Regulatable liver expression of the rabbit apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1) in mice lacking endogenous APOBEC-1 leads to aberrant hyperediting

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Apolipoprotein (apo) B mRNA editing is the deamination of C⁶⁶⁶ to uridine, which results in translation of the apoB-48 protein instead of the genomically encoded apoB-100. ApoB-48-containing lipoproteins are cleared more rapidly from plasma and are less atherogenic than apoB-100-containing low-density lipoproteins (LDLs). In humans, the intestine predominantly produces apoB-48 whereas the liver secretes apoB-100 only. To evaluate a potential therapeutic use for liver-induced apoB mRNA editing in humans, we investigated the efficiency and safety of transgenic expression of apoB mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1) in the absence of endogenous editing in the mouse model. Here we show that regulatable tetO-mediated APOBEC-1 expression in the livers of gene-targeted mice lacking endogenous APOBEC-1 results in

30% apoB mRNA editing. In a time-course experiment, the expression of tetO-APOBEC-1 mRNA was suppressed within 2 days after mice were fed doxycycline and apoB mRNA editing and apoB-48 formation were suppressed within 4 days. However, tetO-APOBEC-1 expression resulted in regulatable aberrant hyperediting of several cytidines downstream of C⁶⁶⁶ in apoB mRNA and in novel APOBEC-1 target 1 (NAT1) mRNA. Several of the cytidines in apoB mRNA were hyperedited to a level similar to that of C⁶⁶⁶, although editing at C⁶⁶⁶ was lower than that in wild-type mice. These results demonstrate that even moderate APOBEC-1 expression can lead to hyperediting, limiting the single-gene approach for gene therapy with APOBEC-1.

Key words: cholesterol, gene therapy, tetracycline.

INTRODUCTION

Apolipoprotein (apo) B plays a central role in lipoprotein metabolism and atherosclerosis. ApoB is present in the plasma in two different forms: apoB-100 and apoB-48, which represents the N-terminal 48% of apoB-100 [1–3]. ApoB-48, synthesized by the human intestine, is necessary for the synthesis and secretion of chylomicrons [4]. ApoB-100, produced in the human liver, is the major protein component of plasma low-density lipoproteins (LDLs) [2]. LDLs transport two-thirds of the plasma cholesterol, and elevated levels of LDL cholesterol are a major risk factor for the development of atherosclerosis. ApoB-100 is also a component of other atherogenic lipoproteins, such as lipoprotein (a) and β -migrating very-low-density lipoproteins (VLDLs). Therefore, ways to lower the plasma apoB-100 and the LDL level have been sought as a therapeutic approach to lower plasma cholesterol levels.

ApoB-48 is produced by a specific deamination reaction of C⁶⁶⁶ in apoB mRNA, generating a uridine [5–8] that changes codon 2153 from a genomically encoded CAA (glutamine) to an in-frame stop codon (UAA) [9,10]. The specificity of the reaction is given by an 11-nucleotide mooring sequence on the apoB mRNA which forms a double-stranded stem with apoB 5' sequences or with the apoB 3' efficiency element [11,12]. ApoB-48 lacks the LDL receptor-binding domain of apoB, which is located in the C-terminal half of apoB-100, and therefore cannot bind to the LDL receptor [13]. Lipoproteins containing apoB-48 possess multiple copies of apoE, which serves as a ligand to

mediate the clearance of particles through the LDL receptor and the LDL receptor-related protein [14–16]. Single copies of apoB-100 and apoE bind to the LDL receptor with similar affinity; however, lipoproteins with multiple copies of apoE bind to multiple receptors, increasing the affinity of the lipoprotein–receptor interaction [17]. As a result, apoB-48-containing particles are cleared from the plasma more rapidly (in minutes) than apoB-100-containing LDL (2–3 days) [18].

