Dissection of the Complex Role of Apolipoprotein E in Lipoprotein Metabolism and Atherosclerosis Using Mouse Models

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Transgenic and knockout mice have been instrumental in delineating the role of apolipoprotein (apo) E in lipoprotein metabolism and atherosclerosis. The severe hypercholesterolemia and premature atherosclerosis of the apoE knockout mouse have been the starting point from which various physiologic processes have been identified in which apoE plays a critical role. These processes include 1) very low density lipoprotein (VLDL) triglyceride production; 2) lipoprotein lipase mediated triglyceride lipolysis; 3) VLDL remnant clearance and intracellular processing; and 4) the efflux of cellular cholesterol. In this review we will discuss the recent insight in the role of apoE in these processes, which has been obtained using a variety of in vivo and in vitro approaches to modify apoE expression and function.

Introduction

Apolipoprotein (apo) E is a 34 kD glycoprotein that is predominantly produced by hepatocytes and to a lesser extent by a variety of other cell types, including macrophages. ApoE is an important constituent of plasma lipoproteins such as chylomicrons, very low-density lipoprotein (VLDL), and their remnants. Three major isoforms of apoE are present in the human population. ApoE3 (Cys112, Arg158) is the most common isoform in humans with an allele frequency of 77% and is considered the wild type form. ApoE4 (Arg112, Arg158) and apoE2 (Cys112, Cys158) both differ from apoE3 by one amino acid substitution and have allele frequencies of respectively 15% and 8% in the Caucasian population [1]. The apoE isoforms function differently in lipoprotein metabolism. In healthy individuals, the presence of apoE4 has been associated with elevated plasma cholesterol and low-density lipoprotein (LDL) levels, whereas the presence of apoE2 has been associated with decreased plasma cholesterol and LDL levels [2].

The best described function of apoE is its role as a ligand for receptor-mediated uptake of chylomicron and VLDL remnants by the liver [3]. Mutations in apoE which affect the binding of apoE to the LDL receptor (LDLR) are associated with Familial Dysbetalipoproteinemia (FD), which is characterized by elevated levels of remnants in the plasma and premature atherosclerosis [4,5]. The majority of FD patients are homozygous for the apoE2 allele; however, only a minor fraction of apoE2 homozygotes develop FD. Apparently, additional environmental and genetic factors are necessary for expression of the disease. A minor fraction of FD patients carry rare variant forms of apoE such as apoE3-Leiden, which is inherited as a dominant trait [6].

The critical role of apoE in lipoprotein metabolism has been confirmed by the severe hypercholesterolemia of apoE knockout (apoE-/-) mice [7,8]. Even heterozygous deficient apoE (apoE-/+) animals are susceptible to diet-induced hyperlipidemia, indicating that the expression level of apoE can be rate limiting for plasma clearance [9]. The apoE-/- mice accumulate VLDL/LDL–sized particles in their plasma that predominantly contain apoB48 and are enriched in cholesterol and cholesterol ester and depleted in triglycerides (TG) [7–9]. The apoE-/- mice were the first well-characterized models to spontaneously develop atherosclerosis without the need for feeding high-fat diets [7, 8]. The development of atherosclerosis in these mice is highly similar to the development of atherosclerosis in humans [10].

Shortly after the generation of apoE deficient mice, transgenic mice were generated, overexpressing dominant apoE mutations such as the apoE2 (Arg112, Cys 142) gene [11] and the apoE3-Leiden gene [12]. Both transgenic mouse models accumulated cholesterol and TG-rich remnant lipoproteins in their plasma, very similar to FD

