Hepatocyte-derived ApoE Is More Effective than Non-hepatocyte-derived ApoE in Remnant Lipoprotein Clearance*

The importance of hepatocyte-derived apolipoprotein (apo) E in the clearance of remnant lipoproteins in the liver is controversial. To address this controversy, we compared remnant clearance in two mouse models in which apoE is primarily derived either from hepatocytes or from an extrahepatic source. Hypomorphic apoE mice universally express reduced levels of apoE in all tissues, with the liver remaining the primary source of apoE. This mouse model of hepatocyte-derived apoE was compared with Apoe^{-/-} mice transplanted with mouse bone marrow as a model of primarily non-hepatocyte-derived apoE. Immunohistochemical analysis of liver sections revealed that only the hepatocyte-derived apoE model had detectable levels of apoE on hepatic sinusoidal surfaces. The non-hepatocyte-derived apoE model with plasma apoE levels similar to those in the hepatocyte-derived model had 2-fold more total plasma cholesterol, 4-fold more total plasma triglycerides, and 8-fold higher levels of apoB48, similar to Apoe^{-/-} mice. Both the hepatocyte-derived and the non-hepatocyte-derived apoE models had delayed clearance of an infused bolus of ^{125}I-labeled remnants compared with wild-type mice. However, after 3 h, plasma remnants reached wild-type levels only in the hepatocyte-derived apoE model, which had accumulated 70 ± 5% of wild-type levels of remnants in the liver while the non-hepatocyte-derived apoE model had accumulated only 38 ± 4%. These results demonstrate the existence of a role for both hepatically derived and localized apoE in remnant clearance. This role likely represents the enrichment of remnants sequestered on hepatocyte, with hepatocyte-derived apoE, facilitating their receptor-mediated internalization.

Plasma remnant lipoproteins are the metabolic end products of intestine-derived chylomicrons and hepatocyte-derived very low density lipoprotein (1–3). Apolipoprotein (apo) E mediates the uptake of remnants in the liver by binding to the LDL receptor (LDLR), the LDLR-related protein (LRP), and heparan sulfate proteoglycans (HSPG) (4–7). Inefficient clearance leads to the accumulation of remnants in plasma and contributes to premature atherosclerosis (8, 9).

The liver is the major source of plasma apoE; extrahepatic tissues, primarily macrophages, contribute up to 10% of plasma levels (10). Hepatocyte-derived apoE has been proposed to promote remnant clearance and uptake through a two-step process referred to as secretion-capture (3, 11, 12). In the first step, a portion of newly secreted apoE interacts with HSPG and remains bound to hepatocyte cell surfaces; the remainder is released into the space of Disse, where it serves to enrich lipoproteins (11–13). The presence of apoE on hepatocytes is thought to promote the trapping, or sequestration, of remnants (6, 11).

In support of this mechanism, distinct dynamic pools of apoE on hepatic cell surfaces have recently been reported in vivo (14). In the second step, sequestered remnants are further enriched with hepatocyte-derived apoE and are internalized into hepatocytes through processes mediated by receptors, including the LDLR and the LRP (7, 15–19), or through interactions with HSPG alone (11, 20, 21). When apoE/HSPG interactions are disrupted in vivo by intravenous heparinase infusion, remnant clearance is severely inhibited, supporting the importance of the secretion-capture role of apoE in remnant clearance (22, 23). The importance of heptically synthesized and localized apoE, and thus the secretion-capture role of apoE, has recently been questioned (24). Moreover, evidence from mouse models expressing apoE exclusively from extrahepatic sources suggests that hepatic apoE expression is not absolutely required for remnant clearance or for normal plasma cholesterol levels (25–27). Finally, it is not clear whether hepatic apoE expression is required for remnant clearance in mice lacking the LDLR (24, 28, 29).