The apoB mRNA-editing enzyme complex is composed of the cytidine deaminase apoB mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1) [19] and at least one other protein. APOBEC-1 is a 229-amino-acid protein that edits C⁶⁶⁶ in the apoB mRNA [20] only in the presence of the APOBEC-1 complementation factor (ACF), a 586-amino-acid RNA-binding protein [21,22]. These two proteins are sufficient for *in vitro* editing of a synthetic apoB RNA substrate. However, other proteins may be involved in the regulation of apoB mRNA editing *in vivo* [23–26]. In humans, APOBEC-1 is expressed only in intestinal enterocytes. Therefore, apoB mRNA editing and subsequent apoB-48 formation are restricted to the intestine. In contrast, ACF is widely distributed and is found in tissues and organs that synthesize little, if any, apoB and have no detectable apoB mRNA-editing activity [21].

Several reports show that overexpression of APOBEC-1 leads to hepatic apoB mRNA editing and lower LDL cholesterol levels. Adenoviral delivery of *APOBEC-1* to the liver of rabbits and liver-specific overexpression of APOBEC-1 in apoB transgenic mice decreased LDL cholesterol levels [27–32]. However,

Abbreviations used: ACF, APOBEC-1 complementation factor; apo, apolipoprotein; APOBEC-1, apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; NAT1, novel APOBEC-1 target 1; tTA, tetracycline-controlled transactivator; VLDL, very-low-density lipoprotein.

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high levels of rabbit *APOBEC-1* cDNA expression in mouse liver resulted in liver dysplasia and hepatocellular carcinomas [32]. In these I20 mice, high expression of APOBEC-1 was driven by the liver-specific human apoE promoter including the hepatic control region [32]. Analysis of apoB mRNA from transgenic mouse livers revealed extensively edited cytidines at multiple sites that did not have a mooring-sequence motif directly downstream [33]. This aberrant editing, which was termed hyperediting, was mainly focused on a stretch of 200 nucleotides downstream of the mooring-sequence motif in apoB and was distributed over the entire coding sequence of a novel mRNA, novel APOBEC-1 target 1 (NAT1) [33,34]. Hyperediting could be mimicked *in vitro* and is thought to result from that loose recognition of RNA features, which are similar to those in the vicinity of the editing site in apoB mRNA [35]. In contrast, low-level expression of rat APOBEC-1 (RE4 mice) efficiently reduced LDL cholesterol levels in human apoB transgenic mice but did not cause pathological changes or hyperediting [29]. In both systems, APOBEC-1 was overexpressed in wild-type mice, which have 60–80% endogenous apoB mRNA editing in their livers. However, humans lack apoB mRNA editing in the liver and produce apoB-100-containing VLDL only. Hence, to evaluate a potential therapeutic use for liver-induced apoB mRNA editing in humans, we investigated the efficiency and safety of transgenic expression of APOBEC-1 in the absence of endogenous editing in the mouse model. This model more faithfully resembles the human situation than wild-type mice to evaluate the extent of induced editing and hyperediting. Therefore, we constructed and characterized transgenic mice with regulatable APOBEC-1 expression but lacking endogenous apoB mRNA editing.

MATERIALS AND METHODS

Vector construction and transgenic mice

Full-length rabbit *APOBEC-1* cDNA [36] was directionally subcloned into pUHG-10 [37–39], containing a minimal cytomegalovirus promoter and the tetracycline-controlled transactivator (tTA)-binding sequences (tetO), with the *Bam*HI and *Xba*I restriction sites to produce the vector *tetO-APOBEC-1*. Transgenic mouse lines (FVB-N) were generated with *Ase*I- and *Xho*I-linearized *tetO-APOBEC-1* (2.5 kb) by pronuclear injection and standard techniques [40]. Genotypes of founder mice were determined by Southern blot hybridization with a ³²P-labelled probe of rabbit *APOBEC-1* cDNA [41]. These *tetO-APOBEC-1* transgenic mice were crossed with *LAP-tTA* transgenic mice [39] to obtain double transgenic trans-heterozygous mice expressing APOBEC-1 in the liver. Subsequent genotyping was done by PCR with primers TAU166 (5'-AACCCGTAACTCGCCCA-GAA-3') and TAL541 (5'-CTTCTCTTTAGCGACTTGA-3') for the *LAP-tTA* transgene (396 bp), and pUGHU (5'-GACCTCCATAGAAGACACCGGACCG-3') and pUGHL (5'-ATACTCTGAGACGCTCATAACCCGGA-3') for the *tetO-APOBEC-1* transgene (587 bp).

