Mechanisms Underlying the Atherosclerosis Risk Associated with Apolipoprotein E Isoforms in Humans

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ABSTRACT

Michael Kelly Altenburg: Mechanisms Underlying the Atherosclerosis Risk of Apolipoprotein E Isoforms in Humans

(Under the direction of Nobuyo Maeda)

Apolipoprotein E (apoE) is a ligand for the low density lipoprotein receptor (LDLR), both of which are well recognized determinants of atherosclerosis risk in humans. In humans the *APOE* gene is polymorphic, with three alleles *APOE2, APOE3*, and *APOE4* coding for the protein apoE2, apoE3, and apoE4. Despite increased LDLR affinity, apoE4 is associated with elevated and apoE2 reduced cholesterol concentration compared to apoE3. Mice that have had their endogenous apoE replaced with the human apoE's do not replicate their human counterparts, and mice with apoE3 and apoE4 are similar to wildtype mice, yet those with apoE2 have drastically elevated lipids, a phenotype observed in only 5% of *APOE2* homozygous humans. Either by adenovirus-mediated or global overexpression, elevated LDLR decreased plasma cholesterol in mice with apoE2, but led to a dramatic accumulation of cholesterol-rich VLDL in mice with apoE4 on a western-type high fat diet.

In addition to the liver, where both apoE and LDLR are highly expressed and contribute to plasma lipoprotein clearance, they are expressed in vascular cells and macrophages. Increases in LDLR expression in macrophages significantly increased cholesterol uptake in culture, more prominently with lipoproteins containing apoE4 than those containing apoE3. In LDLR-deficient mice expressing the human apoE4 isoform, the

replacement of bone marrow cells with those expressing LDLR increased atherosclerotic lesions in a dose-dependent manner compared with mice transplanted with cells having no LDLR. Thus apoE4, but not apoE3, in macrophages enhances atherosclerotic plaque development in mice in an LDLR-dependent manner and this interaction may contribute to the association of apoE4 with an increased cardiovascular risk in humans.

Higher LDLR expression decreased the secretion of apoE4 and increased its degradation in both macrophages and hepatocytes. ApoE localization using apoE-GFP fusion proteins expressed by adenovirus in the liver of apoE-deficient mice revealed that apoE4 accumulated in the space of Disse, but apoE2 did not. Using several genetic approaches, apoE4s association with increased atherosclerosis risk can be replicated in mice. ApoE4 binding to the LDLR in macrophages and localization in hepatocytes both contribute to apoE4 pathogenesis.

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Nobuyo Maeda has been a great friend and mentor throughout my project. Moreover I could never have begun graduate studies without her support. Under her guidance I have had numerous opportunities to work in many different areas, from microarrays and adenovirus gene transfer to mouse models and primary culture, as well as in collaborations outside of the lab and several rewarding mentoring challenges. I also was given tremendous freedom to develop my own direction and now as I conclude this project I truly feel prepared to be an independent investigator. Together Nobuyo and Oliver both lead by example, each with an exceptional focus on science, an open mindedness to people and ideas, and always questioning seemingly established hypotheses. You will often hear either one say the phrase, ” you dont know unless you try” or in response to some widely accepted hypothesis, ”but I don’t really believe that”. Before coming to the lab I was interested in electronics, construction and mechanics and thought biological sciences were limited to describing organisms. I also had little academic aspirations or confidence. But all that changed in their lab, where I could see all the disciplines being used together in down to earth, resourceful, and creative ways.

I am also indebted to Lance Johnson my friend and fellow student who helped with nearly every aspect of this project. While I only began to work with Jose Arbones-Mainar towards the end of my project, Lance, Jose and I are the apoE group in the lab. It is always motivating to work alongside these team players.

I am also thankful to the many Post-Docs (most now assistant professors at UNC or other institutions) who I have worked with in the lab, for sharing their advice, views and experience: Patrick Sullivan, Leighton James, Myron Hinsdale, Suzanne Kirby, Seigo Hatada, Masao Kakoki, Nobuyuki Takahashi, Kathleen Caron, and especially my friends Kumar Pandya and Dominic Ciavatta, both of whom have enthusiaism and vision for molecular biology and were always willing to take the J to IP.

Patrick Sullivan was my introduction to the lab and biomedical science when I applied for a job in the lab several years before beginning my PhD. He taught me the basic principles of solid lab work. "Mike, didn't you write that down?" Patrick made the apoE mice that I used in this project and he and I often discuss results as he continues to use them in Alzheimer's research at Duke Univeristy. When Patrick left, Chris Knouff, an MD-PHD student inherited me along with the apoE project. Chris and I, for the next two years, were a good team together and I will always remember his enthusisam and ambition and that 3 AM is a fine time to start an experiment.

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I owe a lot to Dr. Robert Bagnell and Vicky Madden who taught me much about confocal and electron microscopy. I would also like to thank the DLAM and the vet services at UNC, especially Mike Crawford and Dr. Bellinger. Also, Dorothy Poteat in our own department has kept me in line, enrolled, and hopefully graduated.

Many people ran assays, cut tissues, genotyped mice, ammended protocols, and ordered reagants. Id like to thank Svetlalna Zhilicheva, Shin-ja Kim, Dr. Hyung Suk-Kim and his Taq-Man expertise, Jenny Langenbach's calm can do attitude, Sylvia Hiller, Lonquan Xu,

John Hagaman, Sharome Jordan, John Cowhig, Annette Staton, and Tomoko Hatada. Nobuyo and Oliver's lab has people with exceptional diversity and dedication and are the labs most valuable resource. I hope I haven't left anyone out.

I'd also like to thank my other good friends and fellow students, Raymond Givens and early on Sudi Malloy. Our lunch room debates about politics, medicine and research, and everything else were enlightening. We shared common experiences and learned that sometimes it takes some stubborness to get past the trying times of a project.

My fellow Graduate students are a close knit group and I will miss being a part of their monthly dinners, seminars, and other events where food is often provided. Our program, I, and every student in the Pathology Department owe a huge thanks to Drs. Frank Church and Bill Coleman for their passion for graduate research, support, and advice at all stages. Also, I appreciate the encouragement of our Chairman Dr. J. Charles Jennette who instills a sense of pride in the department and who always makes the students and their research a top priority. He truly reflects my ideal concept of a leader; helping others to be successful. Every student is well served knowing they have support from this dedicated group and the rest of the faculty behind them.

Some of my Committee members and I had a long history together that preceded my graduate work. Suzanne Kirby worked at the bench across from Patrick and she has a great loyalty to the lab, and is someone I know I can turn to for straightforward advice. I worked in Susan Lord's lab very briefly one summer and have always had a personal interest in her field of a coagulation research. John Parks is a leading lipoprotein investigator I met early in my graduate work when he worked alongside us in the lab while on sabbatical from Wake Forest University. Since then he has been my connection outside of the lab to the world of

lipoproteins. Interestingly to me, Patrick Sullivan and my two remaining committee members are all from Michigan, my family's home state. Jon Homeister has been an excellent resource for atherosclerosis and career advice. Chris Mack always asks well reasoned project–advancing questions at our seminars.

I inherited my scientific curiosity from my mom's side of the family; she often tells a story about me sticking a knitting needle in a 220V electrical outlet when I was 4 years old. It would not be the last time I would get shocked or knock out the power to our house. Despite disassembling everything in the house, both my parents always encouraged my experiments and it was this self-teaching and enjoyment of exploration that would be most useful to me in my project. I am thankful for the support of my siblings who are now spread all over the country and sometimes the globe, but most call at least once a week, my sisters Kathleen, Bridget, Molly, and brother Patrick--in that order. I enjoy each of your very different views and you've helped me to put my own life in perspective.

Jen, my whole life changed when I met you. My loving wife got me focused and motivated. Jen also works in Nobuyo and Oliver's lab and has helped me throughout my graduate work. I can't imagine not seeing her every hour of the day as I have been fortunate to do. She has also put up with my often odd work hours and has been a constant source of strength and dedication. She is a wonderful mother to our children Jack and Emily. Together they are my inspiration and the reasons I am the happiest person in the world.

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LIST OF ABBREVIATIONS

Apo	**Apolipoprotein**
AcLDL	**Acetylated Low-Density Lipoprotein**
ARE	**AU-Rich Element**
ABCA1	**ATP-Binding Cassette, sub-family A, member 1**
BM	**Bone Marrow**
CAD	**Coronary Artery Disease**
CE	**Cholesterol Ester**
CETP	**Cholesterol Ester Transfer Protein**
CM	**Chylomicrons**
CMV	**Cytomegalovirus**
CR	**Chylomicron Remnants**
DiI	**1,1'-dioctadecyl-3,3,3'3'-tetramethylindo-carbocyanine perchlorate**
ELISA	**Enzyme Linked ImmunoSorbent Assay**
FPLC	**Fast Protein Liquid Chromatography**
GFP	**Green Fluorescent Protein**
HDL	**High Density Lipoprotein**
HFW	**High Fat Western**
HSPG	**Heparan Sulphate Proteoglycans**
LDL	**Low-density Lipoprotein**

LDLR	**Low-density Lipoprotein Receptor**
LPL	**Lipoprotein Lipase**
LRP	**LDL Receptor-related Protein**
MPM	**Mouse Peritoneal Macrophages**
RCT	**Reverse Cholesterol Transport**
SR-B1	**Scavenger Receptor B-1**
TBARS	**Thiobarbiturate Reactive Substances**
TC	**Total Cholesterol**
TG	**Triglyceride**
TRL	**Triglyceride Rich Lipoprotein**
VLDL	**Very Low-density Lipoprotein**

Chapter I

Introduction

Hyperlipoproteinemia in Human Disease

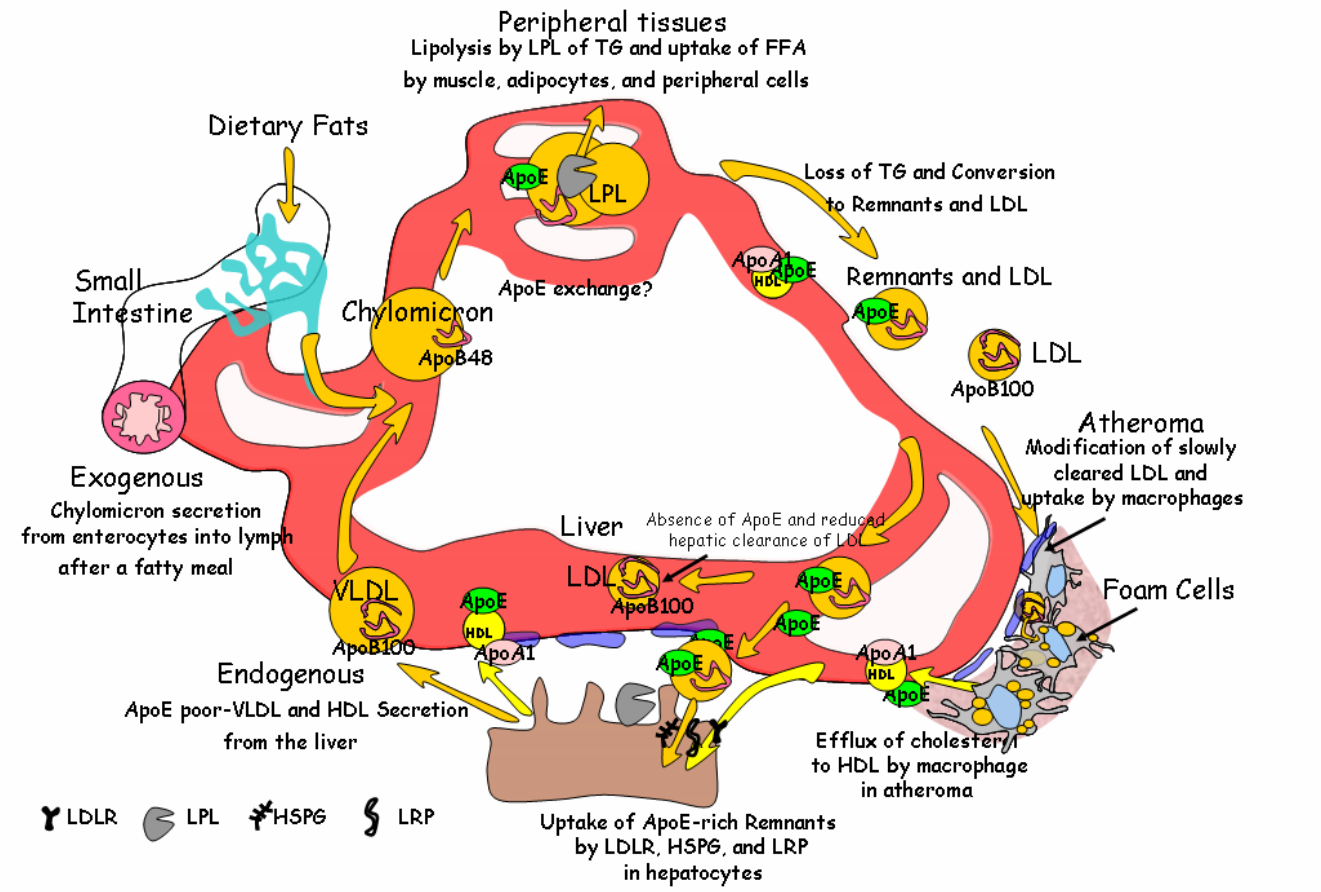
Cardiovascular disease causes the death of approximately 1 million people in the United States each year [1]. According to the American Heart Association, elevated total and LDL cholesterol levels is one of seven primary risk factors linked to coronary heart disease (CHD). It has been estimated that the apolipoprotein E (apoE) locus accounts for as much as 10% of the variation of plasma cholesterol levels and that apoE4 is associated with a modest increase in cholesterol [2, 3]. One study estimated a 40% increased risk for CHD mortality for middle aged male apoE4 carriers compared to apoE3 homozygote or apoE2 carriers [4]. Considering that 25% of the population carries at least one copy of the apoE4 allele, the impacts of its effects are significant. Mice with human apoE that overexpress the LDLR approximate the relationship between apoE and atherosclerosis in humans. Understanding the mechanism of human apoE4-induced atherosclerosis in mice may translate to understanding the disease in humans and discovering therapies for its intervention.

Lipoprotein Metabolism

In mammals, there are two main sources of plasma lipoproteins (Figure 1.1). The absorption of dietary fats in the gut begins the exogenous pathway of lipoprotein formation. The enterocytes of the small intestine package triglycerides, cholesterol, and phospholipids into Chylomicrons (CM), which are then secreted into the mesenteric lymphatics, and empty into the subclavian vein and the circulation via the thoracic duct. In the circulation, CM particles can acquire apoE and the apolipoprotein CI, CII, and CIII (apoCI, apoCII, and apoCIII) from other plasma lipoproteins, such as high-density lipoproteins (HDL) [5]. Lipoproteins undergo processing in the plasma and their triglyceride content is hydrolyzed by lipoprotein lipase (LPL) in the capillary beds into free fatty acids (FFA) and glycerol. Now smaller and more dense, these cholesterol-rich remnants can then be removed from the plasma by apoE binding the low-density lipoprotein receptor (LDLR), heparan sulphate proteoglycans, and the LDL receptor-related protein (LRP) [6]. Several hours after a meal, the CM production by the small intestine is exhausted and the liver becomes the main source of plasma lipoproteins. This endogenous pathway transports lipids from the liver, in humans, as ApoB100-containing Very Low Density Lipoproteins (VLDL) particles that are secreted into the plasma through the space of Disse. Similar to CM's, newly secreted triglyceride-rich VLDL is processed by LPL in the peripheral circulation to VLDL remnants. VLDL remnants can then be removed from the circulation by apoE binding to the LDLR. If not removed, VLDL remnants can be further processed into LDL [7]. These small, dense, cholesterol ester-rich particles do not contain apoE and can be removed only by the lower affinity ApoB100 binding the LDLR [8]. The reduced affinity of ApoB100 LDL results in a greater plasma half-life for LDL than VLDL. ApoE is also a constituent of HDL, a class of plasma lipoproteins that contains apolipoprotein A1 as its constitutive protein instead of

apoB as in the larger lipoprotein classes. HDL is thought to mediate reverse-cholesterol transport (RCT); sending cholesterol from the peripheral tissues back to the liver via the cholesterol ester transfer protein (CETP) or directly through the scavenger receptor B-1 (SR-B1) [9]. A key player in HDL formation is the ATP-binding cassette, sub-family A, member 1 (ABCA1) protein which effluxes cholesterol and other lipids from cells to nascent HDL [10]. However in contrast to the long held RCT hypotheses, recent work with hepatic ABCA1 overexpression or deletion has revealed that the liver may be the source for most of the cholesterol in plasma HDL [11, 12].

Figure 1.1 Review of Lipoprotein Metabolism. Human lipoprotein metabolism is divided into two pathways: the exogenous pathway (left) which after dietary lipid absorption in enterocytes of the small intestine are packaged into apoB48 containing TG-rich large chylomicrons which are secreted into the lymphatic system (blue). During fasting, the endogenous pathway (bottom left), the liver secretes apoB100 containing VLDL (both apoB48 and apoB100 are secreted in mice) which transport lipid derived from the liver. Chylomicrons and VLDL are both TG rich and can undergo lipolysis in the capillaries by lipases such as LPL. While in the circulation these particles aquire apoE as well as various other apoproteins. Loss of TG and surface constituents (phospholipid, apoproteins) decreases the size, and these smaller more dense cholesterol enriched remnants can, if they have sufficient apoE, be cleared by the liver via LDLR, LRP, or HSPG (bottom). LDL in humans does not have apoE and must rely on lower affinity LDLR mediated apoB100 clearance. Increased particle residence time, such as that of LDL or apoE-poor remnants, increases the likelihood of modification and oxidative changes and subsequent uptake by intimal scavenger macrophages in developing atheromas and foam cell formation (lower right). Efflux of cholesterol through ABCA1 and other transporters to HDL can remove excess cholesterol from macrophages delaying lesion development. Most apoA1 and HDL are secreted from the liver (bottom).


Peripheral tissues
Lipolysis by LPL of TG and uptake of FFA
by muscle, adipocytes, and peripheral cells
Dietary Fats
Small
Intestine
Chylomicron
ApoB48
ApoE
LPL
ApoE exchange?
Loss of TG and Conversion
to Remnants and LDL
ApoA1
HDL
ApoE
Remnants and LDL
ApoE
LDL
ApoB100
Atheroma
Modification of slowly
cleared LDL and
uptake by macrophages
Foam Cells
Exogenous
Chylomicron secretion
from enterocytes into lymph
after a fatty meal
Liver
Absence of ApoE and reduced
hepatic clearance of LDL
LDL
ApoB100
VLDL
ApoB100
ApoE
HDL
ApoA1
Endogenous
ApoE poor-VLDL and HDL Secretion
from the liver
ApoE
ApoE
ApoE
ApoA1
HDL
ApoE
Efflux of cholesterol
to HDL by macrophage
in atheroma
LDLR
LPL
HSPG
LRP
Uptake of ApoE-rich Remnants
by LDLR, HSPG, and LRP
in hepatocytes

ApoE in lipoprotein metabolism

ApoE is a 299 amino acid, 34 kDa protein found on the surface of most lipoproteins and is an important ligand for their receptor-mediated endocytosis from the circulation. In humans apoE is polymorphic with three isoforms, apoE2, apoE3, and apoE4, which differ by the amino acids at positions 112 and 158. ApoE2 has two cysteines, while apoE3 has a cysteine at 112 and an arginine at 158, and apoE4 has two arginines. These amino acid changes are responsible for apoE2 having a reduced LDLR affinity and apoE4 an increased affinity compared to apoE3 [13-15]. Paradoxically, however, apoE2 is associated with reduced cholesterol and atherosclerosis risk, except for 5% of homozygotes who develop type III hyperlipoproteinemia [16]. In contrast, apoE4, despite its increased LDLR affinity, is associated with an elevated plasma cholesterol level and atherosclerotic risk [2, 3]. Weisgraber, et al has shown that apoE4 has a higher affinity for large TG rich lipoproteins, especially VLDL, than apoE3 [17]. The increased risk associated with apoE4 is thought to reflect increased intracellular apoE4 mediated lipid delivery, leading to LDLR down-regulation and accumulation of plasma LDL [3, 18, 19]. However, this has not been proven and is difficult to reconcile with both LDL and VLDL being able to bind the LDLR. ApoE is involved in several different lipoprotein metabolic pathways. Secreted apoE can redistribute to chylomicrons, VLDL, remnant lipoprotein particles, and HDL [6, 20]. Lipoprotein-associated apoE can interact with many cell surface molecules, and can facilitate endocytosis via the LDLR, LDLR related protein (LRP), and heparan sulfate proteoglycans (HSPG) [6]. Hepatocytes secrete lipoprotein-free apoE into the space of Disse, where it associates with chylomicron remnants in a process called secretion capture [21]. Accumulation of apoE on lipoprotein surfaces can inhibit lipolysis of triglycerides by lipases [22]. In addition to its

role in the catabolism of lipoproteins, increased apoE secretion is associated with increased VLDL triglyceride production [23]. ApoE can also initiate HDL particle formation [24]. On cell surfaces, LPL mediates the hydrolysis of triglycerides in CMs and their conversion to CMRs. CMRs, which become enriched with HDL-derived apoE, can then be internalized via binding of apoE and LPL to LRP. The lipid core with apoB48 is targeted to late endosomes/prelysosomal compartments. CMR surface remnants containing apoE with LPL and some lipids are retained in early endosomes to mobilize intracellular cholesterol. Some apoE/cholesterol complexes with HDL or lipid poor apoA1 in these peripheral endosomes and can be recycled to the cell surface as apoE containing HDL. In the postprandial state, secretion of apoE-HDL can facilitate the enrichment of CMRs with apoE and thereby ensure the efficient hepatic clearance of CMRs. This HDL mediated transfer of apoE to facilitate CMR or VLDL remnants is likely to depend on apoE isoform differences in receptor binding and recycling. ApoE affinity to the LDLR may be a key regulator of this process.

The LDL Receptor in Lipoprotein Metabolism

The LDL receptor plays a vital role in lipoprotein metabolism. The LDLR is a receptor for lipoproteins containing the ligands apoE and apoB100. After binding the receptor, these particles are endocytosed via clathrin-coated pits. The importance of this receptor is illustrated by the genetic deficiency of the LDLR, resulting in severe increases in low density lipoprotein (LDL) cholesterol and premature atherosclerosis known as familial hypercholesterolemia (FH) [25]. In 1974 Goldstein et al determined that elevated lipids in patients with FH were due to defects in a cell surface receptor that has a high affinity for apoB and apoE, which they named the LDLR [26]. FH patients have an increase in atherosclerosis and early death from myocardial infarction. Goldstein and Brown went on to show that the cellular levels of the LDLR are feed-back regulated at the transcriptional level by concentrations of intracellular cholesterol [26, 27]

Mice lacking LDLR, like FH in humans, have elevated LDL and develop atherosclerosis. Overexpression of the LDLR by adenovirus mediated gene transfer has been shown to reduce the cholesterol in LDLR knock out mice [28].