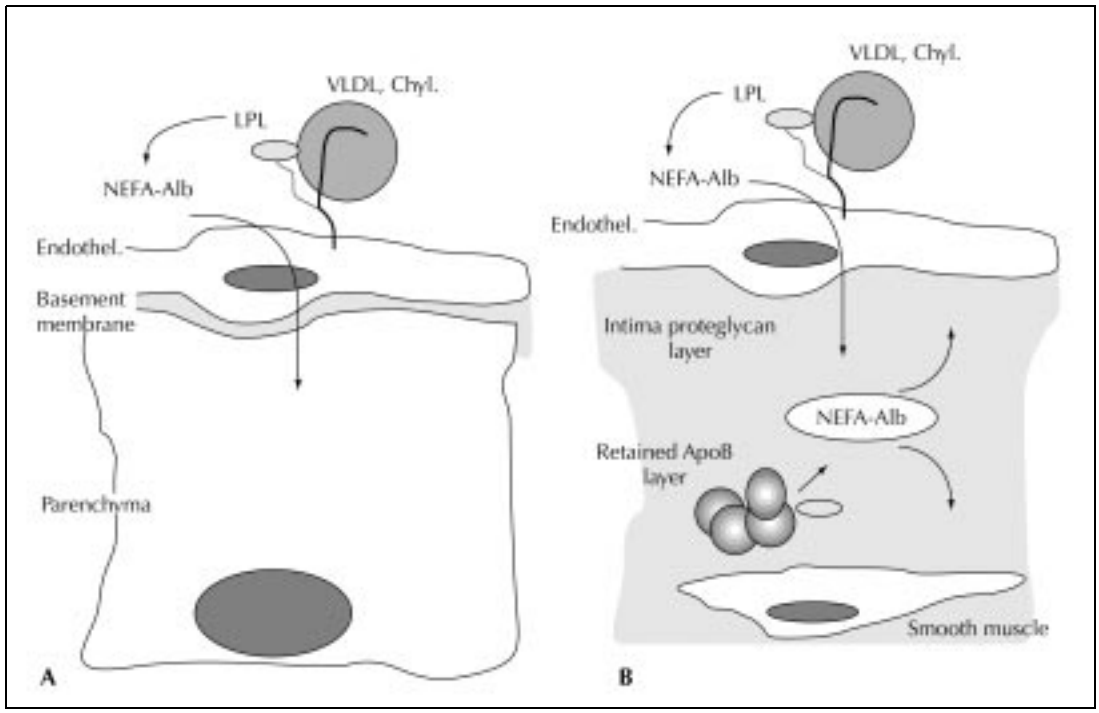

Figure 1. Schematic overview of the processes in which apolipoprotein E has been shown to play a role. Numbers correspond to the sections discussed in this review.

patients carrying this mutation. Further analysis of the apoE3 Leiden mice revealed that, high-fat feeding resulted in premature atherosclerosis [13,14]. The apoE3Leiden mice have a less severe phenotype than apoE-/- mice, but are extremely sensitive to diet-induced hyperlipidemia [15]. Mouse models, such as the apoE-/-, apoE2 (Arg112, Cys 142) and apoE3 Leiden mice, have firmly established the mouse as a model organism for the analysis of the lipoprotein metabolism and atherosclerosis.

A variety of techniques have been applied to vary expression and function of apoE in specific cell types in vivo. These techniques include bone marrow transplantation to modulate gene expression in macrophages, and adenovirus mediated gene transfer to modulate gene expression in the liver. These approaches have revealed additional insight in the complex role of apoE in lipoprotein metabolism and atherosclerosis. A schematic representation of the processes in which apoE is currently thought to play a role is depicted in Figure 1. These processes will be discussed separately below. As we hope to demonstrate, the influence of apoE on the individual processes is dependent both on the quality and quantity of the specific apoE variant. Moreover, processes such as VLDL-TG secretion and remnant clearance require an optimal amount of apoE to function properly.

The Role of Apolipoprotein E in Very Low-Density Lipoprotein Triglyceride Production