Crossing the transgenes into the *APOBEC-1*^{-/-} background

LAP-tTA and *tetO-APOBEC-1* transgenic mice were each crossed with *APOBEC-1*^{-/-} mice lacking endogenous APOBEC-1. Offspring were genotyped for either the *LAP-tTA* or the *tetO-APOBEC-1* transgene. *LAP-tTA/APOBEC-1*^{-/+} were then crossed with each other, and the offspring were genotyped for *LAP-tTA* by PCR and for homozygosity of the APOBEC-1-knockout allele (*APOBEC-1*^{-/-}) by Southern blot hybridization after *Pvu*II digestion [42]. *APOBEC-1* exon 8 was used as a probe which was amplified from genomic mouse DNA with primers

MBECU25 (5'-TTAAGAAGAAAGCAACCTCAACTC-3') and MBEC1859 (5'-ACAACCCCTCAACAGACACAATA-3'). In parallel, *tetO-APOBEC-1/APOBEC-1*^{-/+} mice were intercrossed, and offspring selected that were transgenic for the *tetO-APOBEC-1* allele and homozygous for the *APOBEC-1*-knockout allele. Mating of *tetO-APOBEC-1/APOBEC-1*^{-/-} and *LAP-tTA/APOBEC-1*^{-/-} mice resulted in animals with regulatable APOBEC-1 expression and without endogenous mouse APOBEC-1. APOBEC-1 expression was suppressed by administering 10 µg/ml doxycycline hydrochloride (Sigma) in the drinking water, which was changed every 3 days. Mice were 2–4 months old when used for experiments. All experiments were carried out in compliance with guidelines for the humane treatment of animals from the National Institutes of Health and the University of California.

Northern blot analysis

Total RNA was extracted by Trizol (Gibco-BRL, Gaithersburg, MD, U.S.A.) from mouse livers that were quick-frozen in liquid nitrogen and analysed by Northern blotting as described in [36]. Blots were hybridized with ³²P-labelled rabbit *APOBEC-1* cDNA, stripped and rehybridized with ³²P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantification was performed on a Phosphorimager (Bio-Rad Molecular Imager FX).

Amplification of apoB and NAT1 cDNA

Total RNA was treated with DNase and reverse transcribed with avian myeloblastosis virus reverse transcriptase using gene-specific primers (Life Sciences, St. Petersburg, FL, U.S.A.). Specific regions of apoB and NAT1 cDNAs were amplified by PCR. The primers used to amplify apoB were MBU6629 (5'-CAATGAAAACTCTCTCAACTT-3') and MBL6803 (5'-AA-ATAAATAGAGATTATGGATT-3'). The primers used to amplify NAT1 were D45620-32UP and D45620-146LP [29]. The PCR products were purified over S-300 HR columns (Amersham Biosciences, Piscataway, NJ, U.S.A.), and the purified PCR product used for primer-extension analysis.

Primer-extension analysis

Primer extension was performed essentially as described in [29,35]. Primer Cons51 [35] was used for primer extension of apoB mRNA and primer PE83 [29] was used to detect editing at C³⁷³⁰ in NAT1 mRNA. The extent of editing was determined with a radioanalytic imaging system (AMBIS, San Diego, CA, U.S.A.).

Sequence analysis

The PCR products were TA-subcloned into pCRII vector (Invitrogen, San Diego, CA, U.S.A.), and individual clones were sequenced. Sequence analysis on both strands of the plasmids was performed with Big Dye d-Rhodamine dye terminator mix on a 373 DNA Sequencing System (Applied Biosystems/Perkin-Elmer Corporation, Norwalk, CT, U.S.A.). The sequences were aligned with the DNASTAR software package (DNASTAR, Madison, WI, U.S.A.).