Species differences

Unlike humans, mice do not normally develop atherosclerosis because of key differences in lipoprotein metabolism. For example, the average plasma cholesterol level of wild type mice on a regular mouse normal chow (NC) diet (4.5% fat, 0.022% cholesterol) is approximately 80mg/dl; most of this cholesterol is carried by HDL particles. Indeed mice have very low levels of LDL and other atherogenic lipoproteins, such as remnants of diet-derived chylomicrons and liver-derived VLDL. This is in marked contrast to humans in which most of the plasma cholesterol (200 mg/dL) is associated with apolipoprotein B100 (apoB) containing LDL particles. These differences are often attributed to differences in lipoprotein metabolism between mice and humans. For example, approximately 70% of liver apoB mRNA in mice undergoes post transcriptional editing, with a corresponding production of apoB48-containing VLDL from the liver, while humans do not have hepatic editing activity and secrete only the LDL precursor, apoB100 containing VLDL [4]. While all mammals appear to have apoB editing activity in the small intestine, humans do not edit apoB mRNA in the liver, while mice do [29]. The notation apoB48 indicates that it contains only the N-terminal 48% of the 4536 residues of apoB100. Consequently, apoB48 lacks the putative C-terminal LDL receptor-binding region of apoB100. ApoB48 is generated by a unique RNA editing system where a premature stop codon is created in the apoB mRNA, by demethylation of a cytosine to a uracil residue. This is done by an editing complex that contains apobec-1, the catalytic activity of the protein complex, and RNA binding proteins that increase the specificity [30]. Another important species difference is in transfer of lipids among different lipoprotein classes. Mice lack cholesterol ester transfer protein (CETP) activity which can transfer cholesterol esters from HDL to VLDL [3]. Mice show little to no

activity of this enzyme and this results in the characteristically low levels of LDL cholesterol seen in mice, which instead transport cholesterol mainly in HDL particles and are resistant to atherosclerosis [31]. However, while mice have a less atherogenic profile than humans, the pathways for cholesterol transport and metabolism in humans and mice are sufficiently similar that mice with mutations or deletions in specific lipoprotein metabolism genes can produce human-like hyperlipidemia and atherogenesis.

Genetic Approaches to Studying apoE and the LDLR

Mouse models

Gene targeting has been used extensively over the past two decades to investigate *in vivo* gene function in mice. Targeting has several advantages over traditional transgenic models, in that only mutations to the germ line generated by homologous recombination are selected, giving precise control over target copy number and genome location and expression. This has resulted in "knocking out" specific gene functions by replacing coding gene sequences with a selectable marker gene duplication and gene replacement [32]. Gene replacement by homologous recombination allows one to change specific gene sequences while leaving other regions intact.

In the late 90's our laboratory used gene targeting to replace the murine *apoE* gene with each of the three human apoE alleles[14, 33, 34]. The murine regulatory regions remain intact and the mice produce solely the human apoE isoforms (All mouse models used in experiments and their abbreviated names are listed in Table 1.1 below). The resultant lipid profiles of these mice reflect their apoE LDLR affinity. Mice with apoE2, which has less than 2% the LDLR affinity of apoE3, exhibit hallmarks of type III hyperlipoproteinemia and develop atherosclerosis on a normal chow diet (NC) [34]. Mice expressing apoE3 and apoE4 had a more mild phenotype and, unlike their human counterparts, did not exhibit hyperlipidemia or develop atherosclerosis on NC or high fat western diet (HFW) [14, 33].

Not long after the apoE-targeted mice were characterized we made mice that modestly overexpress a human *Ldlr* minigene in place of the murine *Ldlr*, via enhanced mRNA stability. A fragment containing the human *LDLR* minigene was isolated from plasmid pMY3, which was used for generating *LDLR* transgenic mice by Yokode *et al*, and

was kindly provided by Dr. Masahiro Yokode, [35]. Message stability was increased by deleting two of the three 3' "AU-rich elements" in the 3'UTR that normally destabilize the mRNA transcript [36]. In addition, the human *LDLR* minigene contains the poly(A) addition signal sequences of the human growth hormone gene. Homologous recombination between the endogenous locus and the targeting construct results in a human *LDLR* minigene that is expressed under the control of the endogenous promoter. All 5'-regulatory sequences of the endogenous mouse locus are intact. Heterozygous mice, via stabilization of the *Ldlr* mRNA, express 2.5 fold higher total LDLR than wildtypes [37, 38].

Chimeras were generated from targeted ES cells and bred with C57BL/6 mice to obtain germ line transmission of the modified chromosome. The genotype of the modified allele in the animals was determined by the presence of a 300bp PCR fragment produced by using the N*eo* specific primer.

Mice that expressed the human apoE's were crossed to mice that were heterozygous for the human *Ldlr* minigene that results in the stabilized *Ldlr* mRNA, ultimately generating mice that were homozygous for human apoE's and heterozygous for the stabilized human *Ldlr* (h). All the mutant mouse strains used in this work were individually backcrossed at least 6 generations to the C57BL/6 genetic background before intercrossing. Mice heterozygous for a targeted replacement of the mouse *Ldlr* gene with the stabilized human *Ldlr* minigene ($Apoe^{+/+}Ldlr^{h/+}$) [37] were bred to mice homozygous for replacement of the mouse apoE gene with either the human *APOE*2, APOE*3,* or *APOE*4* allele ($Apoe^{2/2}Ldlr^{+/+}$, $Apoe^{3/3}Ldlr^{+/+}$, and $Apoe^{4/4}Ldlr^{+/+}$) [14, 33, 34]. The littermates generated by crossing $Apoe^{2/2}Ldlr^{+/+}$ (2m) with $Apoe^{2/2}Ldlr^{h/+}$ (2h), $Apoe^{3/3}Ldlr^{+/+}$ (3m) with $Apoe^{3/3}Ldlr^{h/+}$ (3h) and $Apoe^{4/4}Ldlr^{+/+}$ (4m) with $Apoe^{4/4}Ldlr^{h/+}$ (4h) respectively, were used in experiments

as donors of bone marrow cells. Mice with human *APOE*3* and lacking LDLR (3ko) and mice with human *APOE*4* and lacking LDLR (*4ko*) were generated by crossing either $Apoe^{3/3}$ or $Apoe^{4/4}$ mice with $Ldlr^{-/-}$ mice [39], and maintained as $Apoe^{3/3}$ $Ldlr^{-/-}$ (3ko) or $Apoe^{4/4}$ $Ldlr^{-/-}$ (4ko) respectively (see Table 1.1 for genotypes and nomenclature).

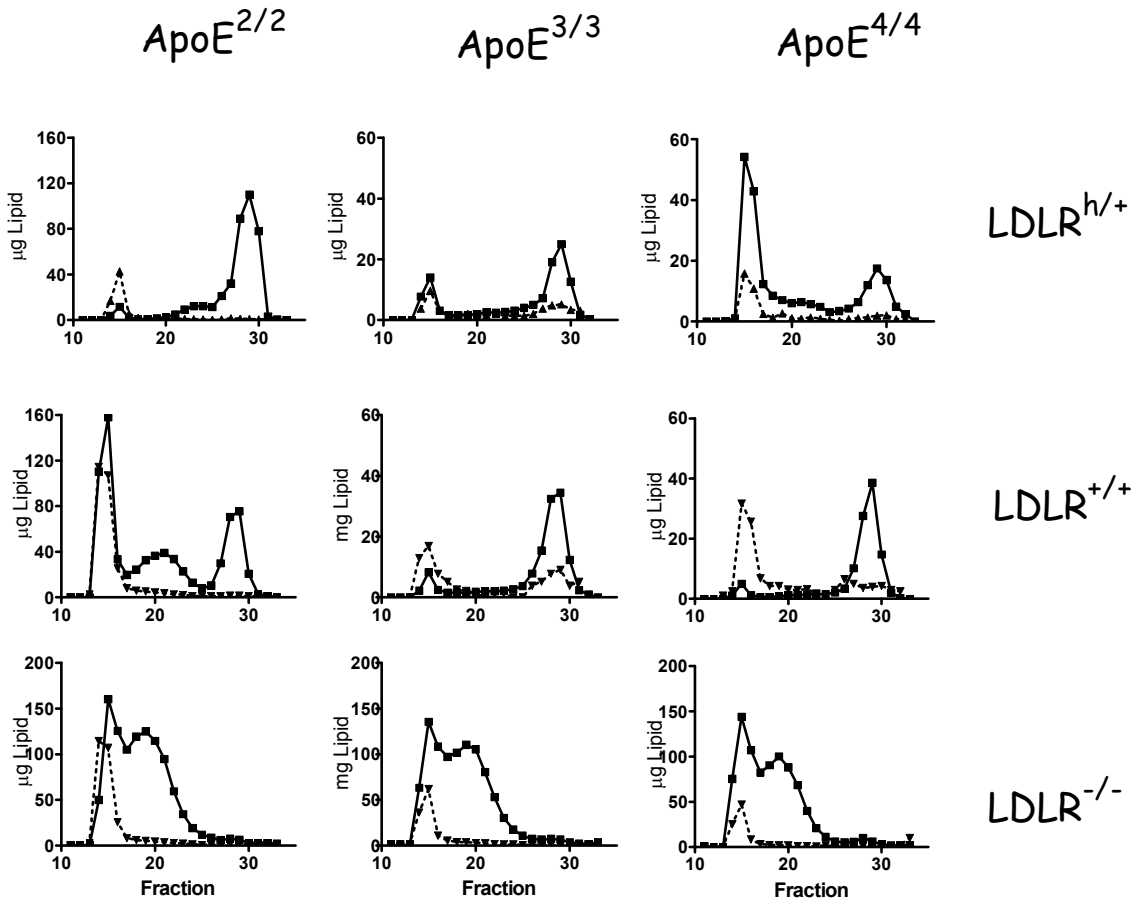
The phenotypes of the replacement mice show that the apoE isoforms behave similarly when physiologically regulated in humans and mice. However, there are some important differences. Although 2m mice exhibit features of type III hyperlipidemia, the phenotype is fully penetrant in these mice, while only 5-10% of 2/2 humans develop type III. The 4m mice have an increase in non-HDL cholesterol compared to 3m mice, but in mice the increase is in VLDL-cholesterol, not in LDL-cholesterol as in humans. Furthermore, steady state apoB48 levels are increased in the 4m mice, while only steady state apoB100 levels are increased in 4/4 compared to 3/3 human subjects. Surprisingly, introduction of the $Ldlr^{h}$ allele into mice expressing human apoE isoforms reproduced the human-like phenotypes associated with apoE isoforms. On a HFW diet, mice with apoE4 overexpressing the LDLR ($Apoe^{4/4}Ldlr^{h/+}$) have increased plasma VLDL/Chylomicron Remnants, decreased HDL cholesterol levels, and develop atherosclerosis, while mice with human apoE3 and the $Ldlr^{h}$ allele ($Apoe^{3/3}Ldlr^{h/+}$) have significantly decreased HDL cholesterols as well as total cholesterol levels and do not develop atherosclerosis. Global overexpression of LDLR in mice with apoE2 ($Apoe^{2/2}Ldlr^{h/+}$) resulted in lower plasma cholesterol and the absence of atherosclerotic lesions [37-40].

Abb	Genotype	TC, Diet	Athero
2m	*Apoe*$^{2/2}$, Human apoE2, wildtype (murine) LDLR	242±59, NC	yes
3m	*Apoe*$^{3/3}$, Human apoE3, wildtype (murine) LDLR	75±26, NC	no
4m	*Apoe*$^{4/4}$, Human apoE4, wildtype (murine) LDLR	73±27, NC	no
2h	*Apoe*$^{2/2}$*Ldlr*$^{h/+}$, Human apoE2, Heterozygous for stabilized (human) LDLR	172±43, HFW	no
3h	*Apoe*$^{3/3}$*Ldlr*$^{h/+}$, Human apoE3, Heterozygous for stabilized (human) LDLR	92±9, HFW	no
4h	*Apoe*$^{4/4}$*Ldlr*$^{h/+}$, Human apoE4, Heterozygous for stabilized (human) LDLR	199±17, HFW	yes
2-	*Apoe*$^{2/2}$*Ldlr*$^{-/-}$, Human apoE2, LDLR Knockout	747±143, NC	yes
3-	*Apoe*$^{3/3}$*Ldlr*$^{-/-}$, Human apoE3, LDLR Knockout	420±77, NC	yes
4-	*Apoe*$^{4/4}$*Ldlr*$^{-/-}$, Human apoE4, LDLR Knockout	340±82, NC	yes
ee	*Apoe*$^{-/-}$, apoE Knockout, wildtype (murine) LDLR	455±82, NC	yes
eehh	*Apoe*$^{-/-}$*Ldlr*$^{h/h}$, apoE Knockout, Homozygous for stabilized (human) LDLR	250±32, NC	yes
eell	*Apoe*$^{-/-}$*Ldlr*$^{-/-}$, apoE Knockout, LDLR Knockout	800NC	yes

TC= Total Cholesterol, NC= Normal Chow, HFW= High Fat Western

Table 1.1 Genotypes and cholesterol levels of mice used in this work.

Figure 1.2 Effect of LDLR level on lipoprotein distribution in mice with human apoE. Mice with apoE2 (left), apoE3 (center) and apoE4 (right) with no LDLR (bottom), wildtype (center), and overexpression of the huLDLR (top). All mice were on a HFW diet. Lipoprotein distributions of cholesterol (solid lines) and triglycerides (dotted lines) within plasma lipoproteins separated by FPLC.


ApoE2/2
ApoE3/3
ApoE4/4
LDLRh/+
LDLR+/+
LDLR-/-
µg Lipid
mg Lipid
Fraction

Adenovirus-Mediated Gene Expression

Adenovirae are excellent tools for *in vivo* gene transfer. This is due to the ability of the adenovirus to infect non-dividing cells and to be concentrated to high titers (greater than 10^{12} plaque-forming units (pfu)/ml). However, adenovirus–mediated expression of a transgene drops *in vivo* after 4-7 days due to host immune response [41]. A low level of adenoviral gene expression induces CD8 T lymphocyte-mediated removal of infected cells and CD4 T cell-mediated facilitation of B cell neutralizing antibody production.

The adenovirae (Ad) are non-enveloped viruses packaged in an icosohedral capsid. The adenovirus genome is composed of eight transcriptional units; six are transcribed in the early phase of viral infection before viral DNA replication. The E1 gene is the first gene transcribed after infection and its product is involved in the preparation of the viral productive cycle through interaction with cellular cyclins and activation of other viral genes through interaction with cellular transcription factors. All the experiments outlined in this proposal utilize first-generation adenoviruses that were generated by deletion of the E1 region as well as the E3 region, which is not required for infection. Recombinant adenoviruses were generated by cloning the gene of interest, with an upstream promoter into a cloning site within the deleted E1 region of a shuttle plasmid. This recombinant plasmid is transformed into bacteria that harbor the remaining adenoviral genome. The two plasmids undergo homologous recombination, resulting in generation of the full length Ad5 genome, with the E1 region replaced by the inserted gene of interest. These recombinant adenoviruses can be propagated in kidney 293 cells in which the missing E1 gene is expressed in trans. The recombinant adenovirus is isolated by CsCl gradient ultracentrifugation, dialyzed, tittered in 293 cells, and typically 1 x 10^9 pfu of virus is injected via tail vein into mice. This

procedure results in greater than 99% reporter gene activity to be found in the liver, with more than 90% of all hepatocytes exhibiting gene expression [42]. However, with visual detection of GFP transgenes in the liver, adjustment of the pfu-injected was necessary to achieve greater than 90% hepatocytes infection and expression.

Bone Marrow Transfer

Atherosclerosis now has established oxidative and immunologic pathways that may have larger roles in foam cell formation and lesion progression [43-45]. Hematopoietic stem cells (HSC), especially monocyte-derived macrophages, are the chief regulators of cholesterol efflux and conversely uptake and foam cell formation. This role in lipid uptake is in addition to their important role in immunoregulation through chemokine secretion. Bone Marrow Transfer (BMT) has been used successfully in many experiments to investigate macrophage-dependant pathways of the atherosclerotic process. Reconstitution of lethally irradiated atherosclerosis models, such as *Apoe*$^{-/-}$, or *Ldlr*$^{-/-}$ mice, with bone marrow cells that are harvested from mice carrying additional mutations allows investigators to separate the function of gene products in the macrophage from their function in the liver and remaining tissues of the recipient animals. This is important given the potential dual roles some proteins play in atherosclerosis development.

ApoE isoforms have established liver-dependant pathways that control atherosclerosis risk. *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ BMT models support liver-independent atherosclerosis changes induced by both proteins. Given their different atherosclerosis and disease associations it seemed logical to investigate if there were additional macrophage pathways behind this risk. Many of the functions of apoE in vascular tissues are isoform-specific and have been implicated as contributing factors to the well-established increased atherosclerosis risk associated with apoE4 in humans. ApoE has been shown to reduce atherosclerosis when expressed in the arterial wall without affecting lipid levels [46]. Human apoE3 expressed in macrophages binds LDLR and is internalized, and a proportion of the internalized apoE is recycled in both hepatocytes and macrophages [47, 48]. The apoE3 and apoE2 proteins carry either one or

two free cysteinyl groups respectively, and may function as a better antioxidant than apoE4 protein which does not have a free cysteinyl group. It has been shown in vitro that lipoprotein oxidation is apoE isoform-dependant [49, 50].

While they do not mimic a common pathologic phenomenon, human hematopoietic mutations occur only after stem cell therapy. BMT is useful for the separation of function and analysis of HSC monocyte-derived macrophages from hepatic and whole body contributions. This is important in delineating the specific contributions of proteins which have roles in inflammation and lipoprotein metabolism in the liver and macrophage such as apoE and the LDLR.

Chapter II

Harmful Effects of Increased LDLR Expression in Mice with Human APOE*4 But Not APOE*3

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Abstract

Objective— Increased expression of the low-density lipoprotein receptor (LDLR) is generally considered beneficial for reducing plasma cholesterol and atherosclerosis, and its downregulation has been thought to explain the association between apolipoprotein (apo) E4 and increased risk of coronary heart disease in humans.

Methods and Results— Contrary to this hypothesis, doubling *Ldlr* expression caused severe atherosclerosis with marked accumulation of cholesterol-rich, apoE-poor remnants in mice with human apoE4, but not apoE3, when the animals were fed a Western-type diet. The increased *Ldlr* expression enhanced in vivo clearance of exogenously introduced remnants in mice with apoE4 only when the remnants were already enriched with apoE4. The rates of nascent lipoprotein production were the same. The adverse effects of increased LDLR suggest a possibility that the receptor can trap apoE4, reducing its availability for the transfer to nascent lipoproteins needed for their rapid clearance, thereby increasing the production of apoE-poor remnants that are slowly cleared. The lower affinity for the LDLR of apoE3 compared with apoE4 could then explain why increased receptor expression had no adverse effects with apoE3.

Conclusions— Our results emphasize the occurrence of important and unexpected interactions between *APOE* genotype, *LDLR* expression, and diet.

Introduction

Apolipoprotein E (apoE) plays a central role in the clearance of atherogenic lipoprotein particles from the circulation [6] The *APOE* gene in humans is polymorphic with 3 common alleles, *APOE*2*, *APOE*3*, and *APOE*4*, which code for apoE2, apoE3, and apoE4. These isoforms differ by the amino acids at positions 112 and 158, where apoE2 has Cys at both sites, apoE4 has Arg at both sites, and apoE3 has Cys-112 and Arg-158. There is a well-established association between the *APOE* polymorphism and the risk for vascular diseases; individuals with *APOE*4* allele have increased plasma cholesterol and an increased risk of atherosclerosis[2, 3]. Although these increases are modest, they have a large impact on the overall human population, because 25% carry 1 or 2 *APOE*4* alleles. However, this association is paradoxical when one considers that apoE4 binds to the LDL receptor (LDLR) with equal or a slightly greater affinity than apoE3, the most common isoform [13-15]. Additionally, individuals homozygous for apoE2, which binds to the LDLR with much less affinity than either apoE3 or apoE4, have low plasma cholesterol and are generally protected from atherosclerosis, except for the 5% to 10% of apoE2 homozygotes who develop type III hyperlipoproteinemia [6]. The present explanation of this paradox is that the high affinity of apoE4 for the receptor leads to increased apoE-mediated cholesterol uptake followed subsequently by downregulation of the *LDLR* gene. This then leads to reduced apoB100-mediated uptake of LDL, accumulation of LDL cholesterol, and the vascular problems [3, 18, 19]. Conversely, the low affinity of apoE2 is thought to lead to upregulation of *LDLR*. Although this explanation seems reasonable given that the loss of function of even one *LDLR* allele leads to markedly elevated plasma LDL and premature atherosclerosis,[25] it has not been proven.

In the present study, we find that, contrary to the expectations of this hypothesis, increased *Ldlr* expression in mice with human *APOE*4* causes severe atherosclerosis with marked elevation of plasma cholesterol when they are fed a Western-type diet. Mice with *APOE*3*, on the other hand, are not harmed by the increase in *Ldlr* expression. Based on these studies, we propose an alternative mechanism that the increased amount of LDLR can trap apoE and deplete the pool of apoE transferable to nascent lipoproteins.

Methods

Mice heterozygous for targeted replacement of the mouse *Ldlr* gene with the human *LDLR* minigene ($Apoe^{+/+}Ldlr^{h/+}$) [37] were bred to mice homozygous for replacement of the mouse apoE gene with either the human *APOE*3* or *APOE*4* allele ($Apoe^{3/3}Ldlr^{+/+}$ and $Apoe^{4/4}Ldlr^{+/+}$).[14, 33]The experimental animals were mostly littermates generated by crossing $Apoe^{3/3}Ldlr^{+/+}$ (*3m*) with $Apoe^{3/3}Ldlr^{h/+}$ (*3h*) and $Apoe^{4/4}Ldlr^{+/+}$ (*4m*) with $Apoe^{4/4}Ldlr^{h/+}$ (*4h*), respectively. Mice with human *APOE*4* and lacking LDLR (*4KO*) were generated by crossing $Apoe^{4/4}$ and $Ldlr^{-/-}$ mice (see Table 2.1 for the definition of the shorthand designation of the mice used in this study). All mice were hybrids between 129 and C57BL/6, having approximately one fourth to one eighth of their genome from 129 and the remaining three fourths to seven eighths from C57BL/6. Twelve- to 36-week-old mice of both sexes were used for experiments that were conducted under protocols approved by the Institutional Animal Care and Use Committees. Littermates were used in each experiment as much as possible. Mice were fed either normal mouse chow (NC) containing 4.5% (wt/wt) fat and 0.022% (wt/wt) cholesterol (Prolab RMH 3000, Agway Inc) or a high-fat Western-type diet (HFW) containing 21% (wt/wt) fat and 0.2% (wt/wt) cholesterol (TD88137; Teklad).

