Liver-specific Inactivation of the Abetalipoproteinemia Gene Completely Abrogates Very Low Density Lipoprotein/Low Density Lipoprotein Production in a Viable Conditional Knockout Mouse*

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Conventional knockout of the microsomal triglyceride transfer protein large subunit (MTP) gene is embryonic lethal in the homozygous state in mice. We have produced a conditional MTP knockout mouse by inserting loxp sequences flanking exons 5 and 6 by gene targeting. Homozygous floxed mice were born live with normal plasma lipids. Intravenous injection of an adenovirus harboring Cre recombinase (AdCre1) produced deletion of exons 5 and 6 and disappearance of MTP mRNA and immunoreactive protein in a liver-specific manner. There was also disappearance of plasma apolipoprotein (apo) B-100 and marked reduction in apoB-48 levels. Wild-type mice showed no response, and heterozygous mice, an intermediate response, to AdCre1. Wild-type mice doubled their plasma cholesterol level following a high cholesterol diet. This hypercholesterolemia was abolished in AdCre1-treated (-/-) mice, the result of a complete absence of very low/intermediate/low density lipoproteins and a slight reduction in the rate of accretion/low density lipoproteins and a slight reduction in the result of a complete absence of very low/intermedi-

Abetalipoproteinemia is an autosomal recessive disorder characterized by the almost complete absence of circulating apolipoprotein (apo) B-containing lipoproteins (1). In addition to the lipoprotein abnormalities, patients with abetalipo-

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§ The abbreviations used are: apo, apolipoprotein; MTP, microsomal triglyceride transfer protein; MTP, MTP large subunit; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; FIAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil.

MATERIALS AND METHODS

Targeting Vector Construction—A mouse 129 strain genomic library was purchased (Stratagene) and screened with mouse MTP cDNA (16). Two overlapping clones encompassing exons 3 to 8 were used to construct two types of targeting vectors. For straightforward replacement-type targeting construct, a neo cassette was inserted into exon 7 of the MTP gene between a Smal and a Xhol site (data available from L. C. upon request). The conditional targeting construct was designed by inserting a neo-loxP cassette in the XhoI site of intron 4 and a loxP fragment in the BamHI site of intron 4 of the MTP (Fig. 1A). A thymidine kinase cassette was ligated to the 5′ end of the construct.

Generation of Germ Line Chimera—An R1 ES cell line was obtained from Dr. Andras Nagy at the University of Toronto. The cells were expanded to passage 14 and used to generate knockout mice as described previously (17). Three positive ES cell clones were injected into blastocysts of C57BL/6J, and chimeric mice were obtained. They were mated with C57BL/6J mice, and germ line transmission was confirmed by Southern blot analysis. Most experiments were conducted on siblings of F2 or F3 mice. The mice were weaned at 21 days and fed either a high fat diet or a control diet, and plasma lipids were measured using a commercial lipid assay (18). MTP expression was determined by Northern blotting and quantitative RT-PCR.

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chow diet (Teklad 7001) or a high fat cholesterol diet (ICN 960393) containing 1.23% cholesterol and 17.84% fat.

**RNA Protection Assay**—Total RNA from the liver and small intestine were isolated using TRIzol (Life Technologies, Inc.). A polymerase chain reaction product containing the first 350 base pairs of the mouse MTP CDS were cloned into pBluescript KS vector and used as a probe for RNA protection assay. The authenticity of the clone was verified by sequencing, and the antisense strand RNA was transcribed by using MAXIscript (Ambion). The assay was done using the RPAII kit (Ambion) with 5 μg of total RNA following the instructions of the vendor’s manual. A β-actin probe was used as an internal control in the assay.

**Western Blot Analysis**—Liver and small intestine were removed, and total proteins were extracted by a Wheaton No. 6 hand-held homogenizer in buffer B (10 mM Hapes pH 7.4, 2.5 mM sodium phosphate monobasic, 250 mM sucrose, 5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride). Microsomal fractions were isolated from extracts of liver and small intestine by ultracentrifugation at 100,000 g × 1 g for 1 h. The pellet was resuspended in buffer B, and the protein concentration was determined by a DC Protein Assay Kit from Bio-Rad. Ten μg of microsomal protein was loaded onto a 4–15% gradient polyacrylamide gel, and a rabbit anti-bovine microsomal protein was loaded onto a 4–15% gradient polyacrylamide gel, and a rabbit anti-bovine microsomal protein was used to measure the MTP-mediated [14C]triglyceride transfer activity as described in Jamil et al. (18).