Analysis of plasma lipids

Lipid levels were measured in fresh EDTA-supplemented plasma from *tetO-APOBEC-1* transgenic mice lacking endogenous APOBEC-1. Blood was collected between 13:00 and 14:00 h after a 5 h fast and was supplemented with EDTA (2.5 mM) and complete proteinase inhibitors (Boehringer Mannheim,

Mannheim, Germany). For FPLC analysis, equal volumes of plasma from six mice were pooled, and 200 μ l was fractionated by Superose 6 chromatography as described previously [43]. In each fraction, cholesterol and triacylglycerol (triglyceride) levels were measured in a 100 μ l sample with a Spectrum Lipid Analyser (Abbot Laboratories, Abbott Park, IL, U.S.A.). The remaining volume of the fractions was pooled according to the major lipoprotein classes [fractions 17–19, VLDL; 20–23, intermediate-density lipoprotein (IDL); 24–29, LDL; and 30–36, high-density lipoprotein] and were concentrated to a volume of 40 μ l with Centricon spin columns. Western blot analysis used 3 μ l.

Western blot analysis

Plasma samples or pooled fractions from the FPLC analysis were separated on SDS/polyacrylamide gels followed by immunoblotting. An affinity-purified polyclonal antibody against mouse LDL was used to detect mouse apoB.

RESULTS

Transgenic mouse line

To generate transgenic mice with regulatable expression of APOBEC-1, a full-length rabbit *APOBEC-1* cDNA was subcloned into the vector pUHG-10. Injection of a linearized fragment of this vector produced seven *tetO-APOBEC-1* transgenic mouse lines. All seven lines were crossed with *LAP-tTA* transgenic mice [39] which express the tTA in their livers controlled by the liver-enriched activator protein promoter [39]. In the absence of doxycycline, tTA supports transcription of

Figure 1 Regulatable APOBEC-1 expression in the liver of transgenic mice

Analysis of rabbit APOBEC-1 expression in the livers of two *LAP-tTA* and *tetO-APOBEC-1* double transgenic mice (lanes 1 and 3) and two non-transgenic littermates (lanes 2 and 4); + indicates that the specific transgene is present in the mouse. (A) Northern blot analysis of total liver RNA detected the $\approx$ 1.5 kb rabbit APOBEC-1 mRNA and mouse GAPDH mRNA as a control for loading. (B) Primer-extension analysis on reverse-transcriptase-PCR-amplified total liver RNA detected editing of C⁶⁶⁶ in the apoB mRNA. The upper band represents edited product uridine, and the middle band represents the cytidine. (C) Mouse apoB was detected in plasma by Western analysis with a rabbit antiserum to mouse apoB. Blood was collected before (Off Dox) and after (On Dox) the administration of doxycycline 10 (μ g/ml) for 6 days in the drinking water. Doxycycline suppressed APOBEC-1 expression resulting in the reappearance of apoB-100 in plasma.

Figure 2 Down-regulation of *tetO-APOBEC-1* expression

tetO-APOBEC-1 mice lacking endogenous APOBEC-1 were given 10 μ g/ml doxycycline (Dox) in their drinking water. At the indicated time points (Days on Dox), blood and liver samples were collected. (A) Northern blot analysis on total liver RNA from individual mice detected the $\approx$ 1.5 kb rabbit APOBEC-1 mRNA and mouse GAPDH mRNA (control for loading). (B) Primer-extension analysis of the editing of C⁶⁶⁶ in apoB mRNA. The samples are cDNAs derived from the liver RNA from individual mice. The upper band represents uridine and the middle band represents the unedited cytidine. (C) Western detection of mouse apoB in pooled mouse plasma from six mice using a rabbit antiserum to mouse apoB. KO, plasma from mice lacking endogenous APOBEC-1 (*APOBEC-1*^{-/-}).

tetO-APOBEC-1. However, in the presence of doxycycline, the tTA protein is inactivated and APOBEC-1 expression is suppressed. In double-transgenic mice, liver-specific transcription of *tetO-APOBEC-1* can therefore be switched on by removing doxycycline from the drinking water and suppressed by adding it to the water.

