A tissue-specific inhibition of apolipoprotein B (apoB) mRNA editing enzyme complex. In humans it is expressed only in the intestine, whereas in mice it is expressed in both the liver and intestine. APOBEC-1 exists as a spontaneous homodimer (Lau, P., P., Zhu, H.-J., Baldini, A., Charnsangavej, C., and Chan, L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8522–8526). We tested the editing activity and dimerization potential of three different mouse APOBEC-1 mutants using in vitro editing activity assay and immunoprecipitation in the presence of epitope-tagged APOBEC-1. One catalytically inactive mutant, mu1 (H81K/C938S/C968S), that retains its capacity to dimerize with wild-type APOBEC-1 was found to inhibit the editing activity of the latter and was thus a dominant negative mutant. Two other inactive mutants that dimerized poorly with APOBEC-1 failed to inhibit its activity. Intravenous injection of a mu1 adenovirus, Admu1, in C57BL/6J mice in vivo resulted in liver-specific expression of mu1 mRNA. On days 4 and 9 after virus injection, endogenous hepatic apoB mRNA editing was 23.3 ± 5.0 and 36.8 ± 5.7%, respectively, compared with 65.3 ± 11 and 71.3 ± 5.2%, respectively, for luciferase adenovirus-treated animals. Plasma apoB-100 accounted for 95 and 93% of total plasma apoB in Admu1 animals on days 4 and 9, respectively, compared with 78 and 72% in luciferase adenovirus animals. Plasma cholesterol on day 9 was 98 ± 17 mg/dl in the mu1-treated animals, substantially higher than phosphat-buffered saline-treated (57 ± 9 mg/dl) or luciferase-treated (71 ± 12 mg/dl) controls. Fast protein liquid chromatography analysis of mouse plasma showed that the intermediate density/low density lipoprotein fractions in the animals treated with the dominant negative mutant adenovirus were much higher than those in controls. We conclude that active APOBEC-1 functions as a dimer and its activity is inhibited by a dominant negative mutant. Furthermore, apoB mRNA editing determines the availability of apoB-100, which in turn limits the amount of intermediate density/low density lipoprotein that can be formed in mice. Liver-specific inhibition of apoB mRNA editing is an important component of any strategy to enhance the value of mice as a model for human lipoprotein metabolism.

The mouse is a useful animal model for lipoprotein metabolism and atherosclerosis (1, 2). Its value as a model for human disease, however, is limited by the fact that there is a substantial difference in lipoprotein metabolism between the two species. One major difference is the presence of high levels of apolipoprotein B (apoB) mRNA editing in the liver in mice but not in humans.

ApoB mRNA editing is a process by which apoB-100 mRNA is converted to apoB-48 mRNA (3, 4) (reviewed in Ref. 5). It involves the conversion of the first base of the codon CAA, encoding glutamine 2153 in apoB-100, to UAA, a stop codon, in apoB-48 mRNA. In humans, editing occurs exclusively in the small intestine but not in the liver. Therefore, the human liver produces apoB-100 and the small intestine produces apoB-48. In mice, apoB mRNA editing occurs in both the small intestine and the liver. Therefore, the amount of apoB-100 produced by the liver is very small, and mice have very low levels of circulating apoB-100; consequently, they have low levels of intermediate density lipoprotein (IDL) and low density lipoprotein (LDL), atherogenic lipoproteins that require apoB-100 as an essential component.

ApoB mRNA editing is mediated by a multiprotein enzyme complex. APOBEC-1 is a cytidine deaminase-like protein that has been identified as a catalytic component of the complex (6–10) that efficiently edits synthetic apoB mRNA in vitro in the presence of complementation factors. Lau et al. (10) showed that APOBEC-1 exists as a spontaneous homodimer. It shows sequence similarity to Escherichia coli cytidine deaminase, which also exists as a homodimer (11). In the case of APOBEC-1, Lau et al. (10) postulated that dimerization may be mediated by hydrophobic interactions in a leucine-rich domain in the C-terminal third of the molecule. It is not known, however, if the active form of APOBEC-1 is in the form of a homodimer.