Lipid and lipoprotein analysis. Animals were fed normal chow (Prolab Rat/Mouse/Hamster 300, Agway, Syracuse, NY) or Western type diet (TD88137, Harlan Teklad, Madison WI). For analysis of plasma lipids, 200-300µl of blood were collected from retro-orbital plexus into tubes containing of EDTA 2mM, gentamicin 1ug/ml and aprotinin 0.1UI (final concentrations). Mice were fasted for four hours unless otherwise stated in the text. Total cholesterol and triglycerides were determined by using reagents from Sigma

(401-25P Infinity Cholesterol reagent and 339-20 Triglyceride (GPO-TRINDER) 20 respectively). HDL was precipitated using the method described[4] and measured using the cholesterol reagent described above. Plasma pooled from at least 6 mice (100 μl) was fractionated by fast protein liquid chromatography (FPLC) using a Superose 6HR column (Pharmacia Biotech Inc. Piscataway, NJ) and lipid analysis of the fractions (500 μl) was performed using Sigma reagents. One ml of pooled plasma from each group was also fractionated by sequential density ultracentrifugation as described.[5] Lipoprotein fractions were isolated by tube cutting and dialyzed against phosphate-buffered saline. Apolipoproteins were separated in a denaturing 3-20% SDS-polyacrylamide gel. Relative apoprotein ratios were determined by image densitometry using NIH Image following Commassie Brilliant Blue staining of the SDS-PAGE, or after Western blotting. Goat anti-human apo E antibody was from Calbiochem (La Jolla, CA), and rabbit anti-mouse apoB was a gift from the late Dr. Harshini de Silva at the University of North Carolina Charlotte. Liver lipids were extracted using an adaptation of the method.

Mice	Genotype	Definition
3m	$Apoe^{3/3}Ldlr^{+/+}$	Mice with human apoE3
3h	$Apoe^{3/3}Ldlr^{h/+}$	Mice with human apoE3 and increased LDLR
4m	$Apoe^{4/4}Ldlr^{+/+}$	Mice with human apoE4
4h	$Apoe^{4/4}Ldlr^{h/+}$	Mice with human apoE4 and increased LDLR
4KO	$Apoe^{4/4}Ldlr^{-/-}$	Mice with human apoE4 and no LDLR
2m	$Apoe^{2/2}Ldlr^{+/+}$	Mice with human apoE2
2h	$Apoe^{2/2}Ldlr^{h/+}$	Mice with human apoE2 and increased LDLR

Table 2.1. Keys to the Shorthand Definition of Mice Described in This Study

Message analysis. RNA was prepared from the livers, harvested from mice fasted for four hours, using Trizol reagent (Life Technologies, Geitesburg, MD) with standard protocols. The primer extension strategy was designed to simultaneously study both messages utilizing a primer (GGAGCACGTCTTGGGGGGACAGCCT) that hybridized to a shared homologous sequence of exon 3 of the LDLR. ddCTP was utilized to terminate extension reaction, resulting in the murine message extension of 5 base pairs while the human message extension is 3 base pairs. The amount of RNA was determined in a phosphoimager analyzer and normalized by the amount of Gapdh mRNA that was simultaneously determined in each reaction.

Remnant clearance. The VLDL fraction (d<1.006)) from chow-fed apoE-deficient mice or chow-fed *4KO* mice and remnant fraction (d<1.02) from *4h* mice on HFW diet were isolated by ultracentrifugation. Approximately 1 mg of VLDL protein was radioidinated with ^{125}I or with ^{131}I. A dose of $5x10^6$ to 10^7 cpm was injected through the tail vein or through the external jugular vein of recipient *4h* and *4m* mice fed HFW for at least two weeks. Mice were bled (50 μl) at time points indicated, and the radioactivity remaining in plasma was measured using a 1272 CliniGamma Gamma Counter (LKB, Turku, Finland) assuming that blood volume is 3.5% of total body weight.

VLDL secretion and fat loading. 16 week old *4m* and *4h* mice were fed HFW diet for two weeks. To examine VLDL secretion, female mice (*n*=8 for each group) were fasted for 4 hours prior to injection of Triton WR1339 (Sigma, St. Louis, Mo) in saline solution at a dose of 0.7 mg/g body weight. For fat tolerance test, female mice (*n*=5 for each group) were fed 0.4 ml of a 1:1 (w/w) mixture of oil olive and dehydrated egg yolk (Sigma, St. Louis, MO)

by oral gavage. Plasma was collected before and at time points indicated for cholesterol and triglyceride measurements. Chylomicron secretion was estimated by fat loading on male mice (*n*=4 for *4h* and *n=4* for *4m*) after *iv* injection of Triton WR1339 to inhibit the clearance.

Atherosclerosis. Mice were fed HFW diet for three months before euthanization with a lethal dose of 2,2,2-tribromoethanol. The heart and the vascular tree were perfused at physiological pressure with 4% phosphate buffered paraformaldehyde, pH 7.4. Morphometric analysis of plaque size at the aortic root was made on four segments using methods described [14, 37].

*Statistical analysis***.** All data were first analyzed with general linear modeling using JMP software (version 5, SAS Institute, Cary, NC) to assess effects of multiple variables of interest (e.g. gender, *Apoe* genotye and *Ldlr* genotype). Where interactions between variables were not significant, reduced models without interactions were used.

Results

Increased *Ldlr* expression causes hypercholesterolemia in mice with human *APOE*4*

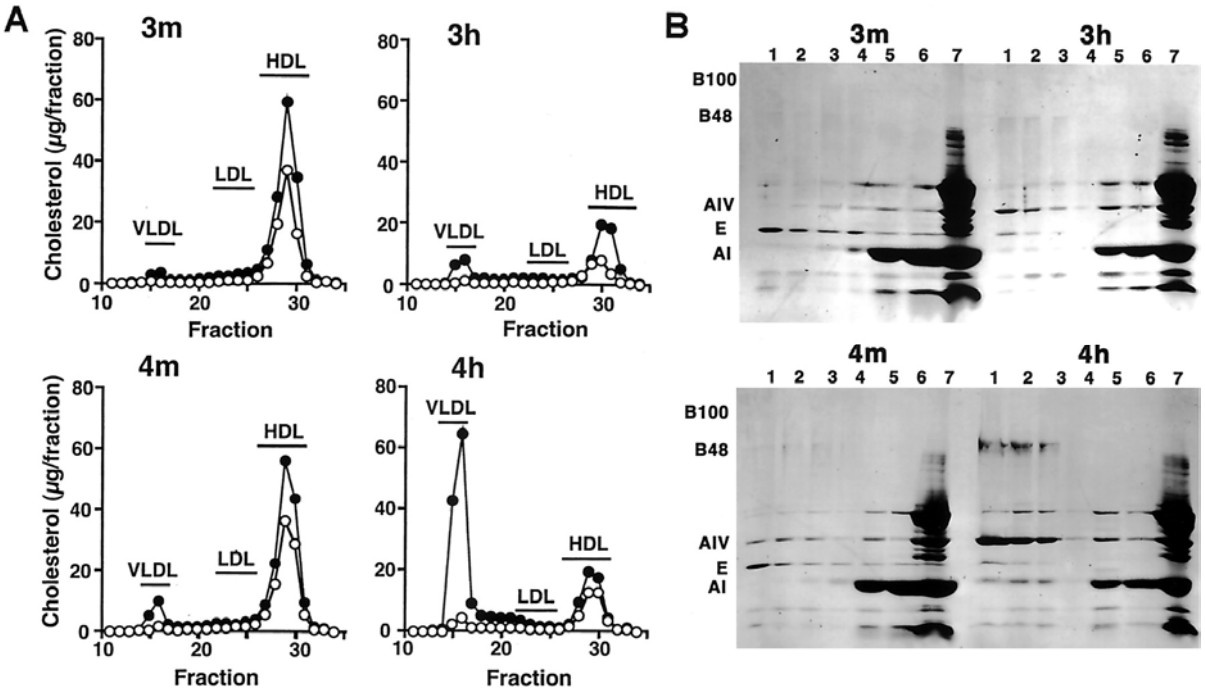
We replaced the endogenous mouse *Ldlr* with a minigene coding for human LDLR ($Ldlr^h$) that produces an mRNA with an increased half-life [37] and introduced 1 copy of this $Ldlr^h$ allele into mice expressing solely apoE3 ($Apoe^{3/3}$) or apoE4 ($Apoe^{4/4}$) [14, 33]. When mice were fed NC, the increase in *Ldlr* expression significantly lowered the plasma lipids in both $Apoe^{3/3}Ldlr^{h/+}$ (*3h*) and $Apoe^{4/4}Ldlr^{h/+}$ (*4h*) mice, relative to the $Apoe^{3/3}Ldlr^{+/+}$ (*3m*) or $Apoe^{4/4}Ldlr^{+/+}$ (*4m*) mice (Table 2). The *Ldlr* genotype had highly significant effects on total cholesterol (TC), triglyceride (TG), and HDL cholesterol (HDL-C) ($P<0.0001$). The effects of the *Apoe* genotype on TC and HDL-C were not significant, but females with apoE4 tended to have higher TG than those with apoE3 whereas males with apoE3 tended to have higher TG than those with apoE4 ($P=0.0002$ for *Apoe*, sex interaction). All classes of plasma lipoproteins including HDL were reduced in mice with increased LDLR, as assayed by FPLC analysis (Figure 2.1A,). Feeding a HFW increased the plasma TC and the HDL-C levels of all of the mice (Table 2.2). Surprisingly, however, the increase in plasma TC was much greater in the *4h* mice than in *4m* mice (120±11 versus 32±5 mg/dL, $P<0.0001$). This increase resulted mainly from a dramatic accumulation of non-HDL particles that elute in the VLDL region during fast performance liquid chromatography (FPLC, Figure 2.1A). In contrast, the *3h* mice on HFW showed only a small increase in non-HDL particles, and they had significantly lower cholesterol levels than the *3m* mice primarily because of reduced HDL. Thus, the *Ldlr* genotype has markedly different effects on the response to HFW in mice with apoE4 compared with those with apoE3.

Table 2.2 Plasma Lipids

Mice Genotype (*Apoe, Ldlr*)	Sex	TC, mg/dL		TG, mg/dL		HDL-C, mg/dL	
		NC	HFW	NC	HFW	NC	HFW
3m (3/3, +/+)	M	95±4 (14)	114±12 (14)	79±6 (14)	49±5 (14)	77±4 (7)	102±16 (9)
	F	66±3 (18)	106±10 (9)	40±4 (18)	39±6 (9)	49±7 (5)	81±10 (9)
3h (3/3, h/+)	M	53±4 (22)	72±8 (12)	44±5 (22)	22±3 (12)	22±2 (5)	31±3 (5)
	F	37±4 (11)	92±9 (7)	27±4 (11)	54±6 (7)	17±2 (7)	30±4 (7)
4m (4/4, +/+)	M	78±3 (30)	124±8 (34)	54±2 (29)	63±5 (27)	68±6 (11)	89±8 (16)
	F	63±4 (16)	114±6 (28)	49±5 (16)	48±4 (26)	51±3 (11)	77±11 (8)
4h (4/4, h/+)	M	52±4 (28)	187±19 (22)	35±2 (29)	41±3 (17)	32±3 (11)	36±4 (6)
	F	53±4 (29)	199±17 (27)	34±4 (29)	41±4 (22)	30±1 (7)	41±5 (4)
Sex effects		P=0.005	NS	P<0.0001	NS	P=0.001	NS
Apoe effects		NS	P<0.0001	P=0.04	NS	NS	NS
Ldlr effects		P<0.0001	P=0.03	P<0.0001	P=0.003	P<0.0001	P<0.0001
Apoe x *Ldlr* interaction		P=0.01	P<0.0001	NS	NS	NS	NS

Values are mean±SE. The effects of sex and 2 genotypes were estimated with three-way factorial analysis of pooled data for both sexes. Numbers of animals are in parentheses.

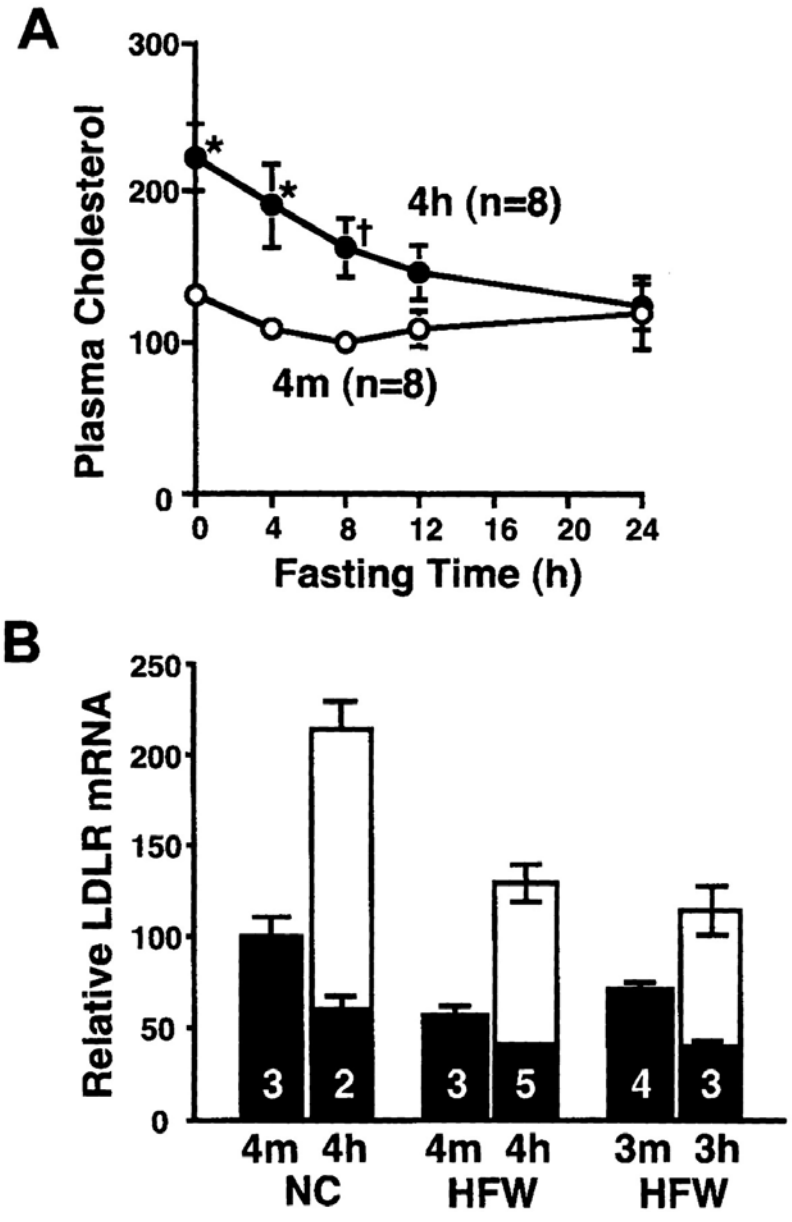
Figure 2.1. Plasma lipoproteins. A, FPLC of plasma lipoproteins of mice fed NC (○) and HFW (•). Plasma was pooled from at least 6 male mice of each genotype. Fractions containing VLDL, LDL, and HDL are indicated. B, SDS polyacrylamide gel electrophoresis. Plasma was collected from mice fed HFW and separated by ultracentrifugation. Lanes 1 through 7 are density fractions $d<1.006$, $1.006<d<1.02$, $1.02<d<1.04$, $1.04<d<1.06$, $1.06<d<1.08$, $1.08<d<1.10$, and $1.10<d<1.21$, respectively. Positions of apolipoproteins are indicated on the left.


A
3m
3h
4m
4h
Cholesterol (μg/fraction)
Fraction
VLDL
LDL
HDL
B
3m
3h
4m
4h
1 2 3 4 5 6 7
B100
B48
AIV
E
AI

The *4h* remnants were mostly in very low to intermediate density fractions (d<1.02 g/mL) by ultracentrifugation and were enriched in TC but poor in TG, with a TC/TG ratio of 5.3 compared with 0.6, 0.6, and 1.2 in *4m*, *3m*, and *3h* remnants, respectively. The apolipoprotein compositions of the VLDL fraction also differed significantly (Figure 1B). Densitometric analysis of at least 4 different preparations of the VLDL fractions showed that the *4h* remnants had a marked reduction of apoE4 (x0.4) but increased apoB48 (x6.7) and apoAIV (x4.7) compared with *4m* remnants. ApoE3 in the *3h* remnants was also reduced (x0.5) compared with *3m* remnants, but the increase in apoB48 (x3) was less prominent. The *4h* remnant fraction contains an average of 4.5 times more apoB proteins than that of the *3h* mice and had a smaller apoE/apoB ratio (8±1% relative to that in *4m*) compared with 36±4% in *3h* fraction (P<0.005). Thus, the remnants of the *4h* mice are cholesterol-rich, TG-poor, and apoE-poor compared with those of the *3h* mice.

The enrichment of apoAIV in the *4h* remnants suggests that they are mainly from intestine-derived chylomicrons. Consistent with this possibility, plasma cholesterol levels declined steadily in fasting *4h* mice but not in *4m* mice (Figure 2.2A) and remnant particles were reduced in *4h* mice fasted for 18 hours or longer. There were no significant differences in the TC and TG content in the livers of these mice. The relative amounts of apoE protein estimated by Western blot analysis indicate that the elevated LDLR expression increases liver-associated apoE by 30% but reduces plasma apoE by 60% regardless of their *Apoe* genotype.

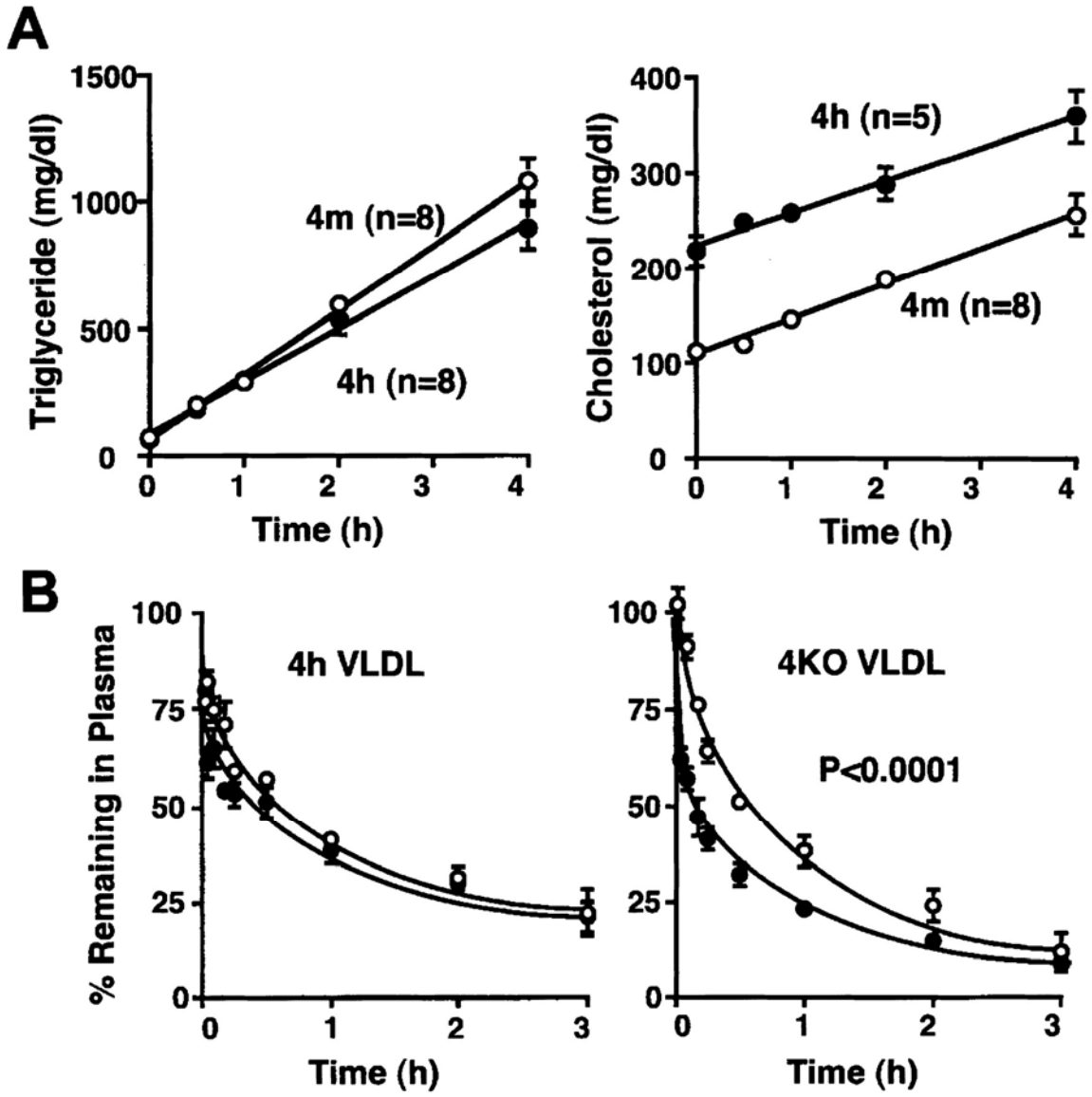
Figure 2.2. Effects of increased LDLR expression on mice with human apoE4 on HFW. A, Fasting effects on plasma cholesterol levels in male *4h* (●, n=8) and *4m* (○, n=8) mice on HFW. Error bars are SEM. $P<0.0001$ by MANOVA. *$P<0.001$ and $P<0.05$ between genotypes by 2-tailed Student's *t* test. B, Liver mRNA levels for the mouse *Ldlr* (black bars) and for the human *Ldlr* (white bars) expressed as percent of the *Ldlr* gene expression in *4m* mice on NC. Numbers of animals (all males) are shown in the bottom of the bars. Error bars are SEM. $P<0.05$ for diet effect, $P<0.0005$ for *Ldlr* genotype effect by 2-way ANOVA. The effect of *Apoe* genotype is not significant.