Regulatable APOBEC-1 expression in transgenic mice

One of seven transgenic *tetO-APOBEC-1* lines showed regulatable APOBEC-1 expression when the *LAP-tTA* transgene was present in the same mouse (Figure 1). In this line, Northern blot analysis detected the $\approx$ 1.5 kb APOBEC-1 mRNA in all livers of double transgenic mice (Figure 1A), and primer-extension analysis showed that hepatic apoB mRNA was edited extensively (Figure 1B). This increased editing of the apoB mRNA reduced the plasma apoB-100 concentration to below detectable levels. The reduction was reversible. ApoB-100 was again observed in

Figure 3 Down-regulation of tetO-APOBEC-1 expression increased LDL cholesterol and triacylglycerols (triglycerides) in mice lacking endogenous APOBEC-1

Representative Superose 6 profile of pooled plasma from six mice. Blood was collected before (none) and during (days) administration of doxycycline (10 µg/ml) in drinking water. (A) *tetO-APOBEC-1/APOBEC-1^{-/-}* mice. Two separate experiments were performed on age- and sex-matched mice. (B) Littermate control mice (*APOBEC-1^{-/-}*) lacking endogenous mouse APOBEC-1.

Figure 4 Liver-derived apoB-48 accumulates predominantly in the IDL and LDL fractions

Pooled plasma from six *tetO-APOBEC-1* mice lacking endogenous mouse APOBEC-1 was separated by Sepharose 6 chromatography and the major lipoprotein classes were pooled. Mouse apoB in these lipoprotein fractions was detected by Western analysis with a rabbit antiserum to mouse apoB. Two independent experiments in the absence of doxycycline (Dox; —) and one control experiment in the presence of doxycycline (+) are shown.

the plasma after suppressing tetO-APOBEC-1 expression with doxycycline (Figure 1C).

Regulatable APOBEC-1 expression in the *APOBEC-1^{-/-}* background

To investigate liver-specific regulatable expression of APOBEC-1 in the absence of endogenous mouse APOBEC-1, we crossed the mice with regulatable tetO-APOBEC-1 expression with *APOBEC-1^{-/-}* mice.

tetO-APOBEC-1 single transgenic mice in the *APOBEC-1^{-/-}* background expressed no APOBEC-1 in liver or intestine, which was shown by a lack of APOBEC-1 mRNA expression and by the lack of apoB-48 in plasma (results not shown). This emphasizes that tetO-APOBEC-1 expression depends on the expression of tTA by the *LAP-tTA* transgene. Kistner et al. [39] have shown that *LAP-tTA*-dependent tetO-luciferase expression was similar to background in the intestine and in a number of other organs but was increased 10000-fold above background in the liver. Therefore, tetO-APOBEC-1 expression and apoB-48 formation is restricted to the liver in *LAP-tTA/tetO-APOBEC-1* double transgenic mice.

Such liver-specific tetO-APOBEC-1 expression of *LAP-tTA/tetO-APOBEC-1* transgenic mice in the *APOBEC-1^{-/-}* background resulted in apoB mRNA editing and subsequent apoB-48 formation in plasma (Figure 2). Feeding mice 10 µg/ml doxycycline in their drinking water suppressed tetO-APOBEC-1 mRNA expression within 2 days (Figure 2A) with the drastic reduction of apoB mRNA editing activity within 4 days (Figure 2B). Following the time course of edited apoB mRNA, the protein apoB-48 was not detectable after 4 days on doxycycline (Figure 2C). APOBEC-1 expression was suppressed as long as doxycycline was fed to the regulatable *tetO-APOBEC-1/APOBEC-1^{-/-}* mice up to at least 30 days.

Effect of APOBEC-1 expression on LDL levels in *APOBEC-1^{-/-}* mice

The lipoprotein profile of *tetO-APOBEC-1/APOBEC-1^{-/-}* mice and littermate controls was investigated off and on doxycycline. Total cholesterol and triacylglycerol levels did not change significantly during doxycycline treatment (results not shown). Plasma

Figure 5 Tet-driven APOBEC-1 expression results in editing of C⁶⁶⁶⁶ and other cytidines in apoB and NAT1 mRNA in the wild-type and *APOBEC-1*^{-/-} backgrounds