We previously reported (30) the generation of hypomorphic apoE (hypoE) mice that express reduced levels of Arg-61 apoE, which is similar to human apoE4 in that it displays apoE4 domain interaction. The reduction in apoE levels is caused by a neomycin (neo) cassette flanked by loxP sites in Apo intron 3. However, the expression pattern of the hypomorphic allele remains normal, with the liver producing the majority of apoE in these mice (30). Excision of the neo cassette by Cre-mediated gene repair restores normal expression of the allele in all tissues.

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1 The abbreviations used are: apo, apolipoprotein; hypoE, hypomorphic apoE; neo, neomycin; FPLC, fast-performance liquid chromatography; HSPG, heparan sulfate proteoglycans; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDLR-related protein; PBS, phosphate-buffered saline.
sues (31). Despite having only 2–5% of wild-type plasma apoE levels, hypoE mice display a nearly normal lipoprotein profile. However, in Apoe<sup>−/−</sup> mice with similar plasma apoE levels after transplantation with varying amounts of wild-type bone marrow, remnants accumulate (32). It was not until plasma apoE levels exceeded 10% of wild-type levels that remnant levels approached normal. The more efficient remnant clearance in hypoE mice suggests the importance of hepatocyte-derived apoE and the secretion-capture role of apoE.

This study was designed to address the controversy concerning the hypothesis that Westernized apoE of both hepatically derived and localized apoE in remnant clearance in <i>in vivo</i> models. To this end, remnant clearance was assessed in hypoE mice (a model of hepato-cell-derived apoE) and in Apoe<sup>−/−</sup> mice expressing similar levels of Arg-61 apoE after bone marrow transplantation (a model of non-hepatocyte-derived apoE). Our results demonstrate that remnant clearance is more efficient in the hepatocyte-derived apoE model and provide <i>in vivo</i> evidence for the importance of hepatically derived and localized apoE in remnant clearance.

**EXPERIMENTAL PROCEDURES**

**Hepatocyte-derived ApoE Mouse Model**—The generation of hypoE mice, or the hepatocyte-derived apoE model, has previously been described (30). Briefly, a neo cassette flanked by loxP sites was inserted into <i>Apoe</i> intron 3 by gene targeting in embryonic stem cells to help follow the replacement of the human equivalent of Thr-61 by an arginine, aimed at creating a model of apoE4 (31). The presence of the neo cassette in <i>Apoe</i> intron 3 results in reduced apoE mRNA levels in all tissues and organs (30). The apoE mRNA levels in the liver, brain, and spleen in targeted mice are ~5% of those in wild-type mice, resulting in plasma apoE levels equal to 0.05–0.12 mg/dl (~2–5% of wild-type levels) as determined by Western blot analysis using mouse apoE standards, with the liver remaining the primary source of apoE. However, following Cre-mediated excision of the neo cassette in targeted mice, normal expression of the Arg-61 apoE allele is restored. The mice were weaned at 21 days of age, housed in a barrier facility with a 12-h light/12-h dark cycle, and fed a Chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO).

**Non-hepatocyte-derived ApoE Mouse Model**—Mice expressing Arg-61 apoE primarily from a peripheral source were generated by transplanting Cre-deleted Arg-61 mouse bone marrow into lethally irradiated Apoe<sup>−/−</sup> mice. Bone marrow was collected by flushing femurs and tibias with RPMI 1640 containing 2% fetal bovine serum and 10 units/ml heparin (Sigma). Cells were washed, counted, resuspended in RPMI, and used immediately for transplantation into lethally irradiated Apoe<sup>−/−</sup> recipient mice. The cells (5 × 10<sup>6</sup> in a volume of 300 µl) were injected into the tail vein 4 h after irradiation with 900 rads from a cesium gamma source.