The role of apoE in VLDL-TG synthesis and secretion has been addressed in apoE-/- mice, which accumulate large amounts of TG in their livers, indicative of a defective TG metabolism. In apoE-/- mice, the VLDL-TG secretion was approximately 50% reduced as compared with wild type control mice. However, the in vitro capacity of hepatocytes to synthesize TG was not affected by the presence or absence of apoE [16••]. The reduction in the hepatic VLDL-TG secretion rate was not due to the hyperlipidemia of the apoE-/- mice, but solely due to the absence of apoE synthesis in the hepatocytes [16]. Nevertheless, very low-level hepatocyte specific expression of a human apoE3 transgene on an apoE-/- background, which was incapable of restoring normolipidemia, resulted in a near normalization of the VLDL-TG secretion rate (Mensenkamp A, Unpublished observation). Moreover, adenovirus mediated gene transfer of human apoE3 to the livers of wild type and apoE-/- mice resulted in a gene dose-dependent increase of the VLDL-TG secretion rate [17•]. A similar effect has been observed by cross breeding a high expressing human apoE3 transgenic mouse onto an apoE-/- background [18•]. Thus, low-level hepatocyte specific expression of apoE3 can rescue the reduced VLDL-TG secretion rate as observed in apoE-/- mice, whereas a high level of apoE3 expression further increases the VLDL-TG secretion rate. Because the apoB production rate of Apoe-/- mice is not different from that of wild type mice (Mensenkamp A, unpublished observation), we speculate that apoE expression is a rate-determining factor in the intracellular assembly of TG into VLDL particles.

A portion of the apoE that is utilized for the synthesis and secretion of nascent VLDL particles may be recycled from internalized apoE. It was demonstrated that the intracellular fate of apoE containing lipoproteins such as VLDL is different from that of LDL after internalization by HepG2 cells [19]. Transport of internalized VLDL to the

lysosomal compartment was retarded as compared with LDL, leading to a higher rate of retroendocytosis. Recently, recycling of apoE has been addressed by following the intracellular fate of internalized apoE in vivo [20••]. It was found that the amount of exogenous apoE found in the Golgi fractions was increased more than 50-fold as compared with exogenous apoB. The preferential association of internalized apoE with the secretory apparatus indicated that recycling of apoE is a physiologic phenomenon in vivo.

The effects of apoE2 and apoE3Leiden expression on the VLDL-TG secretion rate have been investigated by breeding apoE2 and apoE3Leiden transgenic mice onto an apoE-/- background [21]. Interestingly, apoE2 expression was capable of rescuing the VLDL-TG secretion rate of apoE-/- mice to the wild type level, whereas APOE3Leiden was not. The basis for this difference is currently under investigation. Because apoE3Leiden mice accumulate apoE-containing inclusions in the liver, one explanation may be that intracellular processing of apoE3Leiden is defective.

The Role of Apolipoprotein E in Very Low-Density Lipoprotein Triglyceride Lipolysis

It has long been suggested that the hypertriglyceridemia of FD patients homozygous for apoE2 is due to defective lipoprotein lipase (LPL) mediated chylomicron and VLDL-TG lipolysis [22]. In vitro experiments have since shown that VLDL containing a number of apoE variants is relatively resistant to LPL-mediated TG lipolysis [23,24,25•]. Surprisingly, the most common form of apoE, apoE3, also inhibits LPL-mediated TG lipolysis in vitro in a dose-dependent manner [17•,26•,27]. Hence, the inhibitory effect of apoE on LPL mediated VLDL-TG lipolysis is related to the increased apoE content of the VLDL particles and possibly also the specific properties of the apoE variant.

The mechanism of the apoE-mediated inhibition of VLDL-TG lipolysis has been addressed in transgenic mice overexpressing apoE2 and apoE3 and in patients with hypertriglyceridemia [18•,25•]. In all cases the plasma TG levels correlated positively with plasma apoE levels. However, increased plasma apoE and TG levels were correlated with decreased VLDL-apoCII levels and decreased rates of LPL mediated VLDL-TG lipolysis. Since apoCII is an essential cofactor for LPL mediated TG-lipolysis, it was hypothesized that the increased amount of apoE on the VLDL particle resulted in displacement of apoCII [18•, 25•].

The rare apoE variant apoE2 (Lys146ÆGln) gives rise to particularly elevated TG levels in FD patients [28]. To investigate whether apoE2(146) has a variant-specific effect on TG metabolism, adenovirus vectors carrying the apoE2(146) and other apoE variants have been generated (de Beer F, Unpublished observations). By injecting apoE2(146) and apoE3 adenovirus into apoE-/- mice, VLDL could be obtained with highly comparable lipid and apolipoprotein composition. Interestingly, apoE2(146) containing VLDL was more resistant to LPL-mediated TG lipolysis in vitro as compared with apoE3 VLDL. Thus, in addition to a quantitative effect, apoE(146) can have a qualitative effect on LPL-mediated lipolysis.