Total liver RNA was extracted from *tetO-APOBEC-1* mice and *tetO-APOBEC-1/APOBEC-1*^{-/-} mice. The apoB mRNA-editing region and NAT1 were amplified by reverse transcriptase PCR, subcloned into pCRII and sequenced. (A) The percentage of physiological editing at C⁶⁶⁶⁶ and hyperediting at other cytidines in apoB is shown in the wild-type ($n = 16$; black bars) and *APOBEC-1*^{-/-} ($n = 20$; grey bars) backgrounds. (B) The percentage of hyperediting in NAT1 is shown in the wild-type ($n = 20$; black bars) and *APOBEC-1*^{-/-} ($n = 40$; grey bars) backgrounds. (C) Primer-extension analysis of C³⁷³⁰ in the NAT1 mRNA from liver of mice on and off doxycycline. The upper band represents the deamination of C³⁷³⁰ to a uridine (U³⁷³⁰). The middle band represents the unmodified cytidine (C³⁷³⁰). wt, tetO-APOBEC-1 expression in the wild-type background; *APOBEC-1*^{-/-}, tetO-APOBEC-1 expression in the *APOBEC-1*^{-/-} background. Experiments in the absence (-) and presence (+) of doxycycline are shown.

was then analysed by FPLC analysis on a Sepharose 6 column. The FPLC profiles for triacylglycerols and cholesterol for the *tetO-APOBEC-1/APOBEC-1*^{-/-} mice demonstrated low concentrations of IDL/LDL because of the expression of the hepatic tetO-controlled APOBEC-1. However, when mice were fed doxycycline in the drinking water and plasma collection was repeated after 2, 6, 13 and 30 days, a small increase in IDL/LDL cholesterol and triacylglycerols was seen in the *tetO-APOBEC-1/APOBEC-1*^{-/-} mice (Figure 3A). The area under the curve for fractions 20–29 (IDL/LDL) increased by 65% (52–81%) for cholesterol and by 29% (25–31%) for triacylglycerols ($n = 2$; results not shown) within 13 days. This increase was not due to

doxycycline treatment since the FPLC profile for the littermates showed a tendency towards decreased IDL/LDL levels during doxycycline treatment (Figure 3B), with a decrease in the area under the curve for the IDL/LDL fractions of 12% for cholesterol and an increase of 9% for triacylglycerols. Doxycycline and tetracycline treatment was previously shown to decrease VLDL secretion from the liver and to reduce total cholesterol in plasma [44–46]. Therefore, the increase in IDL/LDL in *tetO-APOBEC-1* mice was not due to increased apoB secretion from the liver but due to decreased catabolism of apoB-100-containing lipoproteins compared with apoB-48-containing ones.

Distribution of apoB in the major lipoprotein classes

We then investigated the distribution of liver-derived apoB-48 in the four lipoprotein classes. Fractions 17–36 from the Sepharose 6 columns were examined by Western analysis with a rabbit antiserum to mouse apoB. ApoB-48 was predominantly found in LDL and less in the IDL fraction, similar to the distribution of apoB-100 (Figure 4). The great reduction of apoB-48 from all lipoprotein classes in mice receiving doxycycline emphasizes the complete suppression of APOBEC-1 expression in *tetO-APOBEC-1/APOBEC-1*^{-/-} mice by doxycycline.

tetO-APOBEC-1 overexpression results in aberrant hyperediting

Previously, transgenic mice (I20) expressing high levels of rabbit *APOBEC-1* cDNA in their livers were generated that unexpectedly displayed liver dysplasia, which developed into hepatocellular carcinomas [32]. Analysis of the apoB mRNA and the NAT1 mRNA from transgenic mouse livers revealed extensively edited cytidines at multiple sites that did not have a mooring-sequence motif directly downstream. We examined tetO-APOBEC-1 expression for hyperediting. We amplified the editing region of the apoB mRNA and the NAT1 mRNA by reverse transcriptase PCR and sequenced individual apoB cDNA clones from liver RNA of mice expressing tetO-APOBEC-1 in the wild-type background. As a result, all C⁶⁶⁶⁶ were edited, and several cytidines downstream were hyperedited (Figure 5A). In *tetO-APOBEC-1/APOBEC-1*^{-/-} mice, only 20% of the apoB mRNAs were edited at C⁶⁶⁶⁶, but several cytidines downstream of C⁶⁶⁶⁶ were hyperedited up to 15% (C⁶⁷⁴³, C⁶⁸⁰²). Furthermore, expression of tetO-APOBEC-1 also induced hyperediting of the NAT1 mRNA. Again, hyperediting of NAT1 was higher in the wild-type background than in the *APOBEC-1*^{-/-} background (Figure 5B). When tetO-APOBEC-1 expression was switched off, hyperediting at C³⁷³⁰ in the NAT1 mRNA decreased in parallel to editing at C⁶⁶⁶⁶ in the apoB mRNA and was abolished within 4 days (Figure 5C).