**Immunohistochemistry**—Mice were fasted for 4 h, anesthetized with avertin, and flushed perfused with PBS, pH 7.2, and 3% paraformaldehyde in PBS, pH 7.2, and 3% paraformaldehyde in PBS, pH 7.2, at room temperature for 5 min. Hardened liver lobes were cut into slices 2–3 mm thick and further fixed overnight by immersion in 3% paraformaldehyde in PBS, pH 7.2, at 4°C. Slices were briefly washed in PBS, pH 7.2, drained, placed in molds with TissueTek compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Blocks were held at −70°C until cut into 6–8-µm thick sections on a Leica Frigocut 2800 cryostat and mounted on glass slides.

Slides were immunostained as follows at room temperature unless otherwise indicated. Sections were incubated in sequence with rabbit primary antibodies (1:2000), and bound primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit antibody (Vector). Signals were amplified by incubating membranes with chemiluminescent reagent (Amersham Biosciences) and exposing them to x-ray film (Kodak, Rochester, NY). Signals were quantified with phosphorimaging and quantification software (Quantity One, Bio-Rad).

**Remnant Lipoprotein Clearance Study**—Remnant lipoproteins were prepared from plasma, adjusted to a density of d < 1.04 g/ml, from fasted Apoe<sup>−/−</sup> mice. Plasma was centrifuged in a Beckman ultracentrifuge in a TL-100.3 rotor at 80,000 rpm for 16 h at 8°C. Remnants were isolated and recentrifuged for two additional 16 h periods at d = 1.04 g/ml to ensure removal of plasma albumin. The purity of the remnant preparation was examined by SDS-PAGE and staining with Coomassie Blue, which revealed apoB48 as the major protein component followed by apoA1; apoB100 was present in trace amounts. Remnants were labeled with Na<sup>125</sup>I (Amersham Biosciences) by a modification of the iodine monochloride method first described by McFarlane (33). <sup>125</sup>I-labeled remnants were extensively dialyzed against PBS, pH 7.2, and adjusted to a specific activity of 65,000 cpm/µg protein. Labeled remnants (24 µg of protein in a volume of 400 µl of PBS) were injected into the tail vein of recipient mice (wild-type, hepatocyte-derived apoE model, non-hepatocyte-derived apoE model, and Apoe<sup>−/−</sup> mice, n = 3/group). Blood (50 µl) was collected from the retro-orbital venous plexus into heparinized tubes 1, 5, 15, 30, 60, and 180 min after injection of 125I-labeled remnants. Aliquots of plasma were analyzed for radioactivity on a gamma counter (Packard). Total counts were calculated assuming that plasma can represent 3.5% of total body weight (34). At 180 min, the mice were anesthetized with avertin and flush perfused with PBS, pH 7.2. Whole livers were collected, and the amount of 125I in the liver was determined.

**RESULTS**

**Detection of ApoE on Hepatocyte Cell Surfaces in the Liver**—The impact of the tissue source of apoE on remnant clearance was assessed by studying two mouse models expressing low levels of plasma apoE derived primarily from liver hepatocytes or primarily from a peripheral source. We first examined liver-associated apoE in both mouse models. Like wild-type mice, the hepatocyte-derived apoE model had detectable levels of apoE bound to hepatic sinusoidal surfaces, although at reduced levels (Fig. 1). In contrast, and similar to Apoe<sup>−/−</sup> mice, the non-hepatocyte-derived apoE model had little if any detectable apoE bound to hepatic sinusoidal surfaces. However, unlike Apoe<sup>−/−</sup> mice, the non-hepatocyte-derived apoE model had some apoE-immunoreactive cells in liver sections, which likely represent macrophage-derived Kupfer cells expressing apoE or cells that have taken up apoE from the circulation. Thus, the enrichment of apoE on hepatic sinusoidal surfaces required local apoE expression, present only in the hepatocyte-derived apoE model.

**Plasma ApoE Levels in the Hepatocyte-derived and in the Non-hepatocyte-derived ApoE Models**—Despite having different levels of liver-associated apoE, both the hepatocyte-derived and the non-hepatocyte-derived apoE models expressed similarly low levels of apoE in plasma, corresponding to ~2–5% of wild-type apoE levels in the hepatocyte-derived apoE model and ~2-fold more in the non-hepatocyte-derived apoE model (Fig. 2).