A
Plasma Cholesterol
300
200
100
0
*
*
†
4h (n=8)
4m (n=8)
0
4
8
12
16
20
24
Fasting Time (h)
B
Relative LDLR mRNA
250
200
150
100
50
0
3
2
3
5
4
3
4m 4h
NC
4m 4h
HFW
3m 3h
HFW

The level of mouse *Ldlr* mRNA in the liver of *4h* mice with a single copy of the gene was 61% of that in *4m* mice with 2 copies (100%, Figure 2.2B). The human *Ldlr* message was 155% (total, 216%). The regulatory machinery of the $Ldlr^h$ allele is intact, because HFW downregulated the mouse and human *Ldlr* messages equally to 60% of their levels in mice fed NC. The total *Ldlr* mRNA level in *4h* mice fed HFW was 2-fold higher than in *4m* mice on HFW and a trace higher than in *4m* mice fed NC. Thus, the marked hypercholesterolemia in the *4h* mice fed HFW occurs despite their having high levels of LDLR expression. *Ldlr* expression is similar in the livers of *4h* and *3h* mice on HFW, indicating that the hypercholesterolemia in the *4h* mice, but not the *3h* mice, is not attributable to any differences in the diet-induced downregulation of *Ldlr* expression in the liver.

In Vivo Lipoprotein Metabolism in 4h Mice To estimate the production of VLDL, we injected Triton WR1339 into *4h* and *4m* mice fed the HFW to inhibit lipolysis and the uptake of TG-rich particles. The initial rate of TG accumulation in plasma was not different between the *4h* and *4m* mice (Figure 2.3A). Plasma TC increased steadily and equally in both mice. They also responded similarly to the fat loading. The rates of plasma TG increase after fat loading in *4h* mice treated with Triton WR1339 (604±33 mg/dL per h, n=4) was similar to those in *4m* mice (656±37 mg/dL per h, n=4). Thus, increased LDLR expression seems to have no effect on the chylomicron production. These data suggest that the dramatic accumulation of remnants in the *4h* mice compared with *4m* mice is not attributable to oversecretion of TG-rich particles.

Figure 2.3. In vivo lipoprotein metabolism. A, Triglyceride (left) and cholesterol (right) secretion of *4m* (○)and *4h* (●) mice after inhibition of lipolysis with Triton WR1339. The overall rate of TG secretion was not significantly different between the genotypes by MANOVA. B, Clearance of remnants. Radioactivity remaining in the plasma after injection of I^{125}-labeled VLDL obtained from *4h* remnants into HFW-fed *4h* males (n=10) and *4m* males (n=9) was not significantly different at any time points except at 10 minutes (left, $P<0.05$). Clearance of the ^{131}I radiolabeled apoE4-rich *4KO* remnants (right) in *4h* males (n=5) was significantly faster than in *4m* males (n=5, $P<0.0001$ by MANOVA, and $P<0.01$ at all times up to 120 minutes).


A
Triglyceride (mg/dl)
1500
1000
500
0
4m (n=8)
4h (n=8)
0
1
2
3
4
Time (h)
Cholesterol (mg/dl)
400
300
200
100
0
4h (n=5)
4m (n=8)
0
1
2
3
4
Time (h)
B
% Remaining in Plasma
100
75
50
25
0
4h VLDL
0
1
2
3
Time (h)
4KO VLDL
P<0.0001
0
1
2
3
Time (h)

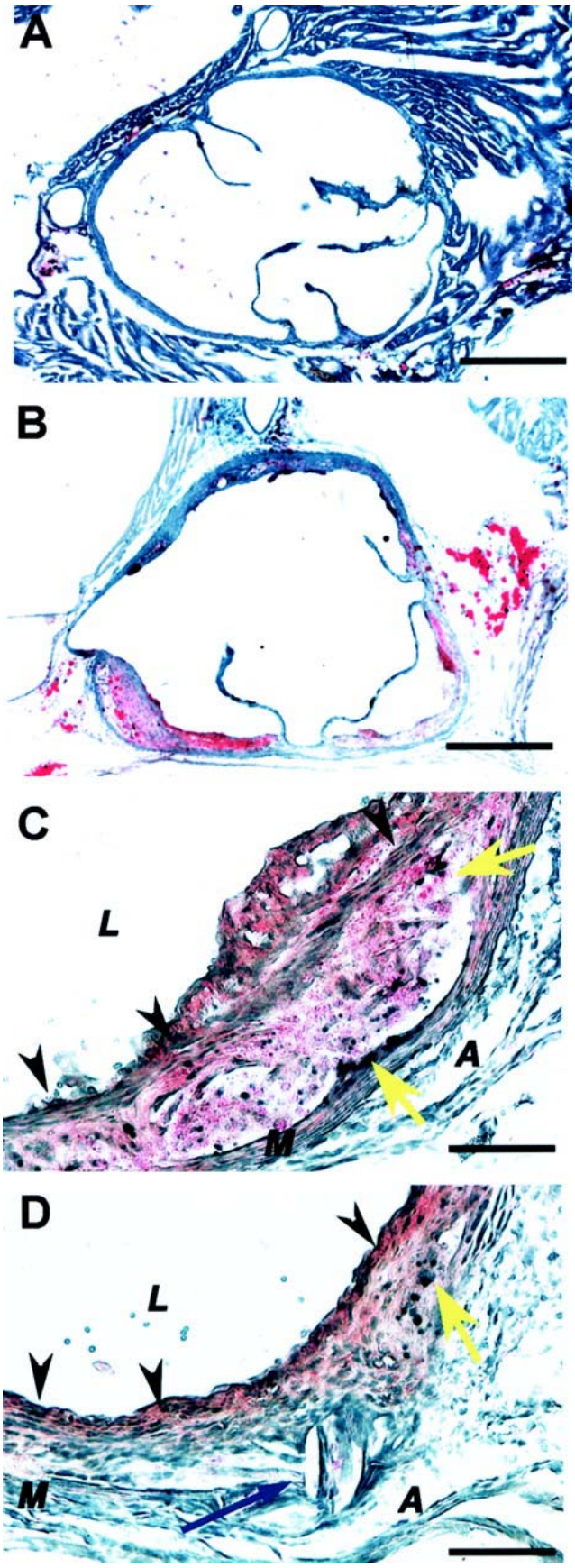
To examine the ability of mice to clear cholesterol-enriched remnant particles, we isolated remnants (d<1.02) from HFW-fed *4h* mice, radiolabeled the particles with ^{125}I, and injected them into the jugular vein of *4h* and *4m* mice fed the HFW diet. Despite the increased *Ldlr* expression in the *4h* mice, removal of the apoE-poor remnants from their plasma (0.04 pools/min) was not different from that in the *4m* mice (0.04 pools/min, Figure 3B, left). Similar results were obtained with remnants (d<1.006) isolated from apoE-deficient mice (not shown). To test whether the *4h* mice having increased LDLR are able to clear apoE4-enriched remnants, we isolated lipoproteins (d<1.006) from mice expressing apoE4 but lacking LDLR (*4KO*) that are markedly enriched with apoE but not with apoAIV (C. Knouff and N. Maeda, unpublished data). The plasma decay of ^{131}I-radiolabeled *4KO* remnants (Figure 2.3B, right) was significantly faster in the *4h* mice (0.07 pools/min) than in the *4m* mice (0.03 pools/min, P<0.0001). Thus, the *4h* mice with genetically increased LDLR expression clear remnant lipoproteins at an enhanced rate as long as these particles are enriched in apoE.

Taken together, these data indicate that the marked accumulation of remnant particles in the *4h* mice is neither because they have increased secretion of nascent TG-rich lipoproteins nor because they have reduced clearance of apoE-poor remnants compared with *4m* mice. The inference from these data is that the conversion of large TG-rich particles to smaller cholesterol-rich remnants is enhanced in the *4h* mice.

Severe Atherosclerosis in 4h But Not 3h Mice on HFW Diet. Accumulation of remnant particles and reduction of HDL-cholesterol in HFW-fed *4h* mice is a high-risk profile for atherosclerosis, even though the average plasma total cholesterol of these mice is only marginally elevated (200 mg/dL). We therefore evaluated the development of

atherosclerosis in mice fed HFW containing 21% fat and 0.5% cholesterol for 3 months. No plaques were found in any of the *3m* mice (5 females) or *4m* mice (8 females and 5 males) on HFW. Similarly, none of the 7 female *3h* mice on HFW developed plaques (Figure 2.4A). In contrast, all of the 15 female and 7 male *4h* mice on HFW developed significant plaques at the aortic sinus area (Figures 2.4B through 2.4D) with average plaque sizes of $59\pm15x10^3$ μm^2 in females and $22\pm7x10^3$ μm^2 in males. Although the numbers and sizes of plaques varied in individual animals, most of the mice had mature complex plaques with fibrous caps, necrotic lipid cores, cholesterol clefts, and calcifications (Figures 2.4C and 2.4D). Thus, mice having human *APOE*4* and a moderately increased amount of LDLR develop significant atherosclerosis when fed a diet similar in composition to that of Western societies.

Figure 2.4. Representative atherosclerotic plaques in the aortic sinus of mice fed HFW for 3 months. A, *3h* female; B, *4h* female; C, *4h* male; and D, *4h* female. Frozen sections were stained with Sudan IVB for lipids and counterstained with hematoxylin. Black arrowheads indicate fibrous caps. Yellow and blue arrows designate calcification and cholesterol clefts, respectively. L indicates lumen; M, media; and A, adventitia. Scale bars=500 μm in a and b and 100 μm in c and d.

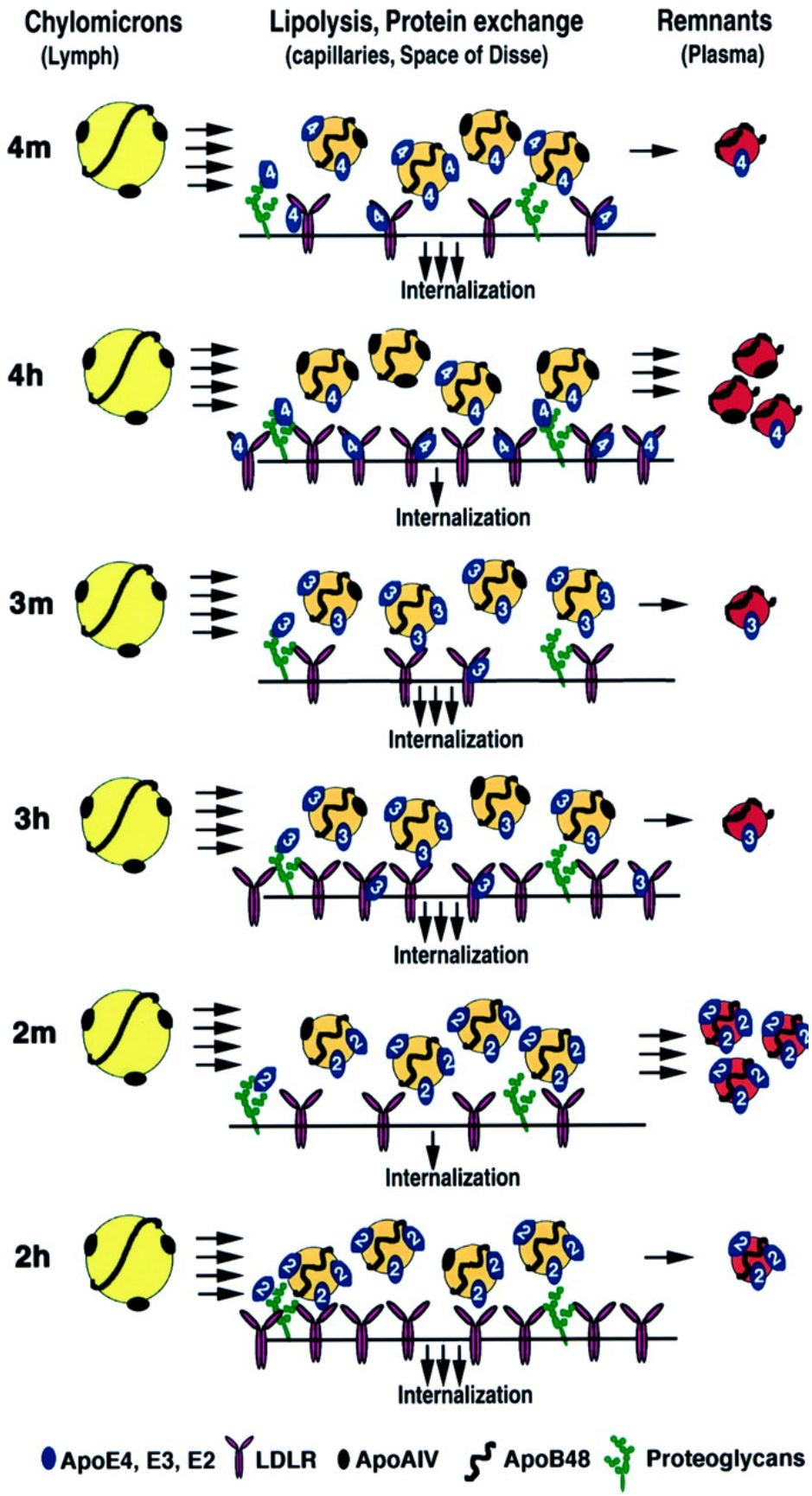

A
B
C
L
A
M
D
L
M
A

Discussion

Our clear demonstration of hypercholesterolemia and atherosclerosis in mice that have human apoE4, but not apoE3, replicates in mice fed a Western diet the paradoxical association between *APOE* polymorphisms and the risk of atherosclerosis in humans. However, contrary to the currently accepted hypothesis that downregulation of *LDLR* in individuals with an *APOE*4* allele is the cause of their high plasma cholesterol levels,[3, 18, 19] we find that these isoform-specific effects are only present when LDLR is increased.

How can increased LDLR expression ever be harmful? We suggest that this is because the LDLR, under some circumstances, traps sufficient apoE and the supply becomes inadequate to process a high dietary intake of lipids. Exchange of apoE onto nascent triglyceride-rich lipoproteins is a necessary prerequisite for their internalization via LDLR . The overall process that we envision is illustrated in Figure 2.5. ApoE4 may be particularly susceptible to this trapping because of its strong affinity for the LDLR. Transient particles that fail to acquire apoE4 are excellent substrates for lipases, and the resulting enhanced lipolysis will increase cholesterol-rich apoE-poor remnant particles in plasma [6]. We suggest that apoE3 is less susceptible to trapping than apoE4, perhaps because of its somewhat lower affinity for the LDLR [13, 14]. Consequently, the *3h* mice have sufficient apoE3 to process nascent lipoproteins for rapid internalization. This differential transfer of apoE3 and apoE4 to lipoproteins can also explain our previous observation that in vivo clearance of exogenously introduced remnants is significantly faster in *3m* mice than in *4m* mice [14].

Figure 2.5. ApoE trapping by the LDLR. Triglyceride-rich chylomicrons (yellow) secreted by the intestine into lymph are remodeled to transient particles (orange) mainly in capillaries and in the space of Disse in the liver. This initial capturing phase is very rapid and does not depend on the LDLR; it probably involves heparan sulfate proteoglycans that facilitate lipolysis and apolipoprotein exchange. Enrichment with apoE is a requirement for the fast internalization of the transient particles primarily via LDLR-mediated endocytosis. Otherwise, the particles are additionally processed to cholesterol-enriched remnants (red) that are slowly cleared from the plasma. The lipid composition of the remnants in plasma depends on both *Apoe* genotype and the amount of LDLR. The processing of liver-derived VLDL particles is likely to be similar to that illustrated for chylomicrons.


Chylomicrons
(Lymph)
Lipolysis, Protein exchange
(capillaries, Space of Disse)
Remnants
(Plasma)
4m
Internalization
4h
Internalization
3m
Internalization
3h
Internalization
2m
Internalization
2h
Internalization
ApoE4, E3, E2
LDLR
ApoAIV
ApoB48
Proteoglycans

Previously we reported that mice expressing solely apoE2 (*2m*) show features typical of type III hyperlipoproteinemia in humans but that increased LDLR expression in these mice (*2h*) completely ameliorates their hyperlipoproteinemia [14, 34] According to our hypothesis, apoE2 with its very low affinity for the LDLR should be virtually free from trapping and should therefore be efficiently transferred to transient TG-rich particles. Although such an apoE2 enrichment of TG-rich particles is likely to increase their LDLR-mediated internalization, it will also severely inhibit lipolysis and could account for the prominent accumulation of TG-rich remnants seen in the circulation of the *2m* mice [14, 34] In the *2h* mice, however, high LDLR expression tips the balance toward more internalization and lowers their plasma cholesterol.

Clearly, additional studies are necessary to refine and test our proposed apoE trapping by the LDLR. For example, we do not know whether it occurs on the cell surface, as illustrated in Figure 2.5, or during intracellular trafficking [51, 52]. The word *trapping* should not be taken too literally; difference in the interaction between apoE and LDLR or their subsequent processing may result not only from differences in binding affinities but also from other properties influenced by the specific amino acids that differ among 3 isoforms. Interactions of apoE with other molecules, such as proteoglycans and hepatic lipase, that are also expressed on the basolateral microvilli of hepatocytes may also influence the apoE interaction with LDLR in an isoform-specific fashion. Published studies have shown that newly synthesized apoE is incompletely secreted and partially degraded in HepG2 cells in culture and a significant portion of apoE synthesized by macrophages undergoes rapid cellular degradation in a non-lysosomal compartment in a sterol-regulated manner [53, 54]. Although the role of LDLR in these processes has not been addressed, LDLR is known to

bind to newly synthesized apoE in macrophages and limits its secretion [55] LDLR expression in macrophages could also contribute to atherogenesis in an apoE isoform–specific fashion. For example, a differential effect on cholesterol homeostasis in macrophages by apoE isoforms with apoE4 being least effective in promoting cholesterol efflux from macrophage has been reported [56]. Additionally, Linton et al have shown that C57BL/6 mice receiving *Ldlr*$^{-/-}$ marrow developed 63% smaller lesions than mice receiving *Ldlr*$^{+/+}$ marrow after dietary atherogenic stimuli, demonstrating that macrophage LDLR affects the rate of foam cell formation under conditions of dietary stress [57].

Some comments are required on the relevance of our findings in mice to the effects of different *APOE* genotypes in humans. We note that *4h* mice preferentially accumulate apoB48-containing lipoproteins of an intermediate density, whereas humans with the *APOE*4* allele mainly have elevated levels of apoB100-containing LDL [3] This is not incompatible with our hypothesis, which predicts that trapping of apoE4 by the LDLR will hinder enrichment of VLDL remnants with apoE4, thereby leading to an increase in their conversion to LDL. Because the clearance of LDL particles mediated by binding apoB100 to the receptor is much slower than apoE-mediated VLDL clearance, we expect that the plasma cholesterol levels in individuals with apoE4 will be increased. Supporting this explanation, an increased conversion of VLDL to smaller remnants and a relative decrease in direct removal of VLDL in *APOE*4* homozygotes compared with *APOE*3* subjects have been demonstrated [58-60].

Our finding that hypercholesterolemia is seen only when the *4h* mice are fed HFW diet is also consistent with observations that human subjects carrying the *APOE*4* allele are more responsive than others to LDL cholesterol–lowering by diet [61, 62] In addition, some

though not all studies have found prolonged postprandial lipemia in normolipidemic subjects that carry *APOE*4 [63, 64]* . Bergeron and Havel have proposed that prolonged residence times of chylomicron and VLDL remnants in persons with *APOE*4* raise the concentration of LDL by increasing the amount of VLDL converted to LDL. We additionally note that an apoE5 variant with lysine in place of glutamic acid at position 3 is also associated with hyperlipidemia and atherosclerosis and it has a twice-normal LDLR binding activity [17]. Finally, some although not all studies have shown that the cholesterol-lowering effects of statins, thought to be primarily mediated by increased LDLR, are apoE isoform–dependent. In these studies, individuals with the *APOE*3/4* and *APOE*4/4* genotypes had significantly smaller LDL cholesterol reductions in response to statin treatment than those with the *APOE*3/3* genotype [65] Clinical studies clearly indicate that statin therapies reduce the risk of cardiovascular disease in humans, including those with apoE4, and no serious adverse effects on plasma lipids have been reported [66, 67]. Nevertheless, our observations suggest the need for additional studies of the interaction between the cholesterol-lowering effect of statins and genetic variations.

In conclusion, our studies demonstrate that, contrary to the presently accepted downregulation of LDLR hypothesis, increased LDLR has harmful effects in Western diet–fed mice expressing human apoE4 and causes marked accumulation of apoE-poor lipoprotein remnants in plasma and severe atherosclerosis. The alternative mechanism of apoE-trapping by LDLR explains our observations and offers a plausible explanation why apoE4, which has a greater affinity for the LDLR than apoE3, is associated with higher plasma cholesterol and a greater risk of atherosclerosis in humans. Our unexpected findings in mice predict important interactions between *APOE* genotype, *LDLR* expression, and diet.

Acknowledgments

This work was supported by grants from NIH (HL42360 to N.M., HL54176 and HL49373 to J.S.P, and HL07115 to L.L.F). The authors thank Dr Tom Smith for the jugular vein injection during the lipoprotein clearance experiments, Drs Greg Shelness, Leighton James, and Oliver Smithies for discussion, and Jennifer Wilder and Ellen Young for technical assistance.

Chapter III

ApoE Trapping by the Low Density Lipoprotein Receptor

Michael K. Altenburg, Sudi I. Malloy, Christopher Knouff, John S. Parks, and Nobuyo Maeda

Abstract

Low-density lipoprotein receptor (LDLR) is critical for plasma lipoprotein clearance and a decreased LDLR expression has been thought to explain the increased plasma cholesterol and atherosclerosis risk in humans with apolipoprotein (apo) E4. However, in mice expressing human apoE4 instead of their mouse counterpart, increased LDLR expression caused accumulation of cholesterol-rich apoE-poor chylomicron remnants, and resulted in significant atherosclerosis. The production rates of chylomicrons and very low density lipoproteins (VLDL) were unchanged, as was the clearance rate of exogenously given apoE deficient remnants. These data suggest an enhanced conversion of nascent lipoprotein particles to cholesterol-rich remnants that are poorly cleared. We hypothesize that the negative effects from elevated LDLR are due to the high binding affinity of apoE4 for the receptor which reduces the availability of apoE4 for transfer to triglyceride rich lipoproteins (TRL), thereby inhibiting their rapid internalization.

Introduction

The low density lipoprotein receptor LDLR plays a pivotal role in the removal of atherogenic lipoproteins from the circulation by removing lipoproteins containing the LDLR ligands apoB100 and apoE [1]. In humans, apoE is polymorphic with three isoforms, apoE2, apoE3, and apoE4. These isoforms differ at positions 112 and 158, with apoE2 having two Cysteines, apoE3 cysteine at 112 and arginine at 158, and apoE4 two arginines. ApoE2 has a reduced LDLR affinity and apoE4 an increased affinity compared to apoE3 [13-15]. Paradoxically, apoE4 is associated with an elevated plasma cholesterol level and atherosclerosis, while apoE2 a reduction in cholesterol and risk in humans [2, 3]. The increased risk associated with apoE4 is thought to be due to elevated intracellular cholesterol from increased apoE4-mediated lipid delivery, leading to down-regulation of the LDLR [3, 18, 19]. Here we describe the over-expression of the LDLR in mice with apoE4 and found increased cholesterol and atherosclerosis.