Moderate expression in the tetO-APOBEC-1 mice

We then compared the expression levels of APOBEC-1 in the *tetO-APOBEC-1* transgenic mice to the high expression levels in the I20 line of mice that developed liver dysplasia and hepatocellular carcinomas [32]. As seen by Northern blot analysis, transgenic rabbit APOBEC-1 mRNA levels were three times higher in the I20 mice than in the *tetO-APOBEC-1* mice (results not shown). I20 mice have three times higher editing activity in their livers than the rat *APOBEC-1* transgenic RE4 mice [29], suggesting that editing activity in the liver of *tetO-APOBEC-1* mice is similar to that of the RE4 mice, which do not develop liver dysplasia and tumours [29].

DISCUSSION

This study shows that regulatable APOBEC-1 expression in the livers of mice lacking APOBEC-1 results in 30 % editing of the apoB mRNA. Furthermore, overexpression of rabbit APOBEC-1 in *APOBEC-1^{-/-}* mice resulted in regulatable aberrant hyperediting of other cytidines in apoB mRNA and in NAT1 mRNA. The level of hyperediting of several cytidines in the apoB mRNA reached 75 % of the level of editing at C⁶⁶⁶⁶, suggesting that overexpressed rabbit APOBEC-1 lost its specificity for editing at C⁶⁶⁶⁶.

Our results with the regulatable *tetO-APOBEC-1* mouse model in the *APOBEC-1^{-/-}* background indicate that hepatic apoB-48 formation reduces plasma LDL cholesterol and triacylglycerol levels. This decrease in plasma IDL and LDL resulted from 30 % editing of the apoB mRNA, emphasizing that omitting mouse-to-mouse differences drastically increases the detection limit for phenotypic changes. However, these results also show that a disproportionately high expression of hepatic tetO-APOBEC-1 is necessary to reach less than half of the apoB mRNA-editing activity of a wild-type mouse. Wild-type mice efficiently edit up to 80 % of C⁶⁶⁶⁶ in liver with low APOBEC-1 expression. In contrast, moderate tetO-APOBEC-1 expression is necessary in *APOBEC-1^{-/-}* mice to edit only 30 % of C⁶⁶⁶⁶. But even this moderate expression of rabbit *APOBEC-1* cDNA resulted in lower specificity for C⁶⁶⁶⁶ than endogenous editing in wild-type mice.

tetO-APOBEC-1 expressed in mice lacking endogenous APOBEC-1 was not specific for C⁶⁶⁶⁶ and deaminated other cytidines in the apoB mRNA. Previously, transgenic mice (I20) expressing rabbit *APOBEC-1* cDNA in the livers unexpectedly displayed liver dysplasia and hepatocellular carcinomas [32]. In these mouse livers, C⁶⁶⁶⁶ in the apoB mRNA was edited to > 95 %, and several other cytidines were edited at high levels in the apoB and the NAT1 mRNA [33,34]. Expression of tetO-APOBEC-1 in the wild-type background resulted in slightly lower levels of editing at C⁶⁶⁶⁶ ($\approx$ 92 %), which were comparable with the levels in the RE4 mice that do not develop liver dysplasia [29]. However, tetO-APOBEC-1 expression in mice lacking endogenous APOBEC-1 resulted in only 30 % editing at C⁶⁶⁶⁶ (Figure 2B). This was supported by sequence analysis of 20 apoB cDNA clones that showed 20 % editing at C⁶⁶⁶⁶. In spite of this low level of editing, several cytidines were hyperedited in the apoB mRNA and in the NAT1 mRNA (Figure 5). Sequence analysis of 20 cDNA clones revealed that several cytidines downstream of C⁶⁶⁶⁶ were edited, including two that were edited to 15 % (C⁶⁷⁴³ and C⁶⁸⁰²). Hence, overexpression of rabbit *APOBEC-1* cDNA in mouse livers lacking endogenous APOBEC-1 results in reduced specificity for C⁶⁶⁶⁶ and hyperediting of other cytidines in apoB mRNA to an extent similar to C⁶⁶⁶⁶.