**FPLC Analysis of Plasma Lipids**—Blood was collected after a 4–5-h fast, and total plasma cholesterol and triglyceride concentrations were measured by enzymatic kits (Sigma Diagnostics). Two hundred μl of pooled plasma from 3–4 animals was loaded on a FPLC system with 2 Superose 6 columns connected in series (Pharmacia FPLC System, Amersham Pharmacia Biotech). 0.5-ml fractions were collected using an elution buffer (1 mM EDTA, 154 mM NaCl, and 0.02% NaN₃, pH 8.2) (19). Lipid contents in individual fractions were determined with enzymatic assays kits (Sigma Diagnostics). Very low density (VLDL), intermediate density (IDL), low density (LDL), and high density (HDL) lipoproteins are well separated by this technique (19).

**Recombinant Cre-Adenovirus Treatment of Floxed MTP Mice**—A replication-defective adenovirus containing recombinant Cre recombinase, AdCrel, was a gift from Dr. Frank Graham (McMaster University, Hamilton, Ontario, Canada) (20). It was amplified in 293 cells and purified as described previously (21). Eight-week-old male mice were injected with 3 × 10⁷ plaque-forming units of AdCrel1 through a jugular vein. The AdCrel1-treated mice were fed either a normal chow or a high cholesterol diet before and after injection. At day 10–21 after adenovirus administration, the mice were sacrificed and studied.

**Primary Hepatocyte Culture**—Hepatocytes were isolated from AdCrel1-treated mice by White’s method (22) except that the perfusion was done on an anesthetized animal through the portal vein instead of an excised liver. A pulse-chase experiment using [35S]methionine was done to determine apoB degradation and secretion in these cells (23).

**Triglyceride Secretion Rate**—Triglyceride secretion in vivo was quantified by the intravenous administration of Triton WR1339 (24). Plasma triglycerides were measured at 1, 2, 3, and 4 h after treatment; the triglyceride accumulation remained linear during this time.

**RESULTS AND DISCUSSION**

As reported by Raabe et al. (15), we produced MTP knockout mice by gene targeting in ES cells and found that inactivation of the MTP locus in mice is embryonic lethal in the homozygous state. We have therefore produced a conditional knockout construct shown in Fig. 1A. It would insert two loxP sequences encompassing exons 5 and 6. Deletion of these exons would be predicted to inactivate the MTP protein because it causes a shift in the translation frame of the mRNA if the remaining exons are correctly spliced into a mutant mRNA. Homologous recombination was verified by digestion of genomic ES cell DNA with BamHI and Southern blot analysis using a probe outside the targeting vector (Fig. 1A). The presence of a diagnostic 4-kb fragment instead of the wild-type 6-kb fragment indicates insertion of the targeted vector by homologous recombination. 14 of 124 clones (11%) that were G418- and FIAU-resistant exhibited a pattern consistent with homologous recombination. 3 of 14 ES cell clones were injected into C57BL/6J blastocysts, yielding 8 chimeric mice with agouti coat color indicating essentially 100% contribution of ES cells. Chimeric males were bred with C57BL/6J females, and germ line transmission was observed in 5% (2/40) of the progeny by Southern blot analysis using a probe on the telomere of tail DNA (Fig. 1B). Heterozygous and homozygous floxed MTP/−/− mice were obtained by cross-breeding. These animals were fertile and produced normal-sized litters. The birth weights, growth, and development of wild-type, MTP+/−, and MTP−/− mice were indistinguishable. The ba-

**TABLE I**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Before AdCrel1 administration (normal chow)</th>
<th>After AdCrel1 administration (high cholesterol diet for 2 weeks)</th>
<th>3 weeks after AdCrel1 administration (high cholesterol diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TG (μg)</td>
<td>CHOL (μg)</td>
<td>TG (μg)</td>
</tr>
<tr>
<td>+/+</td>
<td>34.90 ± 1.31</td>
<td>90.39 ± 5.03</td>
<td>42.89 ± 3.76</td>
</tr>
<tr>
<td>+/−</td>
<td>35.41 ± 2.48</td>
<td>95.37 ± 3.45</td>
<td>43.32 ± 2.73</td>
</tr>
<tr>
<td>−/−</td>
<td>40.67 ± 3.52</td>
<td>85.18 ± 13.47</td>
<td>43.12 ± 4.61</td>
</tr>
</tbody>
</table>

TG, triglyceride; CHOL, cholesterol.

1 Significant difference between +/+ and +/− (p < 0.05).

2 Significant difference between +/+ and −/− (p < 0.01).

**Fig. 1. Floxed conditional targeting of the mouse MTP gene.**

A, exons 3–8 of the mouse MTP gene are indicated by black boxes. A PGKneoA-lox cassette (NEO) was inserted into the Xbal site of intron 6, and another loxP sequence was inserted into a BamHI site of intron 4. A pCM-Tki- poly(A) cassette (TK) was attached to the 5′ end of the targeting construct. loxP sequences are represented by triangles. Restriction enzyme sites: B, BamHI; RV, EcoRV; Xb, Xbal. An EcoRV and BamHI genomic fragment 3′ to the targeting construct (represented by a horizontal bar under Probe) was used as a probe for genotyping. The expected size of the wild-type (6 kb) and the targeted (4 kb) alleles were indicated as double arrowhead lines. B, Southern blot analysis of tail DNA of animals of different genotypes.