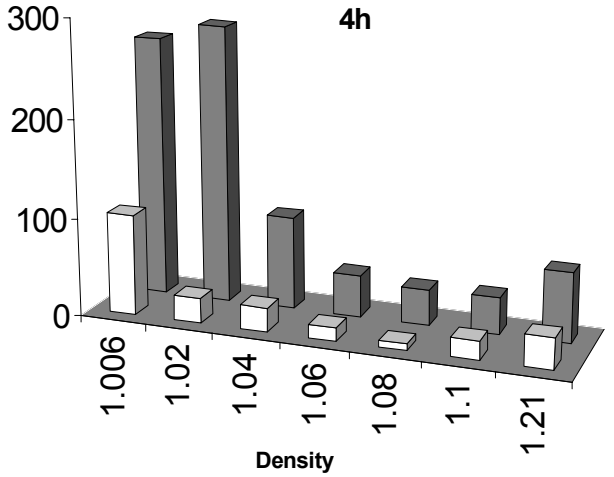
Material and Methods

Mice homozygous for the replacement of APOE*4 allele (*Apoe*$^{4/4}$*Ldlr*$^{h/+}$) were bred to mice heterozygous for targeted replacement of the mouse *Ldlr* gene with the human *Ldlr* minigene (*Apoe*$^{+/+}$*Ldlr*$^{h/+}$)Experimental animals were made by crossing Apoe *Apoe*$^{4/4}$*Ldlr*$^{+/+}$ (4m) to *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ (4h). Twelve to 36 week old mice of both sexes were used for experiments that were conducted under protocols approved by the Institutional Animal Care and Use Committees. Littermates were used when possible. Mice were maintained on either a normal chow (NC) 4.5%(w/w) fat and 0.022% (w/w) cholesterol (Prolab RMH 3000, Agway Inc., Syracuse, NY) or a high fat Western diet (HFW) containing 21% fat and 0.2% cholesterol (TD88137; Teklad, Madison WI).

Results

To examine the effects of LDLR expression level on mice with apoE4, mice homozygous for the replacement of APOE*4 allele (4m) were bred to mice heterozygous for targeted replacement of the mouse *Ldlr* gene with the human *Ldlr* minigene (4h) that results in a stabilized *Ldlr* mRNA [4-7]. Total mRNA for LDLR in 4h mice was 2.3 times that of 4m mice. On normal chow (NC), plasma cholesterol in 4h mice was about one third that of 4m mice, resulting from reduced HDL cholesterol. When fed a high fat Western diet (HFW), 4h mice became markedly hypercholesterolemic with accumulation of apoE-poor, but cholesterol enriched, particles in their plasma predominantly in their chylomicron/very low density lipoproteins (VLDL) remnant ($1.006<d\leq1.02$) fractions [7]. Plasma lipoprotein fractions from 4m mice fed HFW diet did not change before and after fasting (Figure 3.1). In contrast, fasting 18 h almost completely eliminated these 4h remnants, suggesting that these particles are derived primarily from the diet. Interestingly similar prolonged postprandial lipemia has also been observed in humans with apoE4[68].

Figure 3.1. Ultracentrifugation Cholesterol distribution in apoE4 mice with elevated LDLR. (mg/dl) in UC fractions before (shaded) and after an 18 hour fast (white). Plasma was pooled from HFW fed 4m (n=5) and 4h (n=5) and separated by ultracentrifugation into density fractions.


4h
300
200
100
0
1.006
1.02
1.04
1.06
1.08
1.1
1.21
Density

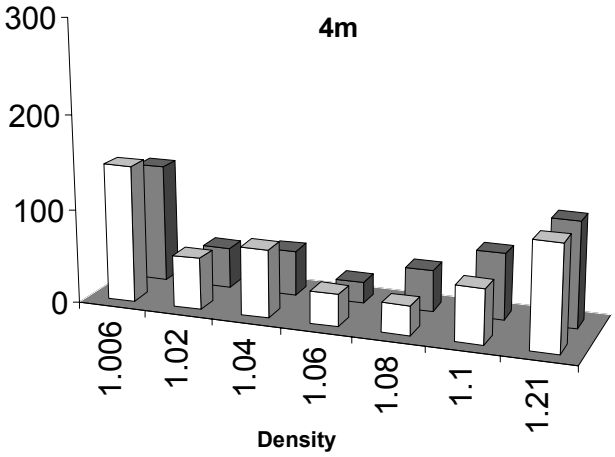
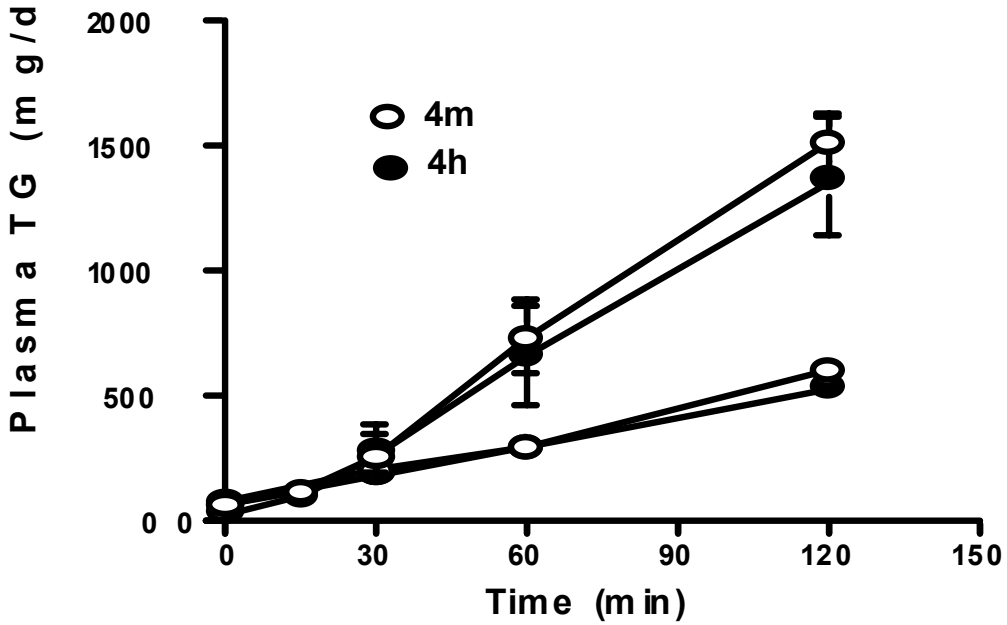
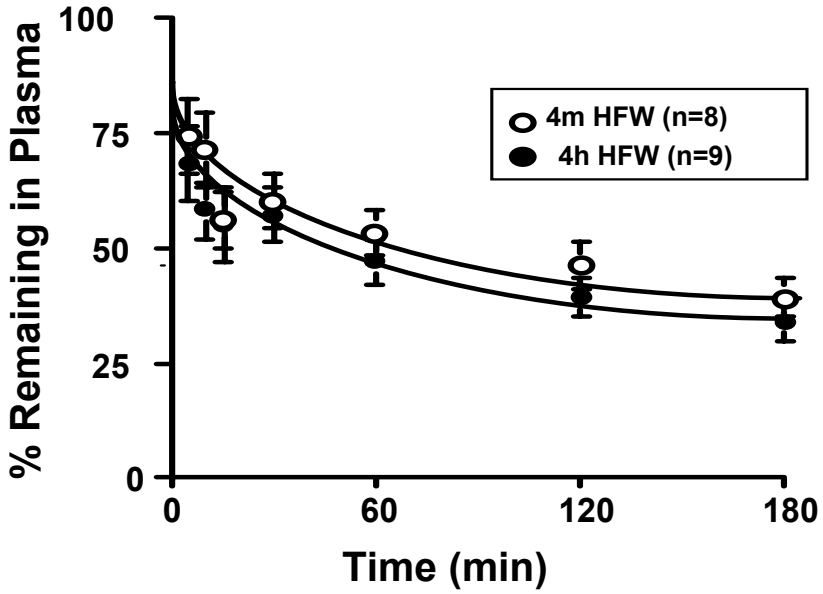

4m
300
200
100
0
1.006
1.02
1.04
1.06
1.08
1.1
1.21
Density

Figure 3.2. Triglyceride secretion of 4m (open symbol) and 4h (closed symbol) mice after inhibition of lipolysis and uptake with Triton WR1339. Mice (*n*=4) after olive oil gavage reflects VLDL and chylomicron secretion (circles). VLDL secretion in mice (*n*=4) fasted 4h (squares). The difference between the top and bottom reflects chylomicron production


Plasma TG (mg/dl
2000
1500
1000
500
0 0
4m
4h
0
30
60
90
120
150
Time (min)

Triglyceride secretion was indistinguishable in 4m and 4h mice, suggesting that the VLDL production from the liver is the same. When mice were gavaged with a 200ul olive oil bolus immediately after Triton WR 1339 injection, the rate of TG accumulation after gavage was also the same in both 4m and 4h mice (Figure 3.2). These results indicate that the chylomicron secretion there was no significant difference in clearance of remnant particles between 4m and 4h mice (Figure 3.3). Therefore, the elevated cholesterol associated with increased LDLR in the 4h mice is not due to delayed clearance of cholesterol-rich apoE-poor remnants. The inference from these data is that the conversion of nascent triglyceride-rich lipoproteins (TRL) to apoE-poor remnants is increased in 4h mice.

Figure 3.3. Clearance of apoE-/- VLDL in 4m and 4h mice. Radioactivity remaining in the plasma after injection of I^{125} labeled VLDL into HFW fed 4m and 4h mice. There is no significant difference in clearance between 4m and 4h mice.

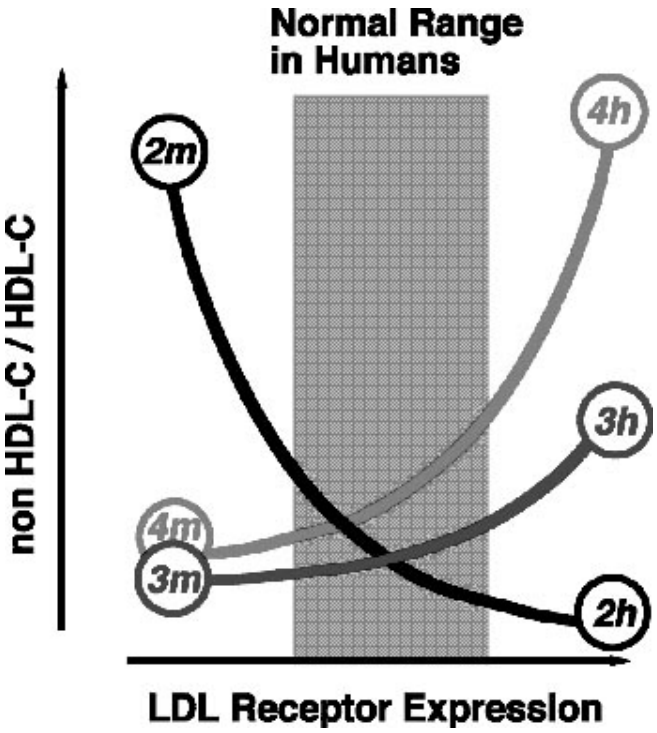

% Remaining in Plasma
100
75
50
25
0
0
60
120
180
Time (min)
4m HFW (n=8)
4h HFW (n=9)

Discussion

ApoE is normally associated with various proteins and proteoglycans on the surface of capillaries and the hepatic space of Disse where it can be transferred to circulating lipoproteins. We propose that the increased LDLR can "trap" apoE protein and limit its transfer to circulating nascent lipoproteins, which in turn reduces the apoE-mediated removal of larger particles and forces their remodeling to slowly cleared remnants. The clearance of these cholesterol-rich apoE poor remnants is equally slow in both 4m and 4h and is seemingly LDLR-independent. The higher affinity of apoE4 for the receptor may make it more susceptible to trapping than apoE3 and apoE2.

Thus mice expressing human apoE isoforms have revealed important interactions between apoE genotype, LDLR expression, and diet. Contrary to the currently accepted hypothesis that increased atherosclerosis risk in humans with apoE4 is due to down-regulation of LDLR, we found that increased LDLR has adverse effects on mice expressing apoE4. Our proposed mechanism of apoE4 trapping by LDLR is likely to be contributing to the paradoxical association between apoE4 and increased atherosclerosis risk. We expect that humans vary in their basal expression of LDLR, and its levels influence the atherosclerosis risk differently depending on the apoE genotype (Figure 3.4). Control of diet would then be more important in individuals with apoE4 than those with apoE2 or apoE3 when the LDLR level is relatively high.

Figure 3.4. Interaction between apoE isoforms and LDLR level. On the left-hand side are mice expressing the different apoE isoforms with a relatively low wild-type LDLR level. On the right-hand side are the same mice with elevated LDLR level. We suggest that the human LDLR level mean is centered between the extremes in our mice


Normal Range
in Humans
non HDL-C / HDL-C
LDL Receptor Expression
2m
4h
3h
4m
3m
2h

Chapter IV

Apolipoprotein E4 in Macrophages Enhances Atherogenesis in a Low Density Lipoprotein Receptor-dependent Manner

Michael Altenburg, Lance Johnson, Jennifer Wilder, and Nobuyo Maeda

Abstract

Apolipoprotein E (apoE) and the low density lipoprotein receptor (LDLR) are well recognized determinants of atherosclerosis. In addition to hepatocytes, where both are highly expressed and contribute to plasma lipoprotein clearance, they are expressed in vascular cells and macrophages. In this study, we examined the effects of human apoE isoforms and LDLR levels in atherogenic pathways in primary macrophages *ex vivo* and atherosclerosis development after bone marrow transfer *in vivo* using mice expressing human apoE isoforms and different levels of LDLR expression. Increases in LDLR expression significantly increased cholesterol delivery into macrophages in culture, and the effects were more prominent with lipoproteins containing apoE4 than those containing apoE3. Conversely, increased LDLR expression reduced cholesterol efflux in macrophages expressing apoE4 but not in macrophages expressing apoE3. Furthermore, apoE3 protected VLDL from oxidation *in vitro* more than did apoE4. In LDLR-deficient mice expressing the human apoE4 isoform, $Apoe^{4/4}$ $Ldlr^{-/-}$, the replacement of bone marrow cells with those expressing LDLR increased atherosclerotic lesions in a dose-dependent manner compared with mice transplanted with cells having no LDLR. In contrast, atherosclerosis in $Apoe^{3/3}$ $Ldlr^{-/-}$ mice, expressing the human apoE3 isoform, did not differ by the levels of macrophage LDLR expression. Our results demonstrate that apoE4, but not apoE3, in macrophages enhances atherosclerotic plaque development in mice in an LDLR-dependent manner and suggests that this interaction may contribute to the association of apoE4 with an increased cardiovascular risk in humans.

Introduction

Elevated levels of cholesterol in plasma, carried by remnant particles of very low density lipoproteins (VLDL) and low-density lipoproteins (LDL), are well known risk factors for atherosclerosis. The bulk of these atherogenic lipoproteins are cleared from the circulation by the LDL receptor (LDLR) in the liver with apolipoprotein E (apoE) and apoB100 as ligands. There are three common apoE isoforms in humans: apoE2, apoE3, and apoE4, each having distinct influences on both lipid metabolism and atherogenesis in humans [6]. Possession of at least one copy of the *APOE*2* allele is associated with higher plasma apoE and triglyceride but lower LDL cholesterol levels and atherosclerosis risk when compared to *APOE*3* homozygotes [6]. In contrast, the presence of at least one *APOE*4* allele is associated with lower plasma apoE, increased LDL cholesterol, and a greater risk of coronary artery disease than *APOE*3* homozygotes. This association is thought to be mainly due to differences in lipoprotein clearance and is counterintuitive considering the LDLR affinity of apoE2 is lower while the affinity of apoE4 is slightly higher than apoE3 [13, 14].

To gain insights into the mechanisms underlying the relationship between atherosclerosis risk and apoE isoform in humans, we previously made mice expressing human apoE2, apoE3, or apoE4 in place of the endogenous mouse apoE [14, 33, 34]. Notably, the atherosclerosis risk associated with the resulting mice was different from that in humans; mice with apoE2 had increased plasma lipids and develop atherosclerosis while mice with apoE3 and apoE4 were normolipidemic and resistant to atherosclerosis. To further test whether the apoE isoform-dependent atherosclerosis risk is affected by the LDLR expression, we also developed mice in which the endogenous mouse *Ldlr* gene was replaced with a gene (*hLdlr*) coding for human LDLR. The transcriptional regulation of the *hLdlr* gene was normal but the steady state levels of

its mRNA in the liver were elevated because the transcripts carry a more stable 3' untranslated region (UTR) sequence than normal. Somewhat unexpectedly, we found that, when this *hLdlr* allele was introduced into mice expressing human apoE isoforms, physiologic overexpression of the LDLR was protective in mice with apoE2 but caused severe atherosclerosis in mice with apoE4, recapitulating the associations between apoE isoforms and atherosclerosis risk seen in humans [39, 69]. These data suggest that the LDLR apoE interaction is central to the increased atherosclerosis risk associated with apoE4.

A substantial portion of the atherosclerosis risk associated with apoE4 is likely due to its hepatic metabolism by the LDLR and the resultant changes to plasma lipids. However, there is ample in-vitro evidence that apoE's interaction with the LDLR in the liver may not be entirely responsible for the risk of coronary artery disease associated with apoE4 in humans [50, 56, 70]. Both ApoE and the LDLR are expressed in many cell types and are thought to play roles in the atherosclerotic process beyond their role in lipoprotein clearance [71]. The effects on atherogenesis of apoE isoforms and levels of LDLR in extrahepatic tissues, for example in macrophages, are difficult to study *in vivo* because their combined effects on plasma lipids are confounding.

Herein, we address the effect of LDLR expression level and human apoE3 and E4 isoforms on lipoprotein uptake and efflux in macrophages isolated from mice having human apoE isoforms and different levels of LDLR expression. We also examined *in vivo* whether the interactions between apoE and LDLR in macrophages, independent of global LDLR expression, affect atherosclerosis after bone marrow transfer (BMT) in mice expressing human apoE3 or apoE4 and lacking LDLR (3ko or 4ko) [72]. We found that the expression of LDLR in macrophages directly correlated with the extent of atherosclerosis in mice with human apoE4. In

contrast, macrophage LDLR expression did not affect atherosclerosis in mice expressing apoE3. These results indicate that apoE4 exerts adverse effects on bone marrow derived cells in the vessel wall in an LDLR dependent manner and may contribute to its pathogenesis.

Material and Methods

Mice - All the mutant mouse strains used in this work were individually backcrossed at least 6 generations to C57BL/6 genetic background before intercrossing. Mice heterozygous for a targeted replacement of the mouse *Ldlr* gene with the stabilized human *Ldlr* minigene ($Apoe^{+/+}Ldlr^{h/+}$) [37] were bred to mice homozygous for replacement of the mouse apoE gene with either the human *APOE*3* or *APOE*4* allele ($Apoe^{3/3}Ldlr^{+/+}$ and $Apoe^{4/4}Ldlr^{+/+}$) [14, 33, 34]. The littermates generated by crossing $Apoe^{3/3}Ldlr^{+/+}$ (*3m*) with $Apoe^{3/3}Ldlr^{h/+}$ (*3h*) and $Apoe^{4/4}Ldlr^{+/+}$ (*4m*) with $Apoe^{4/4}Ldlr^{h/+}$ (*4h*) respectively, were used in experiments as donors of bone marrow cells. Mice with human *APOE*3* and lacking LDLR (*3ko*) and mice with human *APOE*4* and lacking LDLR (*4ko*) were generated by crossing either $Apoe^{3/3}$ or $Apoe^{4/4}$ mice with $Ldlr^{-/-}$ mice [39], and maintained as $Apoe^{3/3}$ $Ldlr^{-/-}$ (3ko) or $Apoe^{4/4}$ $Ldlr^{-/-}$ (4ko) respectively (see Table 3.1 for genotypes and nomenclature). Mice were fed either normal mouse chow (NC) containing 4.5% (wt/wt) fat and 0.022% (wt/wt) cholesterol (Prolab RMH 3000, Agway Inc) or a high-fat Western-type diet (HFW) containing 21% (wt/wt) fat and 0.2% (wt/wt) cholesterol (TD88137; Teklad). The animals were handled under protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina–Chapel Hill.

Table 4.1 Genotypes of mice

Mice	Genotype	Description
3m	*Apoe*$^{3/3}$, *Ldlr*$^{+/+}$	human apoE3
3h	*Apoe*$^{3/3}$, *Ldlr*$^{h/+}$	human apoE3 and increased LDLR
3ko	*Apoe*$^{3/3}$, *Ldlr*$^{-/-}$	human apoE3 and no LDLR
4m	*Apoe*$^{4/4}$, *Ldlr*$^{+/+}$	human apoE4
4h	*Apoe*$^{4/4}$, *Ldlr*$^{h/+}$	human apoE4 and increased LDLR
4ko	*Apoe*$^{4/4}$, *Ldlr*$^{-/-}$	human apoE4 and no LDLR

Peritoneal Macrophage Isolation - Macrophages (MPM) were obtained from the peritoneal cavity of mice 4 days after intra-peritoneal injection of 1 ml of 4% (w/v) thioglycolate (Becton Dickinson). The cells obtained were either directly used for gene expression analyses (below) or washed with Ham's Nutrient Mixture F10 media (F10), spun at 1000g for 5 minutes and plated in 12-well plates at a density of 6 x 10^5 cells/well in F10 media supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were washed 2 hours later to remove non-adherent cells. Cells were cultured in media without FBS for 24 hours prior to experiments.

Lipoprotein Uptake - LDL was isolated from human plasma as described [69]. Mouse VLDL fractions enriched with human apoE3 or apoE4 were isolated from pooled plasma of 3ko or 4ko mice respectively by ultracentrifugation at d<1.006. Lipoproteins were labeled with 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate (DiI C_{18}; Molecular Probes Inc., Eugene, Oregon, USA), as described by Stephan and Yurachek [73]. Macrophages in culture were washed and incubated in F10 medium without FBS for 24h, followed by incubation with medium containing 1µg/ml of DiI -labeled human LDL or mouse VLDL. After 2hrs, cells were washed with fresh medium and fluorescence was observed with an IX70 inverted microscope (Olympus) equipped with a filter set for Texas red (exciter 560/55, dichroic 595, emitter 645/75; Chroma Technology Corp). Fluorescence pixel intensity of each cell was recorded with a SPOT RT Slider digital camera, and analyzed with SPOT version 4.0.9 (Diagnostic Instruments) and ImageJ 1.33u (NIH) software.

