the delayed turnover of LDLR in ARH−/− mice is attributable to low LDL uptake by the liver. Interestingly, incorporation of 3H-CE-LDL into the liver of ARH−/− is significantly higher than that of LDLR−/− (P<0.05). This result implies that the LDLR may function to incorporate LDL in the liver to some extent in vivo even without ARH.

To examine in vitro LDLR activity in the cells of ARH−/− mice, the incorporation of 3H-CE-LDL was measured in primary cultured hepatocytes of ARH+/+, LDLR−/−, and wild-type mice (Figure 5A). The cultured hepatocytes of ARH−/− incorporated a slightly smaller amount of 3H-CE-LDL than that of the wild-type, but much larger than that of LDLR−/−. These findings were confirmed by 125I-LDL incorporation and degradation experiments (Figure 5C, D). The binding of 125I-LDL to the hepatocytes of ARH−/− was larger than that of wild-type (Figure 5B). Recently, Michaely et al26 reported that the number of LDLRs on the cell surface of the lymphocytes in ARH subject was increased 20-fold, and that LDL binding activity was increased two-fold. The hepatocytes of ARH+/+ seemed to remain the characteristics of in vivo status to some extent. To see whether LDL is incorporated into the cell or whether it remains on the cell surface, the cells were incubated with Dil-LDL and observed by using a laser confocal microscopy. The cytosol of the hepatocytes of ARH+/+ mice was fluorescently stained, suggesting that Dil-LDL was internalized, like that of wild-type mice (Figure 6A, E). The results were highly consistent with the findings that the LDLR in the cultured fibroblasts from the homozygous ARH patients normally functioned, despite the fact that LDL clearance in their blood was severely impaired.5 This suggests that the LDLR functions normally in vitro without ARH protein.

The PTB domain of ARH protein has been shown, by pull-down technique, to bind to the FDNPVY sequence of the LDLR protein.8 ARH protein was also reported to interact with clathrin and AP-2, and it is suggested to function as an adaptor protein that couples LDLR to the endocytic machinery. ARH patients have severe hypercholesterolemia caused by delayed LDL clearance in vivo, although they have normal or subnormal levels of LDLR activity in their fibroblasts when measured in vitro.5 However, transformed lymphocytes and monocyte-derived macrophages were unable to internalize LDL in ARH patients.12 Thus, the dependency of LDLR function on ARH protein can be cell-specific. However, we have demonstrated here that hepatocytes do not take-up LDL in vivo without ARH protein, but they normally catabolize LDL in vitro. Thus, the requirement of ARH protein for proper functioning of the LDLR is not cell-specific, but rather may depend on the cellular environment.

Hepatic uptake of 3H-CE from LDLR−/− mice was internalized, like that of wild-type mice (Figure 6A, E). The results were highly consistent with the findings that the LDLR in the cultured fibroblasts from the homozygous ARH patients normally functioned, despite the fact that LDL clearance in their blood was severely impaired.5 This suggests that the LDLR functions normally in vitro without ARH protein.

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Hepatic uptake of 3H-CE from LDLR−/− mice was significantly higher in ARH−/− mice than LDLR−/− mice. This may indicate that LDLR functions to some extent without ARH, even in vivo. Some other adaptor proteins may compensate for ARH by forming an LDLR–clathrin complex, or the condition can be induced so LDLRs can be internalized without forming a clathrin complex. Our in vitro results strongly indicate that these possibilities may be enabled in a certain cellular environments.

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Materials and Methods

Mice

Mice were maintained on a light/dark (12/12 hours) cycle at 24 °C, receiving food and water ad libitum. The animals were subjected to the study under anesthetization with sodium pentobarbital (60 mg/kg). All mice were fed a normal chow, CE-2 (Clea Japan Inc., Tokyo, Japan) containing 0.1% (w/w) of cholesterol and 4.8% of fat. All the animal experimental procedures were approved by the institutional animal welfare committee.

Generation of knockout mouse

To generate ARH knockout mice, mutations were created by insertional mutagenesis using the gene trap vectors developed by Lexicon Genetics Incorporated (Woodlands, TX, USA), based on retroviral-based gene trap technology previously reported.¹ The vector contained a splice acceptor sequence, a galactosidase/neomycin phosphotransferase fusion gene (Beta-Geo), a polyadenylation sequence, a phosphoglycerate kinase-1 promoter (PGK), the first coding exon of the Bruton's tyrosine kinase (BTK) gene and a splice donor sequence. The fusion transcript generated from the PGK promoter between the BTK exon in the vector and the downstream exons of the gene is used to generate a sequence tag of the trapped gene by reverse transcription (RT)-polymerase chain reaction (PCR). This fusion transcript contains stop codons in all reading frames and is therefore designed to not produce a
protein product. The viruses deliver a gene trap construct to the mouse ES cells (129SvEv) that allows the expression of a promoter-less selectable marker gene when the vector has inserted into and trapped exons from a gene. OmniBank Sequence Tag #149604 corresponded to the insertion mutation in the third intron of ARH gene in mouse chromosome 4. The line was obtained from Lexicon Genetics Inc. All experiments were performed with the F2 or F3 generation descendants, which were back-crossed with the C57Bl/6. LDL receptor knockout mice (LDLR\textsuperscript{-/-}) were generated as previously described,\textsuperscript{2} which were backcrossed to C57Bl/6 mice, were used for the study.

RT-PCR

Total RNA was isolated from the liver of wild-type, heterozygous and homozygous mice using the acid guanidium thiocyanate-phenol-chloroform method, as described.\textsuperscript{3} ARH mRNA was reverse transcribed with the ARH specific primer 5'-TTAAGCTTTCAGAAGGTGAAGACGTCATCATC-3'. ARH cDNA was amplified with the same primer and primer 5'-ATTCTAGACATGGACGCGCTCAAGTCGGCG-3'.\textsuperscript{4} GAPDH mRNA was reverse transcribed with the GAPDH specific primer tagged with M13RPI sequence 5'-GTCCAGGAAAAACAGCTATGACCAAAAGTTTGCATGGATGACCTTG-3' (M13RPI sequence tag was underlined). To avoid amplification of GAPDH pseudogenes that occur because of retrotransposition of mRNA from trace amount of contaminating genomic DNA, the GAPDH cDNA was amplified with the GAPDH specific primer
5'-CTTCATTGACCTCAACTACATG-3' and M13RPI primer
5'-CAGGAAACAGCTATGACC-3' after the residual primers in the reverse
transcription reaction were removed with PCR purification kit (QIAGEN, Hilden,
Germany).

Labeling of human LDL with $^3$H-CE

LDL was labeled with $^3$H-CE according to the previously described method$^5$ with a
minor modifications.  Briefly, 5 mg of L-$\alpha$-phosphatidylcholine (Avanti Polar Lipids,
Inc., AL, USA), and 500 $\mu$Ci $^3$H-CE were mixed and dried under nitrogen stream and
sonicated in 10 ml PBS to make cholesteryl ether containing vesicles.  The vesicle
solution was incubated in the presence of 13.4 mg of LDL, 10 ml of LPDS, Aprotinin
and penicillin, at 37 °C overnight.  The incubation mixture was applied to a column of
dextran sulfate-cellulose, kindly provided by Kaneka Corp (Osaka, Japan), and then
washed with PBS.  The labeled LDL was eluted with 0.5M NaCl.

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