**TABLE II**

Plasma lipids in wild-type and floxed MTP knockout mice

The number of mice studied before AdCrel1 administration were 6 each from +/+ and +/− and 4 from −/−; after AdCrel1 treatment there were 4 for each genotype.


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Fig. 2. Cre-recombinase-mediated tissue-specific excision of the floxed (MTP knockout allele). A, the wild-type (top) and targeted (middle) MTP loci are as shown. Cre-recombinase-mediated excision of sequences between the two loxP (triangles) sites would delete exons 5 and 6, intron 5, and part of introns 4 and 6, giving rise to the excision allele shown in the bottom. An EcoRV site was introduced to the 5′ of the loxP sequence when the latter was inserted into the BamHI site of intron 4. A probe spanning part of exon 7 and the adjacent part of intron 7 was used to screen EcoRV-digested genomic DNA. B, Southern blot analysis of the small intestinal (I) and liver (L) DNA from the homozygous (−/−) floxed knockout and the wild-type (+/+) mice 10 days after AdCre1 adenovirus treatment. The 6.5-kb band indicates the presence of the floxed targeted allele without Cre activation of the floxed excision, the 4.5-kb band, the wild-type allele, and the 3.5-kb band, the Cre-mediated excision allele. There is liver-specific excision of the floxed allele following AdCre1 treatment.

Fig. 3. Functional analysis of MTP expression in wild-type and knockout mice 10 days after AdCre1 treatment. All assays were performed in sibling mice with different genotypes. A, levels of MTP mRNA expression in intestine (I) and liver (L) examined by RNase protection assay. A β-actin probe (A) was used for internal control. The first 350 bp of the MTP cDNA was amplified by PCR and used as probe (M) for the quantification of MTP mRNA. Yeast RNA (Y) was used as a negative control. The protected RNAs are indicated by arrows. B, Western blot analysis of the microsomal proteins. A rabbit anti-bovine MTP antiserum (kindly provided by Dr. David Gordon) was used for the analysis, and the 97-kDa MTP band is indicated by an arrow. C, triglyceride transfer activity of microsomal proteins isolated from the liver and small intestine. The activity was expressed as percent of triglyceride (TG) transferred for 100 µg of microsomal protein in 1 h. Four assays were done on each genotype, and the mean and standard deviation are depicted. D, Western blot of total plasma apoB-100 and apoB-48. One µl of plasma was loaded on a 6% SDS-PAGE. Western blot was performed using a goat anti-mouse LDL prepared from apoE-deficient mice.
The mRNA band is essentially undetectable in the liver of Cre-treated MTP−/− mice, whereas the MTP mRNA level in treated MTP+/− mouse liver was only slightly reduced. In comparison, the concentration of MTP mRNA in the small intestine of mice of all three genotypes and in the liver of wild-type mice was unaffected by AdCre1 administration. Therefore, there was good correlation between MTP mRNA expression and the presence of an undisrupted MTP gene, and Cre-induced deletion of exons 5 and 6 led to the absence of detectable MTP mRNA. Since the antisense probe used corresponds to a region of the MTP gene 5' to the missing exons, these results suggest that in the AdCre1-treated mouse liver, if the disrupted MTP gene were transcribed at all, the RNA transcript was so unstable and its steady-state concentration so low that it was undetectable by RNase protection.

To examine whether these changes at the DNA and mRNA levels are reflected at the protein level, we isolated microsomes from the liver and small intestine and performed immunoblot analysis using an MTP antibody. As shown in Fig. 3B, a MTP immunoreactive band was easily detectable in wild-type mouse liver and small intestine, being slightly more intense in the latter. Following AdCre1 treatment there was a reduction in intensity of the immunoreactive band in the liver, but not small intestine, of the heterozygous floxed MTP+/− mice. In the homozygous floxed MTP−/− mice, the Cre gene transfer to the liver completely eliminated the MTP band. Interestingly, there seemed to be a concomitant increase in the intensity of the MTP band in the small intestine following AdCre1 treatment. (MTP has a relatively long half-life (4.4 days in HepG2 cells) (26)). These results indicate that in mice, within 10 days of acute interruption of MTP gene transcription, there is essentially no immunoreactive MTP left in the cells.