**Plasma Lipid and Lipoprotein Levels in the Hepatocyte-derived and in the Non-hepatocyte-derived ApoE Models**—The tissue source of apoE resulted in a marked difference in plasma lipid and lipoprotein levels. Plasma cholesterol levels in the
non-hepatocyte-derived apoE model were 2-fold higher than those in the hepatocyte-derived apoE model (230 ± 50 versus 98 ± 18 mg/dl, n = 6, p = 0.02) and plasma triglyceride levels were 4-fold higher in the non-hepatocyte-derived apoE model than in the hepatocyte-derived apoE model (176 ± 27 versus 45 ± 12 mg/dl, n = 6, p = 0.03). Fractionation of mouse plasma showed a marked difference in the lipoprotein profiles (Fig. 3). The non-hepatocyte-derived apoE mice transported 60–80% of plasma cholesterol as remnant lipoproteins. In contrast, the hepatocyte-derived apoE mice transported only 30–40% of plasma cholesterol as remnant lipoproteins (30), which is more like wild-type mice that transport the majority of their plasma cholesterol as high density lipoprotein (17). Thus, the non-hepatocyte-derived apoE model transports 2–3-fold more remnant lipoprotein-associated cholesterol and less high density lipoprotein cholesterol than the hepatocyte-derived model. Agarose gel electrophoresis of mouse plasma confirmed the more normal lipoprotein profile in the hepatocyte-derived apoE model and the accumulation of remnants in the non-hepatocyte-derived model (data not shown).

The distribution of apoE among the classes of plasma lipoproteins in both mouse models was determined by pooling plasma FPLC fractions into lipoprotein classes, followed by Western blotting. As shown in Fig. 4, apoE was present in the remnant fractions in both mouse models and demonstrates that the distribution pattern of apoE among plasma lipoprotein classes in both mouse models is identical.

SDS-PAGE Western blot analysis of mouse plasma demonstrated that both models accumulate apoB48. The non-hepatocyte-derived apoE model had ~8-fold more plasma apoB48 than the hepatocyte-derived apoE model, but both models had similar levels of plasma apoB100, which was lower than in wild-type mice (Fig. 5). These results are consistent with the more rapid removal of apoB48-containing remnants in the hepatocyte-derived apoE model than the non-hepatocyte-derived apoE model. Taken together, these results demonstrate that remnant clearance is more effective in the hepatocyte-derived
apoE model than in the non-hepatocyte-derived model due to hepatically derived and localized apoE.

**Uptake of 125I-labeled Remnant Lipoproteins in Mice**—The importance of the source of apoE synthesis for remnant uptake was further assessed by measuring the plasma clearance and liver uptake of 125I-labeled apoE-deficient mouse remnants (Fig. 6A). Although both the hepatocyte-derived apoE model and the non-hepatocyte-derived apoE model had an overall delay in remnant clearance relative to wild-type mice, only the hepatocyte-derived apoE model reached wild-type levels at 3 h. Moreover, the livers of the hepatocyte-derived apoE model (n = 3) contained 70 ± 5% of the total radioactivity found in wild-type mouse livers, whereas the livers of the non-hepatocyte-derived apoE model (n = 3) contained only 38 ± 4% after 3 h.

Contrary to wild-type mice, the initial rates of remnant clearance in the hepatocyte-derived apoE and apoE null mice were identical, indicating that the initial rates in these models were not affected by the levels of hepatically localized apoE or by the number of apoE molecules per remnant (Fig. 6A and B). Rather, the difference in the rate of remnant clearance between the two models was apparent only at the later time points diverging after the first 30 min. These results demonstrate that apoE synthesized by liver hepatocytes favors an overall greater capacity for liver-mediated remnant uptake than apoE synthesized by extrahepatic tissue. Moreover, these results support a role for apoE-enrichment of remnants sequestered at the surface of hepatocytes and are consistent with the plasma lipoprotein profiles of fasted mice that show a significant accumulation of remnants in the non-hepatocyte-derived apoE model, but not in the hepatocyte-derived apoE model (Fig. 3).