The crystallographic structure of E. coli cytidine deaminase suggests that the active enzyme functions as a dimer (11). We reasoned that active APOBEC-1 might also function as a dimer and enzymatically inactive APOBEC-1 mutants (e.g. those that have substitutions in the zinc-coordinating residue in the active site (11)) might under appropriate conditions inactivate the wild-type enzyme by forming an inactive heterodimer with...
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MATERIALS AND METHODS

Site-directed Mutagenesis by Polymerase Chain Reaction—Mouse APOBEC-1 mutants (mu1, H61K/C93S/C96S; mu2, APOBEC-1 1–172 that misses the C-terminal 57 residues containing the leucine-rich domain; mu3, L182R/L187R) were produced by the polymerase chain reaction (PCR). In brief, bPhageII KS (Stratagene) containing the wild-type mouse APOBEC-1 cDNA (7) was amplified using the reverse primer (5′-GCCCTGGACGCTCCCGGCTGGAAGCC-3′), forward primer (5′-GCCCTGGACGCTCCCGGCTGGAAGCC-3′), and specific mutagenesis primer m1 (5′-GCCCTGGACGCTCCCGGCTGGAAGCC-3′) in the presence of chicken enterocyte S-100 extracts. Primer sequences were verified by double-stranded DNA sequencing. The mutations are in boldface. H61K (5′-GAAAGCTTGAAGGTTTCAACATTGTTGCTCTGTTTGG-3′; CGTGCCCATG-3′), forward primer (5′-GCCCTGGACGCTCCCGGCTGGAAGCC-3′), and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′) generated by PCR using forward primer and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′) were generated by PCR using forward primer and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′) were generated by PCR using forward primer and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′) were generated by PCR using forward primer and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′) were generated by PCR using forward primer and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′) were generated by PCR using forward primer and 3′ downstream primer (5′-GCCGGAGATTTCCAATGTTGGGATCTGGCCAAAGG-3′). After PCR, the purified products were digested with BamHI and EcoRI and subcloned into the BamHI/EcoRI sites of pBKCVM (Stratagene) or bPhageII KS. DNA sequences were verified by double-stranded DNA sequencing.

In Vitro Translation—RNAs were transcribed from cDNAs subcloned into pBKCVM and capped with T3 RNA polymerase using the MASESAGE mMACHINE kit (Ambion). They were translated in nuclease-resistant 15% agarose gels followed by ultracentrifugation from plasma pooled from four individual animals. For analysis of plasma apoBs, lipoproteins of each FPLC fraction and of plasma were determined enzymatically (Sigma kits 352-50 and 352-50). For analysis of plasma apoBs, lipoproteins of each FPLC fraction and of plasma were determined enzymatically (Sigma kits 352-50 and 352-50). For analysis of plasma apoBs, lipoproteins of each FPLC fraction and of plasma were determined enzymatically (Sigma kits 352-50 and 352-50).

In Vitro ApoB mRNA Editing Assay—The in vitro editing assay was carried out as described previously (12) using 8 fmol of synthetic substrate in the presence of chicken enterocyte S-100 extracts. Primer extension products were fractionated on a 6% polyacrylamide gel, stained with Coomassie Blue, and the editing activity of the translation products was determined. The capacity of various APOBEC-1 mutants to dimerize with epitope-tagged wild-type APOBEC-1 was examined using epitope-tagged wild-type APOBEC-1 (Fig. 1), lane 4. However, a mutant that lacks the leucine-rich region (mu2) or one with mutations in this region (mu3) showed a markedly decreased ability to dimerize with epitope-tagged wild-type APOBEC-1 (Fig. 1, lanes 6 and 8). These results indicate that the leucine-rich region is important for dimer formation.

RESULTS AND DISCUSSION

Heterodimer Formation between Mutant and Wild-type APOBEC-1—We examined the capacity of various APOBEC-1 mutants to dimerize with epitope-tagged wild-type APOBEC-1 (10). mu1 (H61K/C93S/C96S) efficiently formed a heterodimer with wild-type APOBEC-1 (Fig. 1, lane 4). Howtver, a mutant that lacks the leucine-rich region (mu2) or one with mutations in this region (mu3) showed a markedly decreased ability to dimerize with epitope-tagged wild-type APOBEC-1 (Fig. 1, lanes 6 and 8). These results indicate that the leucine-rich region is important for dimer formation.