Cholesterol Efflux- MPM were cultured 24 hrs in F10 medium without FBS. Cells were radio-labeled by incubating with 2 μCi/ml [^{3}H] cholesterol (Perkin Elmer) for 24hrs. They were then washed and further incubated for 24hrs with F10 media with or without 10 μg/ml of apolipoprotein A-I (apoA-I) protein (Sigma). Following incubation, medium was removed, spun at 12,500 g for 15 min, and assayed for radioactivity. Cells were washed with ice-cold PBS, lipids were extracted with isopropanol for 4 hrs, and assayed for radioactivity. Radiolabel in the medium and the cellular isopropanol extract was measured, and percentage effluxed was calculated as the ratio of radioactivity in the medium divided by the total (cells + media). To analyze efflux of cholesterol from cholesterol-loaded foam cells, MPM were incubated with acetylated human LDL (AcLDL) and 2 μCi/ml [^{3}H]-cholesterol for 24 hrs, and efflux was measured after 16 hrs. LDL (density1.019-1.063 g/ml) was acetylated by acetic anhydride [74].

Western blot - To measure ApoE protein content, MPM in culture were washed and cultured in lipid free F10 medium. After 24 hrs, apoE protein amount associated with cells and in the medium was a measured using Western blot with antibody against human apoE (Calbiochem). Protein loading was controlled by the amount of tubulin using anti-tubulin antibody (Sigma) and gel to gel control for densitometric analysis was done with a standard amount of apoE.

Oxidative Stress- VLDL (1 μg protein) from the 3ko or 4ko mice was incubated with 10μM $CuSO_4$ as described [49]. Oxidation and the increase in the formation of conjugated dienes were measured by recording their absorbance at 234 nm every 20 min for 3hrs. The lag time was determined graphically. Amounts of thiobarbituric acid-reactive substances (TBARS)

in plasma, VLDL, and cultured media were assayed as described [49]. MPM were cultured with VLDL isolated from apoE deficient mice (50 μg per ml F10 medium) for 24hrs. Oxidized VLDL was prepared by incubating VLDL in 10μM $CuSO_4$ for 24 hrs.

Gene Expression - RNA was extracted from MPM using RNAeasy kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol, either directly from peritoneal cells of mice that underwent BMT or cultured MPM from mice fed HFW diet. Cultured macrophage cells were incubated with F10 media or F10 media + 50 μg/ml apoE-deficient VLDL for 16hrs. Real-time RT-PCR amplifications (8) were performed in a 96-well plate in the ABI Prism 7700 sequence detector (PE Biosystems) in a total volume of 30 μl, which included 10 μl of RNA sample from the ABI Prism 6700 plus 20 μl of a reaction mixture. Each RT-PCR amplification was performed in duplicate: 30 min at 48°C for the RT reaction, then 10 min at 94°C, followed by a total of 40 temperature cycles (15 sec at 94°C and 1 min at 60°C). During the amplification, the fluorescence of FAM (or TET), TAMRA, and ROX (a passive reference dye) was measured by the 7700 sequence detector in each well of the 96-well plate. To determine total *Ldlr* mRNA levels, a primer probe system specific for murine exon 1, which is also present in mice targeted for the *hLdlr*, was used [37].

Bone Marrow Transfer - Bone marrow cells were collected from the femurs and tibias of donor male mice by flushing with F10 media. Recipient female mice, 6- 8 weeks of age, were lethally irradiated (9.5 Gy), and injected with $2x10^6$ bone marrow cells in 0.2 ml medium through tail veins. For 2 weeks after transplantation, mice were given drinking water acidified to pH 2.0 by HCl and containing 100 mg/L of neomycin. Mice were fed a HFW diet for 3 months.

Plasma Lipids, Lipoprotein Analysis - Plasma was isolated, and total cholesterol (free and esterified), and triglycerides were measured as described previously [39]. Lipoprotein distribution in pooled plasma samples (100 μL) was analyzed by fast protein liquid chromatography (FPLC) using a Superose 6 HR10/30 column (Pharmacia Biotech Inc., Piscataway, New Jersey, USA).

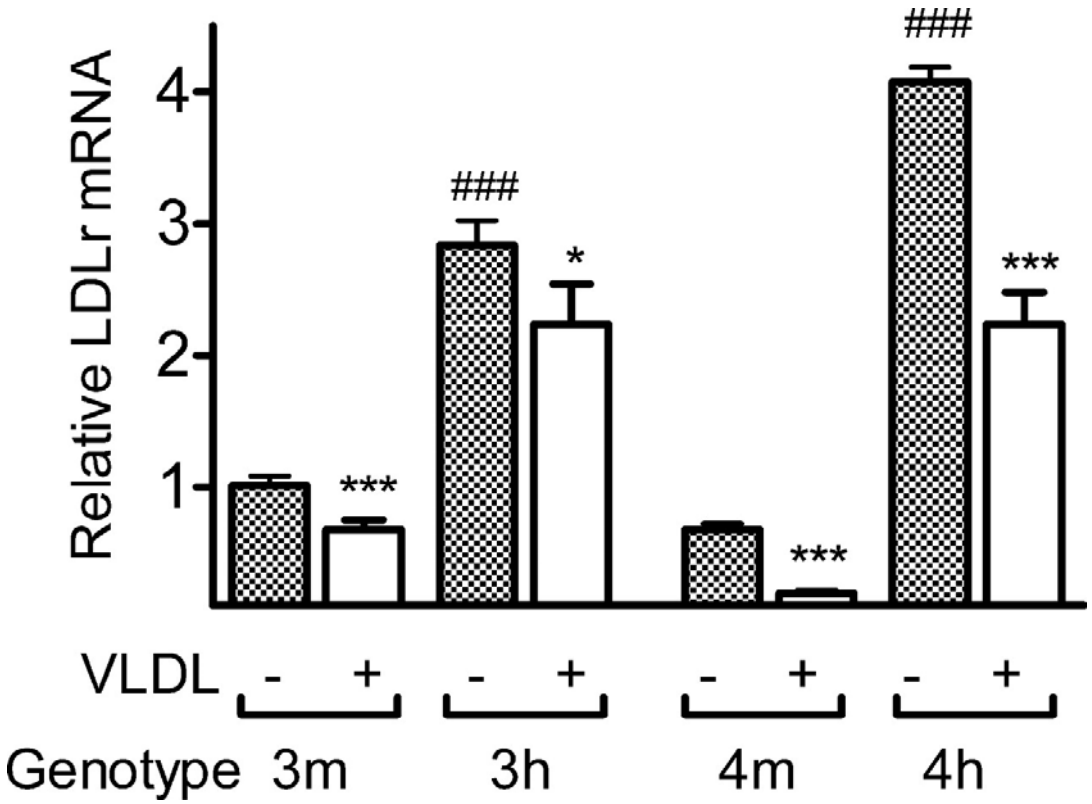
Evaluation of Atherosclerotic Lesions - BM recipient mice were fed a high fat western diet (HFW) for 12 weeks before sacrifice. Mice were perfused through the apex of the left ventricle with 4% paraformaldehyde under physiological pressures. Segments of the aortic sinus were embedded, and sectioned, and the size of atherosclerotic plaques in aortic roots were scored as described previously [75].

Statistical Analysis - The statistical significance of genotype effects were analyzed by using ANOVA (JMP software; SAS Inc., Cary, North Carolina, USA). Tukey-Kramer HSD was used for post-hoc pair-wise comparisons.

Results

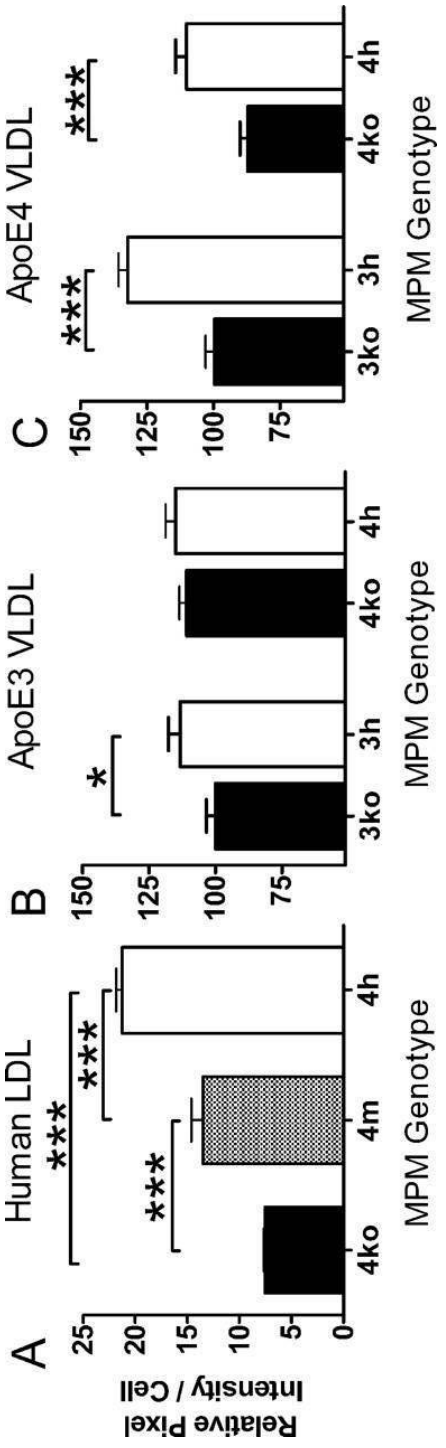
Downregulation of the LDLR Expression by Lipid in Macrophages - Our previous work [37, 39] showed that the 3'UTR modification within the *hLdlr* -allele made its transcripts in the liver more stable than the normal mouse allele, but the *hLdlr* -allele retained the transcriptional repression in response to dietary cholesterol intake. To test whether the expression of the *hLdlr* -allele is also normally regulated in macrophages by exogenous lipid, we isolated MPM from 3m, 3h, 4m and 4h mice (see Table I) and placed them in culture. Both 3h and 4h MPM cells cultured for 16 hrs without VLDL had higher LDLR expression than 3m and 4m MPM cells by approximately 3-4 fold (Fig 4.1, $p \leq 0.0001$). The levels of *Ldlr* mRNA decreased in all MPM cells when they were incubated with apoE-deficient VLDL for 16 hrs, and the decrease was proportional whether cells had wildtype or elevated LDLR. However, cells with apoE4 had a greater proportional decreases in LDLR expression compared to cells with apoE3. Thus the *hLdlr* gene in macrophages retains its normal feedback response to increased intracellular lipid and can be downregulated by high levels of lipid, but its transcripts remain 2-3 fold higher than the wildtype *Ldlr* mRNA.

Figure. 4.1. Expression of the *Ldlr* gene in the primary culture of mouse peritoneal macrophages. MPM from 3m, 3h, 4m, and 4ko mice were cultured in lipid free F10 medium with or without 50 μg/ml of VLDL isolated from apoE-deficient mice for 16 hours. Total mRNA amount for the Ldlr gene was measured by RT-PCR, using the exon 1 sequence that is common between wild type mouse allele and *hLdlr* allele, normalized with the beta actin expression and shown as expression levels of 3m equal to 100%. White bars indicate expression in cells cultured with VLDL and black bars indicate without VLDL. *$p \leq 0.05$, ***$p \leq 0.0005$ for effect of VLDL , and ### $p \leq 0.0005$ for the effect of LDLR with Tukey-Kramer HSD.


Relative LDLr mRNA
4
3
2
1
###
*
###
***
***
***
VLDL - + - + - + - +
Genotype 3m 3h 4m 4h

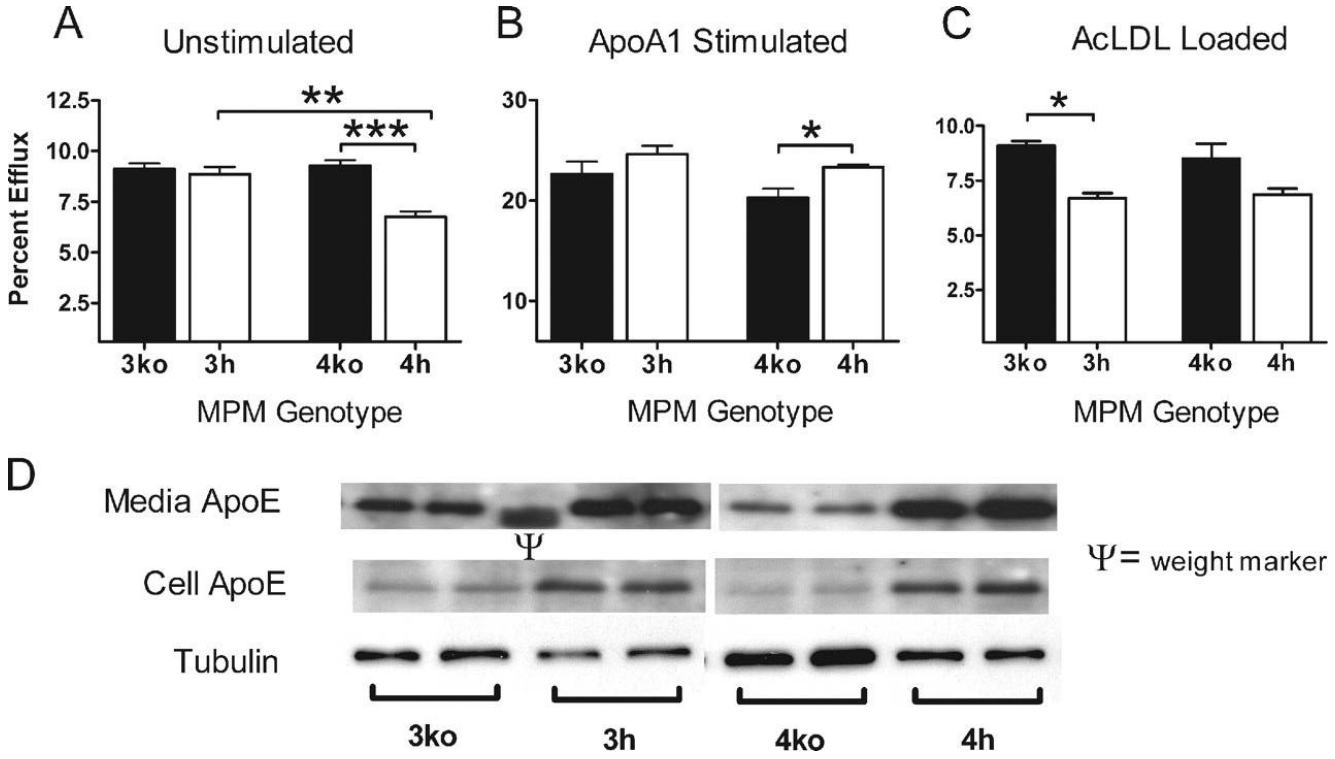
LDLR Expression and Lipoprotein Uptake in Macrophages – To test whether increased expression of the LDLR in macrophages increases uptake of lipoproteins we isolated MPM from 4ko, 4m, and 4h mice and incubated them with DiI-labeled human LDL in culture. After two hours, intracellular fluorescent intensity in 4h macrophages was twice that of 4m macrophages and three times higher than 4ko cells (Figure 4.2A), demonstrating that the LDL uptake by macrophages directly correlates with LDLR expression. In hyperlipidemic mice, as well as humans, lipoprotein particles accumulate not only in LDL fractions but also in VLDL fractions. For example, in the plasma of LDLR -/- mice with human apoE, VLDL particles are enriched with cholesterol and triglyceride, as well as apoE protein [69]. We therefore incubated the MPM with VLDL fractions enriched in apoE3 or apoE4 from 3ko and 4ko mice. Uptake of the mouse apoE3 enriched VLDL particles by 3h MPM was modestly but significantly increased compared to that by 3ko MPM (9%, p=0.02, Figure 4.2B). Uptake of apoE3 enriched VLDL by the 4h MPM was not different from the uptake by the 4m MPM. In marked contrast, both 3h and 4h MPM incorporated significantly more apoE4 enriched VLDL than 3ko and 4ko MPM (33%, $p \leq 0.0001$, and 26%, $p \leq 0.0001$, respectively) (Figure 4.2C). This suggests that the apoE isoform present on the VLDL particle and the LDLR in the macrophages, but not macrophage apoE, influences uptake, and that apoE4 in the particles promotes VLDL uptake more than apoE3. Taken together, the data suggest that increased LDLR in macrophages by mRNA stabilization causes increased uptake of LDL particles as well as VLDL particles, and the VLDL uptake would be more pronounced when the particles contain apoE4 rather than apoE3.

Figure 4.2. Lipoprotein uptake. (A) MPM from 4ko, 4m, and 4h mice were cultured and incubated for 2hr with 1μg/ml DiI labeled human LDL. Cells were washed three times with PBS and fluorescent intensity was measured using NIH Image J. (B) MPM from 3ko, 3h, 4ko and 4h mice were incubated for 2hrs with 1μg/ml each of DiI labeled 3ko VLDL, or (C) 4ko VLDL. Pixel intensities reflecting VLDL uptake were measured from four wells per experiment with cells pooled from at least 3 mice of each group, and averages of three experiments are shown as mean uptake of 3ko cells as 100%. Effect of VLDL $*p \leq 0.05$, $***p \leq 0.0005$ with Tukey-Kramer HSD.


A
Human LDL
Relative Pixel Intensity / Cell
25
20
15
10
5
0
***
***
***
4ko
4m
4h
MPM Genotype
B
ApoE3 VLDL
150
125
100
75
*
3ko
3h
4ko
4h
MPM Genotype
C
ApoE4 VLDL
150
125
100
75
***
***
3ko
3h
4ko
4h
MPM Genotype

Effects of ApoE4 and LDLR Expression on Cholesterol Efflux from Macrophages - To examine whether the different apoE isoforms facilitate cholesterol efflux from macrophages differently and whether the effect is dependent on the levels of LDLR expression in macrophages, we measured cholesterol efflux from MPM isolated from mice expressing human apoE isoforms with high levels of LDLR (3h and 4h) or without LDLR (3ko and 4ko). In unloaded cells expressing, cholesterol efflux was not influenced by the LDLR expression when cells were incubated without apoA-I. In contrast, the LDLR expression had a significant effect when cells expressed apoE4, and the efflux from the 4h cells was 30% less than from 4ko cells ($p \leq 0.0005$, Figure 4.3A). Addition of apoA-I to the culture medium increased cholesterol efflux from cells regardless of apoE isoforms (Figure 4.3B). ApoA-I-mediated efflux from the 4h cells with elevated expression of LDLR was significantly higher than 4ko cells not expressing LDLR. The difference was larger and significant between 4ko and 4h cells ($p<0.003$), than between 3ko and 3h cells (not significant). In contrast, efflux of cholesterol from cholesterol loaded foam cells after incubation with AcLDL depended on LDLR but not on apoE genotype (Figure 4.3C). These data demonstrate that the LDLR expression in macrophages influences the cholesterol efflux in an apoE isoform dependent fashion when macrophages are cultured in lipid free media, and suggest that both apoE isoform and LDLR expression may be important determinants for foam cell formation. However, when cells were loaded with AcLDL, apoE-isoform effects were no longer detectable.

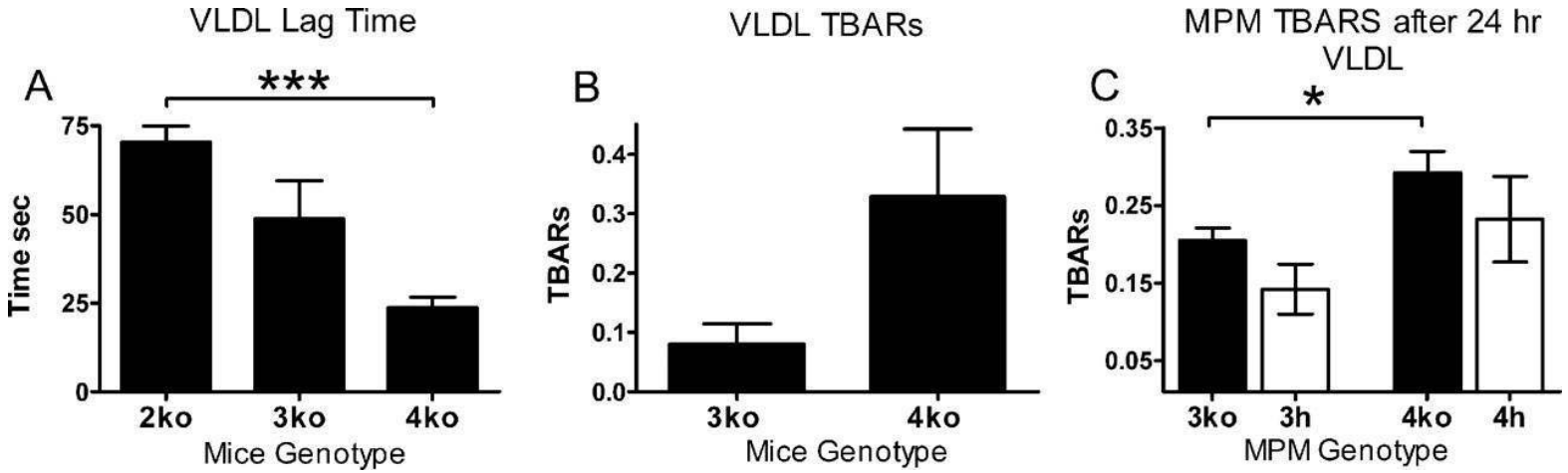
Figure 4.3. Cholesterol efflux from MPM. Thioglycolate-elicited MPM were isolated from mice and incubated with [^{3}H] cholesterol for 24hrs. Cells were then washed and incubated 24hrs in media without FBS (A) or with the same media containing 10 µg/ml of apoA-I for 24 hr (B). Radiolabel in medium and the cellular isopropanol extract were quantified, and percentage efflux was calculated as the ratio of radioactivity in the medium divided by total radioactivity (cells + media) x 100%. Data are mean ± SE for 3 mice of the indicated genotypes assayed in quadruplicate. (C) Cholesterol efflux from MPM loaded with 50 µg /ml AcLDL and [^{3}H] cholesterol for 24hrs. (D) ApoE that is cell-associated or secreted into culture medium from MPM incubated for 24 hrs in lipid free media. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$.