Surprisingly, bone marrow transplantation from wild type mice into apoE-/- mice that also lack the LDLR (apoE-/-.LDLR-/- mice) leads to dramatically increased plasma levels of mouse apoE without leading to hypertriglyceridemia [29•]. A similar plasma accumulation of human apoE3 in Apoe-/-.LDLR-/- mice after adenovirus mediated gene transfer of apoE3, does result in significant hypertriglyceridemia, associated with VLDL that is resistant to LPL mediated TG lipolysis [17•]. Apparently, human apoE3 and mouse apoE differ in their propensity to interfere with VLDL-TG lipolysis. However, it should be noted that a quantitative analysis of the effects of human apoE3 versus mouse apoE is required to firmly establish this.

The Role of Apolipoprotein E in Hepatic Clearance of Very Low-Density Lipoprotein

Evidence to date indicates that two receptors are responsible for plasma clearance of chylomicron and VLDL remnants via apoE; the LDL receptor (LDLR) and the LDL receptor related protein (LRP). Although LDLR deficiency in humans [30] and mice [31] does not lead to the accumulation of remnant lipoproteins in the plasma, antibodies to the LDLR interfere with chylomicron remnant removal in mice [32]. Moreover, mutations in apoE that affect LDLR binding lead to FD, characterized by plasma accumulation of remnant lipoproteins [4]. Thus, the LDLR is involved in remnant clearance.

The fact that LDLR deficiency does not lead to the accumulation of remnants indicates that multiple receptors are involved in remnant clearance. Definitive evidence for the role of the LRP as an alternative route for remnant clearance has recently been obtained by the generation of a conditional knockout mouse model, in which the LRP gene can be deleted from the liver at will [33••]. In the presence of the endogenous LDLR, hepatic LRP deficiency did not lead to the accumulation of lipoproteins in the circulation, but did result in a compensatory up-regulation of the LDLR gene and protein. However, absence of the LRP from the liver of LDLR-/- mice did lead to the accumulation of chylomicron and VLDL remnant sized lipoproteins in the circulation. These data provide direct evidence for a role of the LRP in remnant lipoprotein clearance and indicate that the LDLR is the predominant clearance route for remnants.

The role of the LDLR in the clearance of lipoproteins containing binding-defective apoE variants such as apoE3Leiden and apoE2 has been investigated by cross breeding apoE transgenic mice onto apoE-/- and LDLR-/- backgounds [21,25•,34]. Decreased expression of the LDLR in apoE2 and apoE3Leiden transgenic mice resulted in a Ldlr gene dose-dependent increase of the hypercholes-

terolemia. Moreover, overexpression of the LDLR in APOE2 and APOE3Leiden transgenic mice using adenovirus mediated gene transfer resulted in a near normalization of plasma lipid levels (van dijk, Unpublished observations). Increased LDLR expression in apoE3Leiden mice resulted in a much more efficient reduction of plasma cholesterol level as compared with apoE2 mice. This is in agreement with the poor in vitro binding capacity of apoE2 to the LDLR as compared with apoeE3Leiden [21]. Moreover, the LDL receptor is the predominant pathway for lipoprotein clearance even in the presence of apoE variants that poorly bind the LDLR.

The quantitative requirement for hepatic apoE expression for clearance via the LDLR and the LRP has been addressed using adenovirus mediated gene transfer of apoE3 [17•]. By injecting increasing doses of an adenovirus encoding human apoE3 into apoE-/- and apoE-/-.LDLR-/- mice, it was found that the LDLR and the LRP have quite distinct ligand requirements. The LDLR is capable of efficient remnant clearance in a wide range of apoE3 expression levels, whereas the LRP requires a relatively high apoE expression level for efficient clearance, but is inhibited by excess apoE3 expression. The requirement for relatively high levels of apoE3 on particles for high affinity binding to the LRP is in agreement with in vitro data [35]. However, the lack of LRP mediated clearance at very high apoE3 expression levels was unexpected. Since excess apoE3 expression is associated with a disturbance in VLDL-TG lipolysis, it was concluded that the increased TG levels of the circulating particles were causing the failure of LRP mediated clearance. At present it is unclear what causes the inability of the LRP to clear TG-rich particles. Liver perfusion studies and in vivo clearance studies have suggested that the size of the remnant particle is also an important determinant in LRP recognition [36,37]. One can speculate that particle size and lipid composition directly influence the conformation and accessibility of the receptor-binding domain of apoE on the particle and thus determine the affinity for the LRP.