**DISCUSSION**

This study presents in vivo evidence that demonstrates that the site of apoE synthesis significantly influences remnant clearance and, consequently, plasma cholesterol and triglyceride levels in mice. Hepatically derived and localized apoE in the hepatocyte-derived apoE model was found to be more effective than macrophage-derived apoE in the non-hepatocyte-derived apoE model in promoting the plasma clearance and liver uptake of apoB48-containing remnants. Moreover, the low levels of hepatically derived and localized apoE in the hepatocyte-derived apoE model influenced the late but not the initial part of remnant clearance.

Because the hepatocyte-derived apoE model expresses Arg-61 apoE, the non-hepatocyte-derived apoE model was generated by using Cre-deleted Arg-61 bone marrow to allow for a direct comparison with the hepatocyte-derived apoE model. In these mice, Cre-mediated excision of the neo cassette in Apoe intron 3 results in normal apoE expression levels in all tissues, including macrophages (31). The normal lipid and lipoprotein levels in Cre-deleted Arg-61 mice suggest that Arg-61 apoE and wild-type apoE are equally effective in remnant clearance (31).

Immunohistochemical analysis of liver sections demonstrated that the hepatocyte-derived apoE model contained significant levels of hepatically localized apoE, whereas the non-hepatocyte-derived apoE model did not. The hepatically localized apoE in the hepatocyte-derived apoE model likely originated mostly from hepatocytes, as the expression pattern of the hypomorphic Apoe allele in the hepatocyte-derived apoE model is normal, with the liver producing the majority of apoE in these mice (30). However, we cannot exclude the possibility that some of the hepatically localized apoE in the hepatocyte-derived apoE model originated in the periphery.

The higher plasma lipid and lipoprotein levels in the non-hepatocyte-derived apoE model relative to those in the hepatocyte-derived apoE model, despite similarly low apoE plasma levels, is consistent with a more efficient clearance of remnants in the hepatocyte-derived model. The non-hepatocyte-derived apoE model accumulated 2-fold more plasma cholesterol and 4-fold more plasma triglycerides than the hepatocyte-derived apoE model. The 8-fold greater accumulation of apoB48-containing remnants in the non-hepatocyte-derived apoE model is also consistent with the site of apoE synthesis influencing remnant clearance, despite both models having similar levels of plasma apoE. In the hepatocyte-derived apoE model, low levels of hepatic apoE synthesis overcome to a significant degree the...
deficiency in remnant clearance seen in the non-hepaticity-derived Apoe<sup>−/−</sup> mice. In contrast to wild-type mice and similar to Apoe<sup>−/−</sup> mice, both models had equally low levels of plasma apoB100. The low levels of plasma apoE in the two models apparently failed to sufficiently enrich apoB48-containing remnants with apoE, causing them to become poor competitors with apoB100-containing LDL for binding to the LDLR.

Alternatively, the reduced levels of apoB100 in both models may result from decreased apoB100 secretion by the liver. However, hepatic apoE expression has been reported to influence the production rate of both apoB48 and apoB100 in mice (35). The finding that the levels of apoB48 are far greater in the non-hepaticity-derived apoE model than in the hepaticity-derived apoE model, emphasizes the importance of hepatically derived and localized apoE in remnant clearance, as the non-hepaticity-derived apoE model likely secretes reduced levels of apoB48, similar to Apoe<sup>−/−</sup> mice (35). Thus, a direct assessment of apoB secretion rates in both models will be necessary to confirm this possibility. Lastly, remnant clearance in both models may have been influenced by the recently described recycling pathway of apoE in liver hepatocytes (36). However, as only 6% of internalized apoE was reported to be re-secreted by hepatocytes, the pathway likely plays a minor role in mediating remnant clearance in our models that have very low levels of plasma apoE.