A
Unstimulated
Percent Efflux
12.5
10.0
7.5
5.0
2.5
**
***
3ko
3h
4ko
4h
MPM Genotype
B
ApoA1 Stimulated
30
20
10
*
3ko
3h
4ko
4h
MPM Genotype
C
AcLDL Loaded
10.0
7.5
5.0
2.5
*
3ko
3h
4ko
4h
MPM Genotype
D
Media ApoE
Cell ApoE
Tubulin
Ψ
Ψ= weight marker
3ko
3h
4ko
4h

ApoE secretion from cultured MPM- To address whether cholesterol efflux is related to the intracellular levels of apoE isoforms, we examined the apoE contents associated with cells and in medium 24hrs after culturing in lipid-free F10 medium by Western blot analysis. The intracellular apoE contents in both 3h and 4h cells were consistently higher than in 3ko and 4ko cells (Figure 4.3D). Similarly, apoE protein contents in culture media of 3ko and 4ko cells were lower than those of 3h and 4h cells. Relative amounts between apoE3 cells and apoE4 cells somewhat varied culture by culture, and were not consistently different. We conclude that LDLR expression in MPM significantly affects apoE uptake and secretion, and likely influences the cholesterol efflux.

VLDL Oxidation- We next tested whether lipoprotein oxidation in our mice is apoE isoform-dependent which may be due to the free cysteinyl groups on apoE3 and apoE2 [49, 50]. ApoE2 has two free cysteinyl groups and apoE3 has one, and both have been shown to function as a better antioxidant than apoE4 protein which has no free cysteinyl group. We isolated human apoE-enriched VLDL particles from 2ko, 3ko, and 4ko mice, and tracked conjugated diene formation during Cu^{2+} -mediated oxidation of these lipoprotein particles. The lag time for conjugated diene formation was 24 min in VLDL isolated from 4ko mice compared to 49 min in VLDL from 3ko mice and the longest, 70 min, in 2ko VLDL (Figure 4.4A). Measurement of thiobarbiturate reactive substances (TBARS) after overnight oxidation showed that apoE4-VLDL contained 3-fold more TBARS than did apoE3-VLDL (Figure 4.4B).

Figure 4.4. VLDL oxidation. (A) VLDL from 3ko and 4ko mice (1 μg) was incubated with 10μM Cu^{2+}. Ordinate shows lag time, average of three experiments, until inflection point on 234 nm absorption curves. (B) TBARS after Cu^{2+} oxidation of VLDL isolated from 3ko and 4ko mice. TBARS were measured after 16 hr incubation with 10μM Cu^{2+} at 0.5 mg protein/ml. (C) TBARS in the media of MPM incubated with VLDL in culture. Thioglycolate-elicited MPM isolated from either 4ko or 3ko mice was incubated with 50 μg/ ml of apoE-deficient VLDL. TBARS in media were measured after 24 hrs. $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$ with Tukey-Kramer HSD.


VLDL Lag Time
A
***
Time sec
75
50
25
0
2ko
3ko
4ko
Mice Genotype
VLDL TBARs
B
TBARs
0.4
0.3
0.2
0.1
0.0
3ko
4ko
Mice Genotype
MPM TBARS after 24 hr
VLDL
C
*
TBARs
0.35
0.25
0.15
0.05
3ko
3h
4ko
4h
MPM Genotype

To test whether expression of LDLR in macrophages influences VLDL oxidation in an apoE-dependent manner, we next cultured MPM in medium with apoE-deficient VLDL for 24 hrs, and measured TBARS in media as an indicator for oxidized lipids. The amounts of TBARS were borderline detectable, and were slightly higher in media cultured with apoE4 macrophages than with apoE3 and lower with elevated LDLR than with no LDLR, but neither apoE genotype nor LDLR had statistically significant effects by two-way ANOVA. These data suggest that apoE4 isoform does not protect lipoprotein particles from oxidation as well as apoE3 when a strong oxidative stress is applied *in vitro*. However, effects of apoE isoforms in macrophages on oxidation in culture are not significant, even under conditions of elevated triglycerides and cholesterol. In addition, we did not detect any statistical differences in the amount of TBARS or reduced glutathione (GSH) in the plasma of 3ko and 4ko recipient mice (data not shown).

BMT, and Plasma Lipids- The experiments above indicate that apoE4 proves to be impaired relative to apoE3 in several pathways important to lipid homeostasis in macrophages and perhaps atherosclerosis lesion formation, and the effects are influenced by the levels of LDLR expression in macrophages. To test whether the observed differences could alter overall atherogenesis *in vivo*, we carried out BMT in mice lacking LDLR but expressing human apoE3 (3ko) or apoE4 (4ko). To alter macrophage expression of LDLR, 3ko or 4ko females were lethally irradiated and transplanted with BM isolated from male mice with the same *ApoE* genotype, having no LDLR (3ko, 4ko), wild type LDLR (3m, 4m), or elevated expression of LDLR (3h, 4h). Three months after BMT, PCR of the *Ldlr* gene in the blood cells of the recipient animals showed only the products corresponding to the genotype of the donor cells, and no visible products corresponding to the recipient genotype (ko) were detected in the mice that

received (m) or (h) BM cells (Figure 4.5A). This confirmed that the replacement of the BM derived cells in the recipients by the donor cells was likely complete. The total *Ldlr* mRNA levels in the peritoneal cells isolated from representative recipient mice showed that the macrophages in the 3ko mice receiving 3h BM cells had 4 fold higher *Ldlr* gene expression than the macrophages in the 3ko mice receiving 3m BM cells. Similarly, total expression of the *Ldlr* gene in macrophages from the 4ko mice receiving 4h BM cells was 4 fold higher than in those receiving 4m cells (Figure 4.5B). Thus the levels of *Ldlr* mRNA in mice that received 3h or 4h bone marrow are elevated compared to those in mice that received wildtype LDLR to similar degrees as the MPM cells in culture as shown in Figure 4.1A.

Feeding HFW diet increased plasma cholesterol levels approximately 3-fold in both 3ko and 4ko mice regardless of donor *Ldlr* genotype (Table 4.2). Both 3ko and 4ko mice have elevated cholesterol in VLDL fractions as well as LDL fractions. HDL-cholesterol were very low in both 3ko and 4ko mice on HFW diet, and the plasma apoA-I levels were not different between them (not shown). The transfer of bone marrows with differing expression levels of LDLR did not cause any significant changes in plasma lipoprotein distribution in either the 3ko or 4ko mice by fast protein liquid chromatography analyses (Figure 4.5C). We conclude that the increased macrophage LDLR expression is not sufficient to alter plasma VLDL clearance in the LDLR -/- mice with apoE3 or apoE4. Consistent with previous work, the LDLR expression in BM-derived cells had no influence over plasma lipid profiles [57].

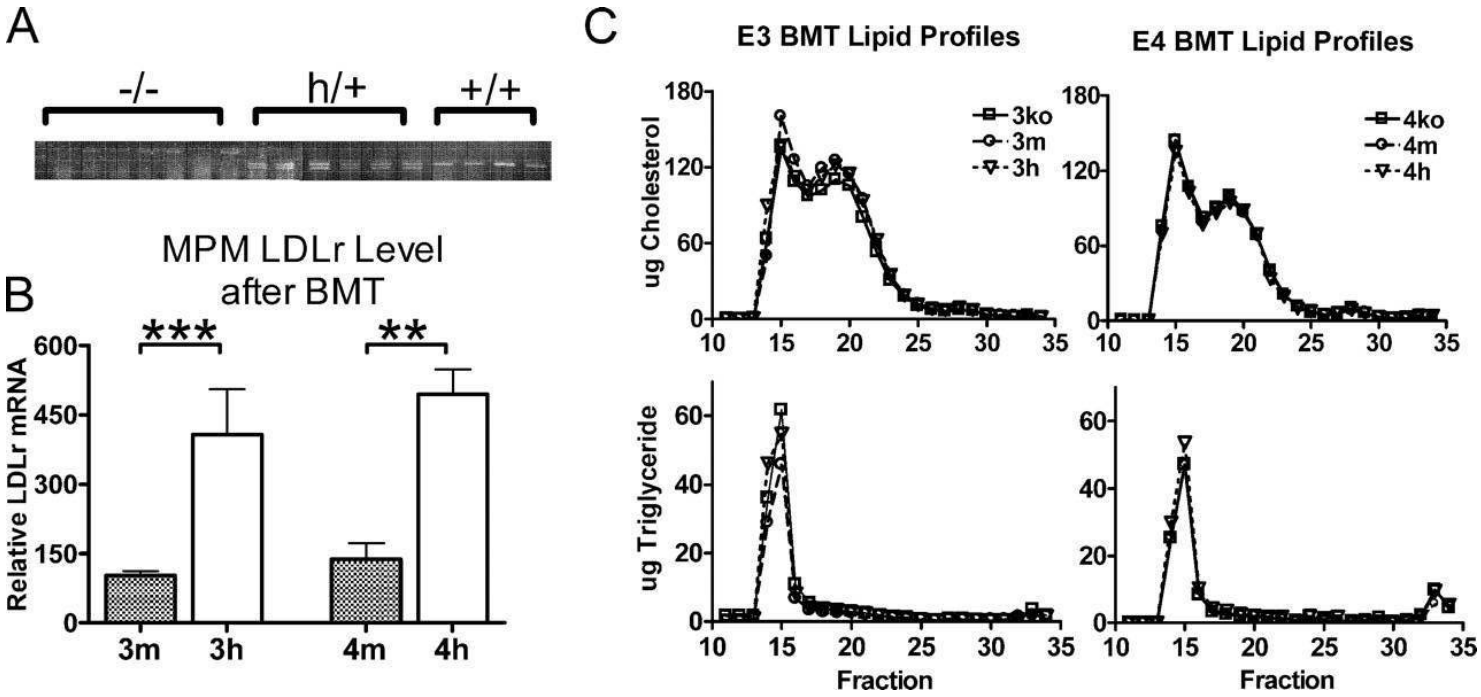
Table 4.2.Plasma lipids expressing human apoE3 or apoE4 after BMT and fed HFW diet

BM Recipient	3ko	3ko	3ko	4ko	4ko	4ko
BM Donor	3ko	3m	3h	4ko	4m	4h
Number	15	15	15	14	15	12
TC (mg/dl)	1,324±57	1,313±46	1,394±23	1,245±49	1,217±43	1,184±97
TG (mg/dl)	186±61	177±43	221±16	111±17	95±22	133±17

Table 4.3. Lipids and atherosclerosis in LDLr-/- mice expressing human apoE3 or apoE4 fed a NC diet

Mice	3ko	3ko	4ko	4ko
gender	Male	Female	Male	Female
Number	11	12	12	12
TC (mg/dl)	241±18	386±12	313±25	427±32
TG (mg/dl)	109±12	102±6	225±22	114±2
Lesion size ($10^3 \mu m^2$)	13.4±3.1	55.5±7.2	12.0±2.7	71.0±14.4
Occlusion (%)	1.3±0.3	5.1±0.5	1.0±0.2	5.4±0.8

Figure 4.5. Bone Marrow transfer. (A), Verification of bone marrow engraftment. DNA was isolated from buffy coat cells of recipient mice, and *Ldlr* genotype was determined by multiplex PCR. First seven lanes are recipients of ko cells showing a single PCR fragment. Next six lanes are recipients of hLdlr cells showing two bands; the upper band corresponds to the wild type mouse *Ldlr* allele and the lower band corresponds to the *hLdlr* allele. The last four lanes are recipients of m-Ldlr cells showing only the wild type allele. No bands corresponding to the ko allele was detected in the recipients of h or m cells. (B), Macrophage *Ldlr* mRNA level. Thioglycolate-elicited peritoneal cells were collected from bone marrow transfer recipients. Real-time quantitative PCR was used for *Ldlr* mRNA levels using exon 1 sequence that is common to *hLdlr* and wild type alleles. Averages from mice are shown relative to the expression of 3m-donor as 100. $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$. (C), Lipoprotein distribution in plasma isolated from the 3ko or 4ko recipient mice that received BM cells with different *Ldlr* expression levels. Plasma (100 μl) pooled from at least five mice of each group was size-fractionated on an FPLC Superose 6 column, and the cholesterol and triglyceride contents of each fraction were measured with an enzymatic assay.

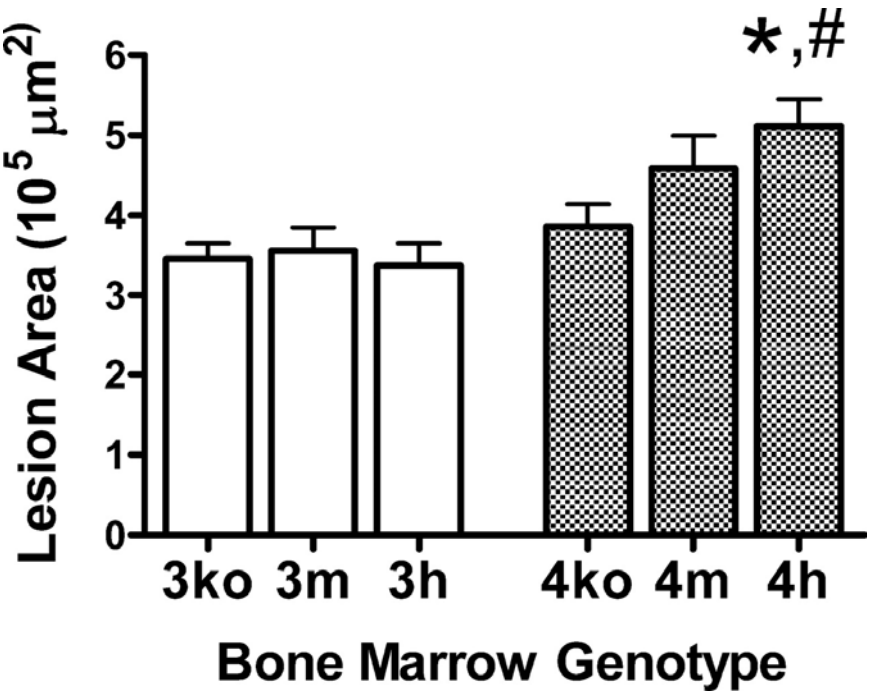

A
-/-
h/+
+/+
B
MPM LDLr Level
after BMT
***
**
Relative LDLr mRNA
600
450
300
150
0
3m
3h
4m
4h
C
E3 BMT Lipid Profiles
E4 BMT Lipid Profiles
ug Cholesterol
180
120
60
0
3ko
3m
3h
4ko
4m
4h
10
15
20
25
30
35
ug Triglyceride
60
40
20
0
Fraction

Macrophage LDLR Expression and Atherosclerosis in Mice with ApoE4 – Both 3ko and 4ko mice develop lesions in the aortic sinus as summarized in Table 4.3. Feeding the recipient mice with HFW diet for 12 weeks increased atherosclerotic plaque size in both 3ko and 4ko mice which received either control 3ko or 4ko BM. There were no significant apoE genotype effects on the lesion area (346 ± 19x10^3 μm^2, n=12, in 3ko vs. 386 ± 28x10^3 μm^2, n=14, in 4ko, p=0.3; Figure 4.6).

We also found no change in the lesion area of 3ko mice as macrophage LDLR level increased (Figure. 6). Thus 3ko mice that received 3m and 3h BM had a mean area of 356 ± 28x10^3μm^2 (n=13), and 338 ± 28x10^3 μm^2 (n=13) respectively p=0.9. In contrast, 4ko mice developed larger lesions as their macrophages expressed increasing amounts of LDLR. Thus 4ko mice that received 4m BM and 4h BM had mean lesion areas of 459 ± 41x10^3 μm^2 (n=15) and 511 ± 34x10^3 μm^2 (n=13), respectively. The difference between the lesion sizes in mice that received 4h donor cells and 4ko donor cells was significant ($p \leq 0.05$). When all six groups were analyzed together, the difference in atherosclerosis between 3ko mice with 3h BM and 4ko mice with 4h BM was also significant ($p \leq 0.01$).

Taken together, our data demonstrate that there is an interaction between the apoE genotype and LDLR expression of macrophages in determining atherosclerosis development. In the LDLR-/- mice, increased expression of LDLR in BM derived cells has no effect when animals are expressing apoE3, while it is detrimental in mice expressing apoE4.

Figure 4.6. Atherosclerotic lesions**.** Mean aortic sinus atherosclerotic lesion areas of 3ko mice that received 3m, 3h, and 3ko BM cells and of 4ko mice that received 4m, 4h, and 4ko BM cells are shown. *$p \leq 0.05$ (4ko vs 4h) , # $p \leq 0.01$ (3h vs 4h) with Tukey-Kramer HSD.


*,#
Lesion Area (10^5 µm^2)
0
1
2
3
4
5
6
3ko
3m
3h
4ko
4m
4h
Bone Marrow Genotype

Gene Expression in the Peritoneal Macrophages of Mice after BMT- To gain insight into the mechanism of the interaction between macrophage LDLR expression and apoE expression in atherosclerosis development, we isolated MPM from mice receiving BM transfer 4 days after stimulation with thioglycollate. Quantitative RT-PCR of peritoneal cells showed that the expression of genes coding for monocyte chemotactic protein-1 (MCP1), CD36, LDLR, apoE, and scavenger receptor A (SR-A) were not significantly different among recipients who received the donor cells with different LDLR levels (Table 4.4). Thus the effect of apoE4 isoform and LDLR level in atherogenesis does not appear to involve pathways affecting expression of MCP1, CD36, or SR-A in macrophages. In contrast, ABCA1 expression was increased in MPM that expressed elevated LDLR compared to ko MPM regardless of apoE genotype, although the increases was statistically significant only in 3h cells compared to 3ko cells. The up-regulation of ABCA1 in 3h and 4h macrophages may indicate higher intracellular cholesterol content in these cells relative to 3ko and 4ko cells [76, 77]. This is also consistent with an increased cholesterol efflux from 3h and 4h macrophages compared to 3ko and 4ko macrophages when exogenous apoA-I acceptor protein is given to the culture (Figure 4.3B).

Table 4.4. Gene expression of peritoneal macrophage isolated from BM recipients

Recipient	3ko	3ko	3ko	4ko	4ko	4ko
macrophage	3ko	3m	3h	4ko	4m	4h
Number	6	7	8	6	2	8
ABCA1	100±9	126±22	210±32*	111±14	76±4	156±20
MCP1	102±8	74±17	97±11	79±22	107±22	62±33
CD36	100±23	102±27	118±25	102±12	70±6	111±12
SR-A	104±13	64±10	72±23	67±14	78±7	52±10
ApoE	101±7	125±27	116±21	119±30	108±44	156±28

Table 4.4. Relative mRNA levels of the genes were calculated by the ratio of expression to the levels of β-actin mRNA. Values are mean ±SE and relative to 3ko which was adjusted to 100. ABCA1 levels 3h cells were significantly different from 3ko. *$p \leq 0.05$

Discussion

ApoE, as a ligand for the removal of lipoproteins, is generally considered atheroprotective; however, the extent to which depends on its isoform, concentration, and location [3, 6, 16, 78]. In this study, we tested the interaction of apoE isoforms with LDLR on several atherosclerosis pathways in macrophages. We then used BMT in mice to investigate whether their interaction in macrophages affect atherosclerosis development. Our results showed that expression of macrophage LDLR in the presence of apoE4 enhanced atherosclerosis. In contrast, these same LDLR increases in mice with apoE3 did not affect atherosclerosis. Thus, our results demonstrate, for the first time in animal models, that some of the risk associated with human apoE4 isoform may be due to its interaction with the LDLR in macrophages as well as with hepatic LDLR.

We found that apoE isoforms expressed by the macrophages had only a modest, nonsignificant effect on apoA-I-stimulated cholesterol efflux from MPM, but that the levels of LDLR expressed by the macrophages significantly increased apoA-I-assisted cholesterol efflux from apoE4 macrophages and less so from apoE3 macrophages. Cholesterol efflux, when apoA-I was not present, was generally low but affected by LDLR expression only in apoE4 macrophages and in an inverse direction. Thus, high LDLR expression did not alter efflux from macrophages with apoE3, but significantly inhibited efflux from macrophages with apoE4. A likely explanation for this is that apoE4 is more efficient than apoE3 for capturing and re-uptake of cholesterol containing particles by macrophages. Alternatively, the possible increased amount of intracellular apoE4 compared to apoE3 may retard cholesterol secretion with the endogenous apoE thus yielding the apparent difference in efflux. In contrast, in cholesterol-loaded foam cells, high LDLR expression inhibited efflux in both apoE3 and apoE4 macrophages. The interaction between LDLR and apoE4 resulted in significant effects only in un-loaded cells in which LDLR expression is high. The difference in efflux between apoE3 and E4 is small in loaded foam cells, in which expression of LDLR is down regulated. In the present work, we observed that cultured MPM expressing LDLR have more cell-associated apoE than MPM without LDLR regardless of apoE genotype.

Nevertheless, the difference between apoE3 and E4 in intracellular trafficking and accumulation has been established [79] and may be contributing to the difference in cholesterol efflux. Supporting this possibility, we reported earlier, in collaboration with Dr. Mazzone and his colleagues at the University of Illinois, that MPM with increased LDLR expression showed reduced apoE secretion compared to MPM with basal LDLR expression and that the relative reduction was greater with apoE4 than with apoE3. Further studies are necessary to elucidate the possible relationships between cholesterol efflux and intracellular localization of apoE3 and apoE4 in MPM. [80].

Increased cholesterol uptake increases ABCA1 expression and efflux to acceptor proteins [76, 77]. Thus we observed that macrophages isolated from Ldlr-/- mice receiving BM cells with high Ldlr gene expression showed elevated ABCA1 expression. This increase in ABCA1 expression likely accounts for the increased apoA-I-mediated cholesterol efflux in the presence of LDLR in macrophages. In addition, the presence of a large amount of apoA-I could interfere with the apoE-mediated re-uptake of lipid particles. Our observation of isoform-specific effects of apoE on cholesterol efflux is consistent with previous reports by other investigators. For example, Cullen *et al.* observed using macrophages isolated from human subjects with different apoE isoforms, that apoE4 macrophages effluxed less cholesterol than apoE2 or apoE3 and suggested that a combination of enhanced uptake and degradation of apoE4 by macrophages leads to lower cholesterol efflux [56]. Similarly, in a murine macrophage cell line, Hara *et al*. demonstrated that adenovirus-mediated expression of apoE isoforms reduced cellular cholesterol in a manner inversely proportional to the LDLR affinity of apoE [81]. Effects of LDLR were not investigated in either study by Cullen *et al*. or Hara *et al*.

The apoE3 and apoE2 proteins carry either one or two free cysteinyl groups respectively, and may function as a better antioxidant than apoE4 protein which does not have a free cysteinyl group. It has been shown *in vitro* that lipoprotein oxidation is apoE isoform-dependent [49, 50]. Similarly, we show that lipid peroxidation *in vitro* by Cu^{2+} of mouse VLDL particles enriched with apoE4 is more susceptible to lipid peroxidation than particles with apoE3 or apoE2. However, we were not able to demonstrate the antioxidant effects of apoE *in-vivo*. Antioxidant effects of apoE *in vivo* are likely to be small.

Nevertheless, differences in the antioxidant capacity of apoE isoforms could be significant when excess oxidative stress is applied, such as in lipid-loaded macrophages in the atherosclerotic plaques.