In vivo uptake of remnants via the LRP has been postulated to occur after enrichment with apoE that is bound to cell surface heparan sulphate proteoglycans (HSPG) in the space of Disse. The physiologic relevance of the so-called secretion re-capture process [38–40] has recently been addressed [29•]. Bone marrow transplantation of wild type donor mice to apoE-/-.LDLR-/- recipient mice resulted in macrophage specific expression of apoE and plasma apoE levels up to 16-fold those of wild type mice. Nevertheless, plasma cholesterol levels were not reduced as compared with apoE-/-.LDLR-/- mice that received apoE-/- bone marrow. Adenovirus mediated gene transfer of the LDLR in the apoE-/-.LDLR-/- mice reconstituted with wild type bone-marrow did result in a dramatic reduction of the hypercholesterolemia. Thus, it was concluded that expression of apoE specifically in the hepatocytes is required for remnant clearance via the LRP and not for clearance via the LDLR. Because hepatocyte derived apoE is present at high levels on the surfaces lining the space of Disse, this was interpreted as evidence for the secretion–recapture model for LRP mediated clearance.

The interaction of remnant lipoproteins with HSPG is an additional process in which apoE could play a role. The binding of hepatocyte derived apoE to HSPG in the space of Disse is required for the enrichment of remnants with apoE and subsequent uptake via the LRP (the secretion–recapture process). In addition, remnants bound to HSPG via their apoE moieties can be internalized directly, although this is a slow process [41]. Different apoE isoforms have different affinities for HSPG [42,43] and could thus determine the efficiency of LRP mediated remnant removal [44]. Both hepatic lipase (HL) and LPL have been shown to function as bridging molecules, increasing the uptake of lipoproteins in vitro. Whether apoE plays a direct role in the stimulation of uptake by LPL and HL is still a matter of controversy [45].

Interestingly, it was recently demonstrated that human apoE3 functions somewhat differently from murine apoE in the lipoprotein metabolism of the mouse. Using a knock-in approach to replace the endogenous mouse apoE gene with the human apoE3 gene, it was shown that human apoE3 preferentially associated with larger lipoproteins such as chylomicron and VLDL remnants, whereas mouse apoE preferentially associated with HDL [46•]. On a high fat-diet a marked increase in plasma cholesterol (but not plasma triglycerides) of apoE3 knock-in mice was observed, associated with elevated plasma remnant levels. The basis for the difference in the physiologic behavior of human apoE3 versus mouse apoE remains to be elucidated. However, this difference may extend to rat apoE, which was shown to reduce plasma cholesterol levels in LDLR-/- mice [47], in contrast to human apoE3 in LDLR-/- mice (van Dijk, unpublished observations).

The Role of Apolipoprotein E in Atherosclerosis

Hyperlipidemia is one of the main driving forces in atherogenesis. Increased plasma lipid levels lead to the accumulation of lipoproteins in the vascular intima. These intimal lipoproteins can be modified and activate surrounding cells such as vascular endothelium and smooth muscle cells (SMC). Via activated endothelium, circulating monocytes will be recruited into the intima and differentiate into macrophages. These macrophages will internalize the (modified) lipoproteins and ultimately become foam cells. Subsequent events in atherogenesis include SMC proliferation/migration, fibrous cap formation, cholesterol deposition, and necrosis [48].