There are two explanations for the loss of specificity for C⁶⁶⁶⁶ when tetO-APOBEC-1 is expressed. First, mouse ACF or other regulating auxiliary proteins may bind less efficiently to the heterologous rabbit APOBEC-1 and form editing complexes with altered stoichiometric composition, which could reduce specificity for the editing site. Editing activity in transgenic mice expressing rat genomic *APOBEC-1* clones was similar to that in *tetO-APOBEC-1* mice, suggesting that the rat sequence, which is phylogenetically closer to mouse than to rabbit, conserved its specificity in mouse liver. However, this explanation is not supported by experiments in rat McArdle cells expressing rat or human *APOBEC-1*, where promiscuous editing depended solely on the expression level of APOBEC-1 [47]. These cell-culture experiments may not be directly comparable with APOBEC-1

overexpression in transgenic animals because there are marked differences between promiscuous editing in McArdle cells and hyperediting by rabbit APOBEC-1 *in vivo* and *in vitro* [33,35,47,48].

The second explanation is that overexpression of the rabbit *tetO-APOBEC-1* cDNA could result in altered stoichiometric composition of the editing complex and lead to aberrant protein processing. The subcellular distribution of APOBEC-1 under physiological conditions is not known because endogenous APOBEC-1 cannot be detected by immunohistochemical methods. Cellular subfractionation and analysis of apoB pre-mRNA suggest that APOBEC-1 is localized in the nucleus under physiological conditions and that apoB mRNA editing is a predominantly nuclear event [49]. However, when tagged APOBEC-1 was overexpressed in cells containing auxiliary proteins, the enzyme was localized in the nucleus and cytoplasm, suggesting that overexpression results in redistribution of APOBEC-1 to the cytoplasm [50]. Considering that cytoplasmic localization of APOBEC-1 upon overexpression coincides with hyperediting of apoB mRNA [51], one could argue that cytoplasmic APOBEC-1 is responsible for aberrant hyperediting. Therefore, hyperediting may only occur if a threshold level of APOBEC-1 expression is reached and APOBEC-1 is redistributed to the cytoplasm. As mentioned before, the editing activity in the *tetO-APOBEC-1* mice is comparable with that in the RE4 mice, but only APOBEC-1 overexpression from the cDNA construct in *tetO-APOBEC-1* mice resulted in aberrant hyperediting. Therefore, transcriptional control through regulatory elements in the genomic P1 clone (RE4) may limit expression to sub-threshold levels. This possibility is supported by the disproportionate relationship between APOBEC-1 expression and apoB mRNA editing in transfected cells [52] and by the observation that apoB mRNA editing is regulated in mouse and rat liver by nutritional and hormonal factors [53–57]. For example, the level of apoB mRNA editing was 30 % in fasting mice, but increased to 90 % when mice were fed a high carbohydrate diet [53,55]. This regulation of apoB mRNA editing is thought to be controlled by the abundance or distribution of APOBEC-1 [1,21] which presumably is tightly controlled by genetic and environmental factors.

Therefore, tight regulation of APOBEC-1 expression seems to be required for potential therapeutic use of apoB mRNA editing to lower LDL cholesterol. Co-expression of ACF and tetO-APOBEC-1 in transgenic animals will determine whether safe and efficient editing of C⁶⁶⁶⁶ is solely dependent on the stoichiometry of the editing complex or whether co-expression still results in aberrant protein processing and hyperediting through subcellular mislocalization of the editing complex.

In summary, we present a liver-specific tetO-APOBEC-1 expression system in mice lacking endogenous APOBEC-1 that results in regulatable apoB mRNA editing and in regulatable hyperediting of other cytidines in both apoB and NAT1 mRNA. Phenotypic analysis of the plasma cholesterol and triacylglycerol levels indicated a reduction in IDL and LDL fractions when APOBEC-1 was expressed.

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