Directly comparing remnant clearance in both the hepatocyte-derived and non-hepaticity-derived apoE models confirmed the conclusions drawn from the steady-state plasma lipid and lipoprotein levels, which indicate that hepaticity-derived apoE is more effective than non-hepaticity-derived apoE in remnant clearance. When compared with wild-type mice, the initial phase of remnant clearance was similarly reduced in both mouse models and comparable with apoE null mice. As the initial phase likely reflects the binding and sequestration of remnants on hepatocyte cell surfaces in the space of Disse, the levels of apoE molecules per remnant as well as the levels of heptatically localized apoE in the hepaticity-derived apoE model do not appear to contribute significantly to this process. Moreover, the differences in lipoprotein pool sizes in the models did not affect the rapid initial clearance of remnants. However, the near normal level of remnant clearance after 3 h only in the hepaticity-derived apoE model, suggests that heptatically derived and localized apoE contribute substantially to a slower component of remnant clearance, which likely represents liver-uptake. Indeed, livers from the hepaticity-derived apoE model contained 70 ± 5% of normal 121I levels, whereas livers from the non-hepaticity-derived apoE model contained only 38 ± 4%.

In the hepaticity-derived apoE model, apoE-poor remnants sequestered on hepatocytes likely become enriched with newly secreted apoE in the space of Disse or by the passive exchange from existing heptatically localized apoE, allowing for accelerated receptor-mediated internalization through the LDLR and the LRP or through HSPG alone. However, in the non-hepaticity-derived apoE model, a large proportion of sequestered apoE-poor remnants likely redistribute to and accumulate in the circulation due to the absence of apoE-enrichment in the space of Disse and inefficient receptor-mediated uptake.

Studies of remnant clearance in an isolated mouse liver perfusion model have recently questioned the importance of hepatic synthesis and localization of apoE and thus the importance of the secretion-capture role of apoE in remnant clearance (24). Livers from Apoe<sup>−/−</sup> mice cleared an infused bolus of apoE-containing rat chylomicron remnants as efficiently as those from wild-type mice, which, unlike livers from Apoe<sup>−/−</sup> mice, had abundant levels of apoE localized on hepatocyte cell surfaces (24). More recent data from this model suggested that apoE/LRP interactions on hepatocyte cell surfaces are not required for efficient remnant uptake (29). Rather, apoE/LRP interactions were proposed to mediate the direct sequestration and internalization of remnants.

Interestingly, and in parallel to the results observed in the mouse liver perfusion model (24), the absence of hepatically localized apoE in both the non-hepaticity-derived apoE model and in Apoe<sup>−/−</sup> mice did not influence the early phase of remnant clearance relative to the hepaticity-derived apoE model. Indeed, all three of these mouse models displayed similarly delayed initial rates of remnant clearance relative to wild-type mice. However, a shortcoming of the liver-perfusion model is that the results cannot necessarily be extrapolated to explain the steady-state levels of remnants in mice. For example, in Apoe null mice, the perfused liver effectively cleared an infused bolus of remnants, whereas at steady-state the mice accumulate remnants in plasma. In contrast, our in vivo models directly focus on the steady-state levels of remnants in mice.

In conclusion, this in vivo study in mice expressing apoE from hepatic versus extrahepatic sources demonstrates and underscores the importance of heptatically derived and localized apoE for efficient remnant clearance by the liver. Because remnant clearance operates close to its sub-optimal level in the hepaticity-derived apoE model, the model should be informative in revealing the relative contributions of the LDLR and the LRP in remnant clearance in the context of low levels of plasma apoE. The hepaticity-derived apoE model can also serve to determine the contribution of other proteins known to be ligands for remnant clearance such as hepatic lipase (37, 38).

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