A number of steps in the initiation of atherogenesis could be modulated by apoE. Because apoE-/- mice readily develop atherosclerosis, apoE is not required for entry of lipoproteins into the intima. Similarly, the uptake of lipoproteins into macrophages apparently does not require

apoE. However, these observations do not exclude the possibility that apoE can modulate both vessel wall entry and macrophage uptake of lipoproteins. Similar conclusions can be drawn for apoE specific lipoprotein receptors on the vessel wall, such as the VLDL receptor (VLDLR) [49,50] and the LDLR. Atherogenesis occurs both in VLDLR-/- mice [51] and in LDLR-/- mice [31]. Again, although these receptors are not required for atherogenesis, they may very well modulate the process.

Because macrophage derived foam cells in atherosclerotic lesions express apoE and increased apoE expression enhanced cholesterol efflux from a macrophage cell line in vitro, it was hypothesized that macrophage apoE might play a direct role in the initiation and progression of atherosclerosis [52]. To test this hypothesis in vivo, transgenic mice have been generated expressing very low levels of human apoE3 exclusively in macrophages [53]. These mice were crossbred onto the apoE-/- background. As compared with cholesterol matched apoE-/- mice, the macrophage-specific apoE3. ApoE-/- mice showed a significant reduction in atherosclerosis. Thus, macrophage specific expression of apoE3 decreased atherogenesis of apoE-/- mice. One underlying mechanism is the increased capacity of plasma containing very low levels of apoE to accept and esterify cellular cholesterol from cholesterol laden cells [54•]. Thus, plasma apoE levels that did not result in a reduction of plasma cholesterol levels did restore the capacity of apoE deficient plasma to accept cholesterol.

The reverse experiment has been performed using bone marrow transplantation of apoE-/- donor mice into wild type recipient mice. After bone marrow transplantation, all macrophages in the recipient mice lacked apoE expression, which did not affect the plasma lipid values. In one set of experiments this resulted in an approximately ten-fold increase in atherosclerosis [55], whereas in a different set of experiments this resulted in an approximately 50% decrease in atherosclerosis [56••]. Currently, there is no explanation for these contrasting observations. Various parameters, including diet, timing of the diet, duration of the experiment, and the sex of the animals differed between both sets of experiments. However, it may be clear that the role of apoE in atherogenesis is not straightforward. Additional processes in which apoE plays a role could underlie the contrasting observations after bone marrow transplantation. For example, apoE has been shown to have antioxidant properties in vitro [57]. The oxidative status of the animals may have differed between the two sets of experiments and could thus modulate the development of atherosclerosis.

Conclusions

As reviewed above, apoE plays a crucial role in a number of major steps in the metabolism of VLDL and VLDL remnants, and in the development of atherosclerosis. These individual processes are indicated schematically in Figure 1: 1) in the production of VLDL, functional apoE is necessary for the addition of TG to nascent VLDL particles. A very low level of hepatocyte specific apoE expression is required for this function. However, overexpression of apoE has an accelerating effect on the VLDL-TG secretion rate; 2) the LPL mediated lipolysis of VLDL-TG is affected by both apoE quantity as well as apoE quality. Excess apoE and specific apoE variants will decrease the efficiency of VLDL-TG lipolysis; 3) apoE functions as a ligand for receptor mediated clearance of remnants by the liver, which in mice is predominated by the LDLR. Clearance via the LDLR can be modulated by mutations in apoE, but is relatively insensitive to variations in the apoE quantity and lipid composition of the particle. In comparison with the LDLR, clearance via the back-up receptor LRP seems much more sensitive to apoE quantity and lipid composition of the particle; 4) macrophage apoE is directly involved in atherogenesis. In addition, a very low level of plasma apoE significantly enhances the capacity of plasma to accept cellular cholesterol.

Thus, in addition to the classic role of apoE as a ligand for receptor mediated uptake by the liver, apoE plays a role in virtually every additional step in the metabolism of VLDL and VLDL remnants. Moreover, apoE seems to play a direct role in atherogenesis. Although these observations provide some insight into the net effects of variation in apoE expression and function on lipoprotein metabolism and atherosclerosis, the majority of observations lack mechanistic explanations. Thus, future research on apoE is aimed at dissecting the precise role of apoE in the individual subprocesses of the VLDL metabolism and atherogenesis.

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