Capacity of Mutant APOBEC-1s to Inhibit the Editing Activity of Wild-type APOBEC-1—In order to study the effect of heterodimer formation on apoB mRNA editing activity, individual mutant and wild-type APOBEC-1s were translated in vitro and the editing activity of the translation products was determined. By itself, mu1, in which the 3 zinc-coordinating residues were mutated, displayed no detectable editing activity in vitro. Increasing the ratio of mu1 mRNA in the presence of a constant amount of wild-type APOBEC-1 mRNA produced an APOBEC-1 product that had lost >95% of its activity in the in vitro editing assay.
vitro editing assay (Fig. 2). Both mu2 and mu3 also had essentially no editing activity by themselves (<5% editing activity compared to that of wild type). The presence of increasing amounts of mu2 or mu3 translation products did not affect the editing activity of wild-type APOBEC-1 (Fig. 2). These results suggest that only a mutant that can efficiently dimerize with the wild-type APOBEC-1 is able to inhibit the editing activity of the latter. This supports the hypothesis that active APOBEC-1 functions as a dimer.

Effect of mu1 Gene Transfer—Normal C57BL/6J mice were treated with an intravenous injection of Admu1, an adenoviral vector containing mu1, or AdLuc, an adenovirus containing luciferase cDNA. Liver and blood samples were obtained at days 4 and 9 after treatment. The highest level of mu1 mRNA was detected in the liver on day 4. It decreased substantially but was still readily detectable on day 9 (Fig. 3A). In contrast, mu1 mRNA was not detectable in the small intestine either on day 4 or day 9. This agrees with our previous experience (13,16) and that of others (reviewed in Ref. 19) that intravenous administration of adenoviral vectors in mice in vivo targets the transgene to the liver almost exclusively. To determine whether the Admu1 effectively inhibited endogenous apoB mRNA editing, we measured the relative amounts of apoB-100 and apoB-48 mRNAs by primer extension (Fig. 3B). On day 4 following adenovirus administration, the hepatic apoB mRNA from Admu1 animals contained 23.3 ± 5.0% (n = 4) and that from AdLuc animals contained 65.3 ± 11% (n = 4) edited mRNA. On day 9, the proportions of edited mRNA were 36.8 ± 5.7% for Admu1 animals (n = 3) and 71.3 ± 5.2% for AdLuc animals (n = 4). The ratio for PBS-treated animals was 69.3 ± 4.3% (n = 4). Therefore, Admu1 but not AdLuc administration markedly reduced the amount of edited apoB mRNA in the liver. The amount of edited apoB mRNA in the small intestine in Admu1-treated animals was 89.5 ± 3.5% (n = 4) and 88.4 ± 2.2% (n = 4) for days 4 and 9, respectively, compared with AdLuc values of 92.7 ± 2.2% (n = 4) and 94.7 ± 0.48% (n = 4) for these two days; it was 93.8 ± 0.7% (n = 4) for PBS-injected controls. Thus, adenovirus-mediated transfer of a dominant negative mutant APOBEC-1 in vivo selectively inhibited apoB mRNA editing in the liver. Furthermore, this maneuver resulted in a significant increase in apoB-100 level (15.2 ± 4.0 mg/dl in Admu1, 2.84 ± 0.5 mg/dl in AdLuc, and 3.63 ± 2.1 mg/dl in PBS animals, p < 0.0005, on day 9) in Admu1-treated animals.

Effect of Inhibiting Hepatic ApoB mRNA Editing on Plasma ApoB-100 and ApoB-48 and Plasma Lipids—The ratio of apoB-

100 and apoB-48 protein was examined in mouse plasma by SDS gel analysis (Fig. 3C). Pooled plasma from four AdLuc-treated mice on days 4 and 9 contained 78 and 72%, respectively, of the total apoB as apoB-100. The proportion of apoB-100 increased to 95% at day 4 and remained at 93% at day 9 in Admu1-treated animals.

There were significant differences in plasma lipids among the three groups following adenovirus treatment. In Admu1 animals, plasma cholesterol (day 9) and triglyceride (both day 4 and day 9) were almost double the values in the other two groups (Table 1).