We determined the MTP activity in the microsomes isolated from AdCre1-treated wild-type, floxed heterozygous MTP+/−, and floxed homozygous MTP−/− animals. The results shown in Fig. 3C reveal that the MTP triglyceride transfer activity of microsomes isolated from the liver of heterozygous floxed MTP+/− (3.7%) mice is reduced to about half that of the wild-type (7.3%), and the activity of the homozygous floxed MTP−/− mice is almost down to background level (1.3%). In contrast, the intestinal MTP triglyceride transfer activity in all three groups of animals is very similar (7.8%, 8.6%, and 7.7%, respectively, for wild-type, heterozygous, and homozygous knockout animals). Therefore, in the liver and small intestine there is good correlation between MTP protein expression and MTP functional activity, which suggests that MTP, and not protein-disulfide isomerase, is limiting under these conditions.

The plasma lipid levels of the different types of mice before and after AdCre1 treatment are shown in Table I. Before adenovirus administration, the mice were on a fat-free cereal diet, and their plasma cholesterol and triglyceride levels were similar in the three groups of animals. Following AdCre1 injection, there was a significant reduction in the plasma cholesterol concentration in the homozygous MTP−/− mice, which was significantly lower than that in wild-type animals. The cholesterol level in the heterozygous animals was intermediate between those of wild-type and homozygous knockout animals. The plasma triglyceride level was not statistically different between the three groups of animals, although it tended to be lower in the homozygous mice.

Because functional MTP is required for apoB biogenesis (12, 14), we analyzed the plasma for apoB-100 and apoB-48 expression by immunoblot analysis. We took plasma from these mice 2 weeks following AdCre1 treatment. In animals that were on regular chow (Fig. 3D, left panel), there was no difference in wild-type and heterozygous knockout animals; both had clearly detectable apoB-48, but barely detectable apoB-100. In homozygous knockout animals, apoB-100 was undetectable and apoB-48 was markedly reduced and barely detectable. In wild-type animals that were fed a high cholesterol diet (Fig. 3D, right panel), plasma apoB-100 and apoB-48 were clearly detected on the blot, with apoB-48 being a much more intense band than the apoB-100 band. In comparison, the AdCre1-treated heterozygous floxed MTP−/− mice had mildly reduced apoB-48 and markedly reduced apoB-100 bands. When we analyzed the plasma from AdCre1-treated homozygous floxed...
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From the liver. As shown in Fig. 4, diet during this experiment, the secretion came exclusively from the liver and intestine. Because the animals were on a fat-free Triton treatment reflects the triglyceride secretion rate from MTP−/− mice. The accumulation of plasma triglyceride following Triton WR1339 inhibits the catabolism of triglyceride-rich lipoproteins. The absence of detectable lipoproteins in the VLDL and IDL/LDL fractions. There was a slight reduction in the HDL peak. Therefore, the markedly reduced apoB-100 production resulting from the nearly complete inhibition of MTP activity in the MTP−/− mice was sufficient to abrogate a VLDL/IDL/LDL response to high cholesterol diet feeding. The heterozygous MTP+/− mice, which had intermediate MTP mass and MTP activity (Fig. 3), displayed an intermediate lipoprotein phenotype (Fig. 4A, middle panel). The plasma lipoprotein triglyceride was entirely in the VLDL region (Fig. 4A, bottom panel) in wild-type animals. It was essentially abolished in MTP−/− mice.

To explore the mechanism of the marked VLDL deficiency in the MTP−/− mice, we measured the rate of triglyceride production in these animals. The intravenous administration of Triton WR1339 inhibits the catabolism of triglyceride-rich lipoproteins. The accumulation of plasma triglyceride following Triton treatment reflects the triglyceride secretion rate from the liver and intestine. Because the animals were on a fat-free diet during this experiment, the secretion came exclusively from the liver. As shown in Fig. 4B, the triglyceride secretion rate in MTP−/− mice (0.09 ± 0.04 mg/min/100 g) was reduced to less than one-tenth that in the wild-type controls (1.02 ± 0.19 mg/min/100 g). The rate in heterozygous knockout mice was intermediate (0.52 ± 0.07 mg/min/100 g). Thus, the absence of VLDL/IDL/LDL in the knockout animals was a result of failure of production, not increased catabolism. Pulse-chase experiments on cultured hepatocytes isolated from wild-type and heterozygous knockout animals revealed that there was complete failure of secretion of apoB in MTP−/− animals, which would account for the absence of VLDL production in these animals. Albumin production was normal (Fig. 4C).

In conclusion, we have produced a viable abetalipoproteinemia gene knockout mouse model using a Cre/loxP strategy. We found that the liver-specific disruption of the MTP gene was sufficient to completely abrogate the plasma VLDL/LDL response to a high cholesterol diet. Because conventional knockout of the MTP gene is embryonic lethal in the homozygous state, the conditional knockout mice will be a valuable model for studying the metabolic defect and pathophysiology of abetalipoproteinemia as well as the role of MTP in apoB biosynthesis and the biogenesis of apoB-containing lipoproteins in vivo.

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