In order to determine which lipoprotein fractions were affected by Admu1 injection, plasma lipoproteins from day 9 samples were fractionated by FPLC and their cholesterol content determined (Fig. 4). It is evident that there is no significant difference in the very low density or high density lipoprotein fractions between Admu1-treated and the AdLuc- or PBS-treated control samples. However, compared with controls the
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TABLE I
Plasma lipids (mean ± S.D.) in adenovirus-treated and PBS-injected (control) mice

|       | Day 4                        | Day 9                        |
|-------|-----------------------------|------------------------------|
|       | Control (8)                 | Admu1 (15)                  | AdLuc (14)                  | Control (8)                  | Admu1 (13)                  | AdLuc (11)                  |
|       | mg/dl                        | mg/dl                        | mg/dl                        | mg/dl                        | mg/dl                        | mg/dl                        |
| Cholesterol | 61 ± 6                      | 80 ± 17                   | 59 ± 6                      | 57 ± 9                      | 98 ± 17                   | 71 ± 12                      |
| Triglyceride | 63 ± 20                      | 143 ± 30                   | 93 ± 31                      | 60 ± 8                      | 131 ± 29                   | 104 ± 25                      |

* The number in parentheses indicates the number of animals.

|       | p < 0.005 versus control. |
|-------|----------------------------|
|       | p < 0.0005 versus Admu1.  |
|       | p < 0.0001 versus control.|
|       | p < 0.05 versus Admu1.  |
|       | p < 0.005 versus control.|

The experiments above lead us to the following conclusions.

(i) Active APOBEC-1 works as a dimer and can be inhibited by a dominant negative mutant. Here we show that a mutant APOBEC-1 (H61C/C98S/C98S) with its zinc-coordinating residues mutated is totally inactive. This is consistent with previous observations (8, 20, 21) and with the catalytic role of the homologous residues in E. coli cytidine deaminase (11). The other two mutants, mu2 and mu3, which had mutations in the leucine-rich region, were also enzymatically inactive. The loss of catalytic activity in these mutants is probably related to their inability to efficiently form dimers, the active form of the enzyme. The fact that the mutations in these dimerization-incompetent APOBEC-1s involve residues in the leucine-rich domain indicates that this region is important for dimer formation. In any case, only mu1, which is competent in dimerization, acts as a dominant negative mutant when coexpressed with wild-type APOBEC-1 (Fig. 2). Therefore, these experiments support the contention that the active form of APOBEC-1 is a homodimer (10).

(ii) Liver-specific inhibition of APOBEC-1 action leads to elevated IDL/LDL cholesterol. We hypothesized that the low IDL/LDL cholesterol observed in mice compared to humans is partly the result of the diversion of apoB mRNA to the production of predominantly apoB-48, severely limiting the amount of apoB-100, which is essential for the formation of IDL/LDL. Here we showed that hepatic APOBEC-1 action in mice was inhibited by adenovirus-mediated transfer of a dominant negative mutant. As a result of the decrease in apoB mRNA editing, the plasma apoB-100/apoB-48 ratio was greatly increased and the plasma IDL/LDL cholesterol was much higher in these animals than in the luciferase- or PBS-treated controls. We have repeated the adenovirus experiments and consistently observed the IDL/LDL elevation (data not shown). We conclude that the availability of apoB-100 (indirectly determined by apoB mRNA editing efficiency in the liver) limits the amount of IDL/LDL that can be formed in mice. It is interesting that in APOBEC-1 knockout mice that do not edit apoB mRNA either in the liver or small intestine there is little (22) or no change (23, 24) in plasma IDL/LDL. The difference in phenotype between the animals reported in this study and the complete knockout mice may be related to the fact that 1) the liver-specific nature of APOBEC-1 inactivation and the normal editing in the small intestine may in some way contribute to enhanced very low density lipoprotein and subsequently IDL/LDL production, and/or more likely 2) the acute inhibition of APOBEC-1 action by adenovirus-mediated gene transfer does not allow sufficient time for the animals to marshal a compensatory response that may play a role in minimizing the lipoprotein changes in the APOBEC-1 gene knockout animals. In any case, future experiments aimed at “humanizing” the mouse should include the permanent liver-specific inactivation of apoB mRNA editing, which would greatly enhance the value of this animal as a model for human lipoprotein metabolism and atherosclerosis.

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