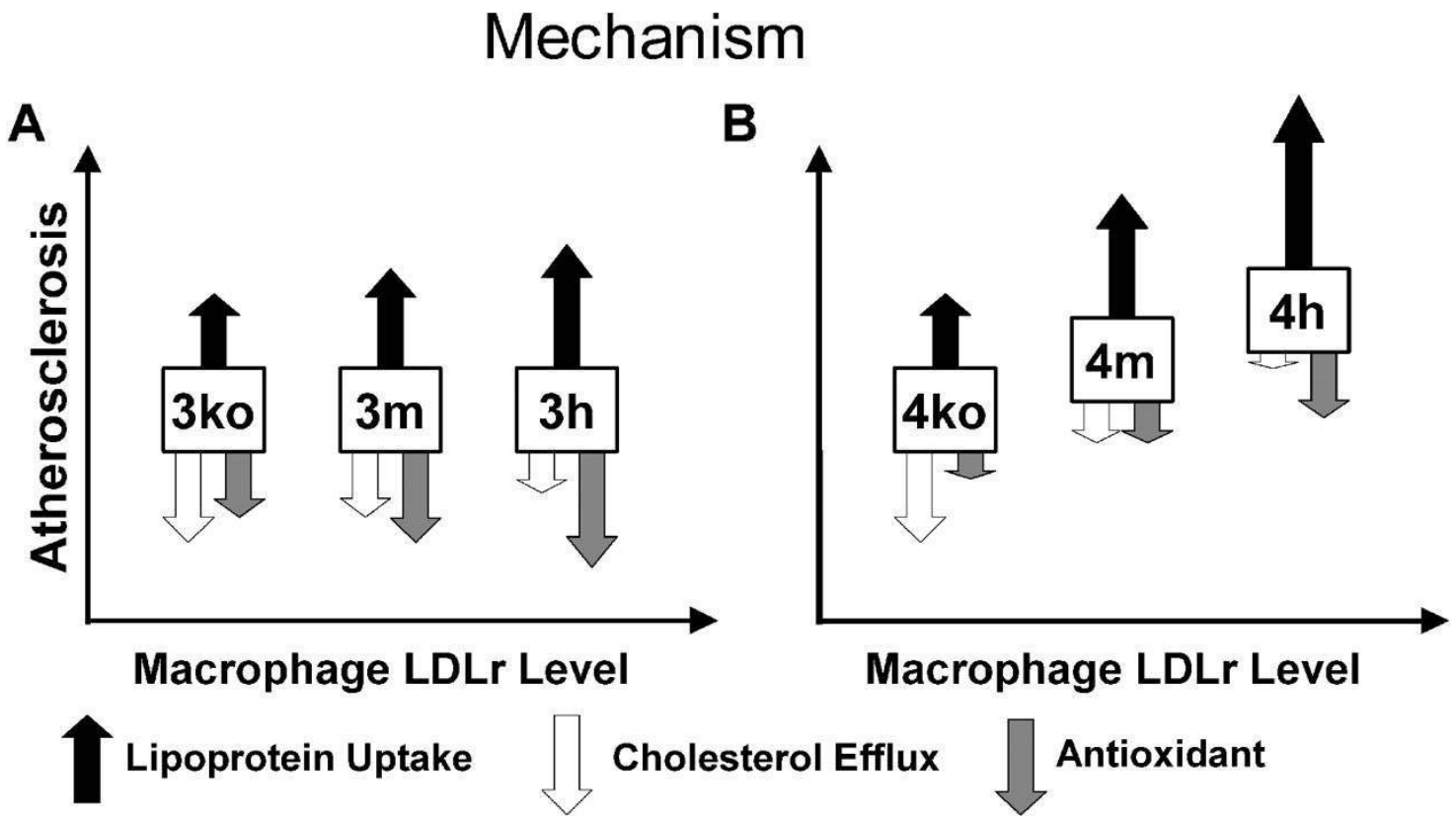
Many of the functions of apoE in vascular tissues are isoform-specific and have been implicated as contributing factors to the well-established increased atherosclerosis risk associated with apoE4 in humans. For example, apoE has been shown to inhibit smooth muscle cell proliferation, with apoE4 being less effective than apoE2 or apoE3 [82]. This anti-proliferative function may also be related to isoform dependent nitric oxide release [83]. ApoE has been shown to reduce atherosclerosis when expressed in the arterial wall without affecting lipid levels [46]. Human apoE3 expressed in macrophages binds LDLR and is internalized, and a proportion of the internalized apoE is recycled in both hepatocytes and macrophages [47, 48]. Consistent with its increased LDLR affinity, apoE4 recycling is impaired relative to apoE3 in hepatocytes [79]. Decreased recycling of apoE4 in the subendothelial space of blood vessels could result in prolonged retention and modification of lipoproteins. In contrast, apoE3 and apoE2 may exert their anti-atherosclerotic effects multiple times if recycled back to the intimal space.

Macrophage LDLR metabolizes LDL and VLDL/chylomicron remnants [6, 20, 74, 84], and uptake of LDL by macrophages through the LDLR can induce foam cell formation in culture [84, 85]. Thus previous studies have shown that WT mice that received BM cells with WT LDLR develop larger diet-induced plaques compared to the WT mice that received cells lacking LDLR [57, 85]. However, macrophage expression of the LDLR did not significantly increase atherosclerosis in the hyperlipidemic setting of LDLR-/- mice recipients when their macrophages expressed WT LDLR [57, 71, 86]. All of these experiments used mice with wild type mouse apoE and LDLR. We now show, in the present paper, that the pro-atherogenic effects of human apoE4 isoform are LDLR expression dependent even under the severe hyperlipidemic setting. Mice with apoE3 showed no difference in atherosclerosis when macrophage LDLR expression level was changed under the same hyperlipidemic condition.

Together, our results show that there is an important interaction between the apoE genotypes and LDLR expression levels in macrophages in determining cholesterol regulation in the cell (Fig 4.7). Thus the higher LDLR binding affinity of apoE4 increases cholesterol delivery to macrophages compared to

apoE3. This effect is enhanced when lipoprotein particles are enriched with apoE4. The pro-atherogenic effects of apoE4 are further enhanced by a reduction in sterol efflux that is also apoE4 specific and LDLR dependent. This is particularly important when cholesterol acceptors such as HDL and apoA-I are reduced in hyperlipidemia. Additionally, the reduced capacity of apoE4 to protect lipoproteins from oxidation compared to that of apoE3 could increase the atherogenicity of apoE4 containing lipoproteins. Although differences in the individual effects of apoE4 compared to apoE3 are small, the combined effects are of importance and likely contribute to the adverse effects of apoE4 on plaque development in an LDLR dependent fashion in macrophages. A similar mechanism is likely to operate in humans during the development of atherosclerosis.

Figure 4.7. Hypothetical interactions between macrophage LDLR and apoE isoforms in mice that underwent BMT. Increasing LDLR results in increased macrophage uptake of apoE containing VLDL and LDL (solid arrow). The relative uptake of apoE3 containing lipoproteins (A) is less than the uptake of apoE4 containing lipoproteins (B). Elevated LDLR reduced efflux in macrophages that were loaded with cholesterol as well as unloaded apoE4 macrophages (open arrow). ApoE3 has greater antioxidant capacity than apoE4. The antioxidant capacity increases as more apoE is retained by LDLR (shaded arrow). Overall the combined effects are balanced in apoE3 macrophages and its atherogenicity is not influenced by LDLR expression. In contrast, the combined effects are not balanced in apoE4 macrophage and the atherogenicity is increased as LDLR expression increases.


Mechanism
A
Atherosclerosis
3ko
3m
3h
Macrophage LDLr Level
B
4ko
4m
4h
Macrophage LDLr Level
Lipoprotein Uptake
Cholesterol Efflux
Antioxidant

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Chapter V

ApoE binding the LDLR in vivo: VLDL clearance varies inversely with LDLR affinity

Michael Altenburg, Lance Johnson, Dominic Ciavatta, Jose Arbones-Mainar, Jennifer Wilder and Nobuyo Maeda

Abstract

Apolipoprotein E (apoE) and the low-density lipoprotein receptor (LDLR) are both well-recognized determinants of plasma cholesterol levels. In this study, we investigated the contribution of the LDLR expression to the localization of human apoE-isoforms in the liver of mice. Adenoviral overexpression of the LDLR decreased plasma cholesterol in mice with human apoE2, but led to a dramatic accumulation of cholesterol-rich VLDL in mice with human apoE4 on a western-type high fat diet. Primary hepatocytes with apoE2 secreted more apoE into medium than the hepatocytes with apoE4. Higher LDLR expression decreased the secretion of apoE4 and increased its degradation. ApoE4-GFP fusion protein expressed by adenovirus in the liver of apoE-deficient mice accumulated in the space of Disse, but apoE2-GFP protein did not. Additionally, LDLR levels significantly influenced the amount of apoE4-GFP on the hepatocyte surface. While the liver of mice with apoE2 and high LDLR avidly internalized DiI-labeled VLDL, internalization was slower in the liver with apoE4 and high LDLR despite that the DiI-VLDL accumulated quickly on the hepatocyte surface. These data indicate that apoE4 co-localizing with the LDLR on the surface of hepatocytes is a poorer mediator of VLDL uptake than apoE2 despite its higher LDLR binding-affinity.

Introduction

The low-density lipoprotein receptor (LDLR) plays a pivotal role in clearance of lipoproteins thereby reducing plasma cholesterol, a leading determinant of atherosclerosis susceptibility [6]. Lipoprotein clearance is mediated through the binding to the LDLR of apolipoprotein E (apoE) and apolipoprotein B (ApoB), constituents of lipoproteins, in the liver where most of LDLR, apoB, and apoE production is found [15] [16].

ApoE is a 34kDa exchangeable protein associated primarily with Triglyceride Rich Lipoproteins (TRL) and HDL [6]. In humans the *APOE* gene is polymorphic, resulting in production of three common isoforms apoE2, E3, and E4. These isoforms differ from one another by an amino acid difference at two positions, E2 having a Cys at both position 112 and 158, E3 having an Arg at 112 and a Cys at 158, and E4 having Arg at both positions. These structural changes alter the LDLR binding affinity; apoE4 binds LDLR with highest affinity while apoE2 has drastically reduced binding compared to the other two isoforms [13, 14, 87]. There is a well-established association between apoE-isoforms and different plasma lipid/lipoprotein phenotypes and coronary artery disease risk in humans. Thus, although a majority of individuals homozygous for apoE2 have slightly higher plasma triglycerides and lower LDL cholesterol, 5-10% of them develop type III hyperlipoproteinemia characterized by significantly elevated plasma levels of triglycerides and cholesterol [6]. In contrast, possession of an apoE4 allele is associated with high LDL cholesterol, low plasma triglycerides and increased risk of atherosclerosis [2, 3, 6, 16, 19, 71]. The molecular mechanisms whereby the apoE isoforms and the LDLR affect these associations are still not clear, and mice with the wild type *Apoe* gene replaced with human *APOE*2, APOE*3* and *APOE*4* alleles do not simply replicate these phenotypes. Thus all mice expressing apoE2 exhibit type III

hyperlipoproteinemia and develop atherosclerosis even on normal, low cholesterol and low fat diet, while those expressing apoE3 or apoE4 are normolipidemic and resistant to atherosclerosis development [14, 33, 34].

Previously we have shown that the *Ldlr*^{h} allele coding for human LDLR, which has a truncation of the 3'-untranslated region, resulted in increased mRNA stability and 2-3 fold elevation of expression in mice [37-40]. Surprisingly, introduction of the *Ldlr*^{h} allele into mice expressing human apoE isoforms reproduced the human-like phenotypes associated with apoE isoforms. On a HFW diet, mice with apoE4 over-expressing the LDLR (*Apoe*$^{4/4}$*Ldlr*$^{h/+}$) have increased plasma VLDL/Chylomicron Remnants, decreased HDL cholesterol levels, and develop atherosclerosis, while mice with human apoE3 and the *Ldlr*^{h} allele (*Apoe*$^{3/3}$*Ldlr*$^{h/+}$) have significantly decreased HDL cholesterols as well as total cholesterol levels and do not develop atherosclerosis. Global over-expression of LDLR in mice with apoE2 (*Apoe*$^{2/2}$*Ldlr*$^{h/+}$) resulted in lower plasma cholesterol and the absence of atherosclerotic lesions [37-40]. We hypothesized that the adverse effects of increased LDLR expression in mice with apoE4 is because the high affinity of apoE4 for the LDLR limits its availability for enrichment on larger TRL particles consequentially promoting a rapid conversion of these particles to poorly cleared smaller, cholesterol enriched remnants.

This hypothesis predicts the following. First, the over-expression of LDLR in the liver of mice with human apoE isoforms will replicate the phenotypes seen in mice with global LDLR expression. Second, over-expression of apoE4 protein will overcome the hyperlipidemia in *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice. Third, a majority of apoE4, but not apoE2, proteins will co-localize with LDLR in the liver cells and accumulate proportionally to the LDLR expression levels. In the present study we have tested these predictions using adenoviral mediated gene transfers. We

show here that LDLR expression in the liver is sufficient, and likely responsible, for the hyperlipidemia in the *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice. We also show that a substantial amount of apoE4 and to a lesser extent apoE3, but not apoE2, is co-localized with LDLR to the surface of hepatocytes. This interaction with the LDLR increases the association of apoE4 with hepatocytes, limits apoE4 secretion, and enhances its degradation in primary cultured hepatocytes.

Material and Methods

Mice- ApoE-deficient mice (*Apoe*$^{-/-}$), LDLR-deficient mice (*Ldlr*$^{-/-}$), mice homozygous for replacement of the mouse apoE gene with either the human *APOE*2, APOE*3* or *APOE*4* allele (*Apoe*$^{2/2}$. *Apoe*$^{3/3}$, and *Apoe*$^{4/4}$) and mice over-expressing the human *LDLR* minigene (*Ldlr*$^{h/+}$) were individually backcrossed at least 6 generations to C57BL/6 genetic background [14, 33, 34, 37]. Mice with various combinations of the *Apoe* and *Ldlr* loci were produced by intercross of these mutants for the present work as listed in Table 5.1. Mice were fed either normal mouse chow (NC) containing 4.5% (wt/wt) fat and 0.022% (wt/wt) cholesterol (Prolab RMH 3000, Agway Inc) or a high-fat Western-type diet (HFW) containing 21% (wt/wt) fat and 0.2% (wt/wt) cholesterol (TD88137; Teklad). The animals were handled under protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina–Chapel Hill.

Table 5.1 Genotypes and Descriptions

Mice Genotype	Description
Apoe$^{2/2}$	human apoE2
Apoe$^{2/2}$ *Ldlr*$^{h/+}$	human apoE2 and increased LDLR
Apoe$^{3/3}$	human apoE3
Apoe$^{3/3}$ *Ldlr*$^{h/+}$	human apoE3 and increased LDLR
Apoe$^{4/4}$	human apoE4
Apoe$^{4/4}$ *Ldlr*$^{h/+}$	human apoE4 and increased LDLR
Apoe$^{4/4}$ *Ldlr*$^{-/-}$	human apoE4 and no LDLR
Apoe$^{-/-}$	no apoE
Apoe$^{-/-}$ *Ldlr*$^{h/h}$	no apoE and increased LDLR

Culture of primary mouse hepatocytes—Mice were anesthetized using avertin (2,2,2-tribromo ethanol), the portal vein was cannulated with a 24-gauge plastic cannula, and the liver was perfused with Ca^{2+}/Mg^{2+}-free Hanks' Balanced Salt Solution containing glucose (10 mM) and HEPES (10 mM) at a flow rate of 3 ml/min for 10 min. The perfusion was continued for another 10 min with the same solution containing collagenase (0.05%, type I, Sigma). The liver was removed from the animal, minced in phosphate buffered saline (PBS), and the dissociated cells were dispersed by shaking followed by filtration through 100-µm nylon cell strainers (Falcon). The liver capsule and dish were rinsed in Dulbecco's modified Eagle's medium (DMEM) containing 0.02 µg/ml dexamethasone, 100 units/ml of penicillin and 100 µg/ml of streptomycin. The cells were pelleted by gravity sedimentation in Percoll for 5 min. at 4 °C. The medium was aspirated, leaving 5 ml total volume, and fresh medium was added to 10 ml. The cells were resuspended and viability was assessed by trypan blue exclusion. The yield of hepatocytes ranged from $3x10^6$ to $6x10^6$ cells/g of liver, and viability was greater than 80%. The cells were plated onto 60-mm mouse collagen IV-coated dishes (Falcon) at a density of $1.2x10^6$ viable cells/dish in 2 ml of the above medium containing 10% FBS unless otherwise stated.

Quantitation of apoE and apoA1— The medium from the primary hepatocytes was centrifuged at 13,000 x *g* for 10 min at 4 °C. Cell associated proteins were isolated from primary hepatocytes that were washed 3 times in PBS and resuspended in 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, and 1% Triton X-100 on ice for 10 min. After centrifugation for 10 min with 13,000 x *g* at 4 °C, the supernatants were harvested and cell associated protein concentrations were determined by Bradford assay (Bio-Rad). Cellular proteins and media were separated by 4–15% SDS-PAGE gradient gels (Bio-Rad), and immunoblotted using goat anti-

human apoE antibody (Calbiochem). After incubation with peroxidase-conjugated rabbit anti goat IgG antibody (Calbiochem), the reaction product was visualized using the ECL system (Amersham Biosciences). Additionally apoE and apoA1 were quantitated by enzyme-linked immunosorbent assay (ELISA). 96 well immunoplates were coated with 1.5 μg/ml mouse monoclonal anti-human apoE antibody (calbiochem) or anti-apoA1 in 0.2 M Na_2CO_3/$NaHCO_3$ buffer.

^{35}S labeling of primary mouse hepatocytes-- Primary hepatocytes were cultured for 16 h in low glucose DMEM containing 1% bovine serum albumin. The cells were pulsed with 0.5 ml medium containing ^{35}S methionine (100 μCi/ml Amersham). After a 60 min pulse, the medium was removed, and the cells were washed twice with PBS and chased for 1 and 4 h in fresh medium with excess cold methionine. ApoE was immunoprecipitated from the media using a goat anti-human polyclonal antibody (Calbiochem). The precipitates were dissolved in sample buffer and separated by SDS-PAGE. The gels were dried and signal was visualized by a Fla-3000 phosphoimager (FujiFilm).

Adenoviruses— The adenoviral vector containing the human LDLR cDNA (Ad-LDLR, [28]) was a gift from Dr. Joachim Herz at the University of Texas, Southwestern Medical Center. The plasmid vectors containing cytomegalus viral promoter-driven cDNA for fusion proteins, ApoE2-GFP, apoE3-GFP, and apoE4-GFP, were provided by Dr. Robert DeKroon at Duke University. These vectors express fusion proteins with EGFP (enhanced green fluorescent protein) attached to the C terminal end of each apoE isoform [88]. Adenoviral vectors encoding apoE2-GFP, apoE3-GFP, and apoE4-GFP were made using the AdEasy adenoviral system

(Stratagene) according to the manufacturer's instructions. Ad-LDLR and Ad-apoE-GFP were amplified in 293 cells, purified by CsCl density gradient ultracentrifugation and dialyzed against 10 mM Tris pH 8.0, 2mM MgCl2, 4% sucrose. Recombinant adenovirus stock stored at –80 °C was diluted with PBS and $1x10^9$ PFU in 0.2 ml of adenovirus was injected to a mouse via tail vein.

Plasma lipoprotein analysis, DiI labeling and injection. Plasma was isolated, and total cholesterol and triglycerides were measured as described [69]. Pooled plasma samples (100 µL) were fractionated by fast protein liquid chromatography (FPLC) using a Superose 6 HR10/30 column (Pharmacia Biotech Inc). Plasma HDL was determined by precipitation with dextran sulfate (Sigma) according to Warnick et al [89]. For precipitation with dextran sulfate and $MgCl_2$ was dissolved in PBS. 5 microliters of dextran sulfate (1.0 mg/ml) was mixed with 5 µl of plasma and incubated at room temperature for 5 min and subsequently centrifuged at 3,000 *g* for 10 min. HDL cholesterol was determined from the supernatant. The VLDL fraction was isolated from pooled plasma by ultracentrifugation at d<1.006 g/ml and labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI C_{18}; Molecular Probes Inc.), as described by Stephan and Yurachek [73]. DiI-labelled VLDL (100 µg protein) was injected into tail veins of mice, and livers were fixed with PFA either 10 or 20 min later. DiI-labeled VLDL remaining in the plasma was determined using a microscope fluorometer (Olympus FV500 with a SPOT 2 digital camera) at 2min 10 min and 20 min using a modification of the fluorometric procedure described by Lorenze et al to DiI flouresence [90].

Tissue processing for immunohistochemistry and confocal analyses. Animals were given a lethal overdose of 2, 2, 2-tribromo ethanol. Livers were perfused through the portal vein at 2 ml/min with 10 ml's of 4% paraformaldehyde, excised from animals and further fixed overnight in 4% paraformaldehyde. Liver sections were paraffin embedded and 5 μm thick sections were made for immunohistochemistry. Slides with consecutive liver sections were incubated overnight at 4 °C with either goat anti-human apoE antiserum or goat anti-LDLR antibodies (1:1000, Calbiochem) followed by an incubation with FITC conjugated anti-goat (1:500, Santa Cruz). Slides were cover slipped after application of Vectashield anti-fade mounting medium (Vector H-1000, Vector Laboratories,) and were observed with an IX70 inverted microscope (Olympus) equipped with a filter set for FITC (exciter 560/55, dichroic 595, emitter 645/75; Chroma Technology Corp), and images were captured with a SPOT 2 digital camera (Diagnostic Instruments) and analyzed with SPOT version 4.0.9 (Diagnostic Instruments) and ImageJ 1.33u (NIH) software. For confocal microscopic analysis of apoE-GFP expressing tissues, 100 μm-thick sections were cut with a vibratome and stored free-floating in PBS at 4º C. Before analyses with an Olympus FV500 confocal microscope, individual sections were treated with sodium borohydride (1mg/ml in PBS) for 30 minutes at room temperature, and then washed in PBS three times for five minutes each at room temperature to reduce fixative-induced fluorescence. Individual vibratome sections were also stained with fluorescent (TRITC or AlexaFluor633) labeled Wheat germ agglutinin (WGA) (50 micrograms/ml) for 30 min. For GFP fluorescence, the 514 line of an Argon laser was used for excitation and the 535-565 band-pass filter was used for emission. To acquire TRITC labeled WGA images, the 543 line of a Helium/Neon-green laser was used for excitation and the 660 band-pass filter for emission (Cy5). GFP and TRITC

fluorescence images were scanned independantly with only their respective excitation and emission frequencies to eliminate bleed through.

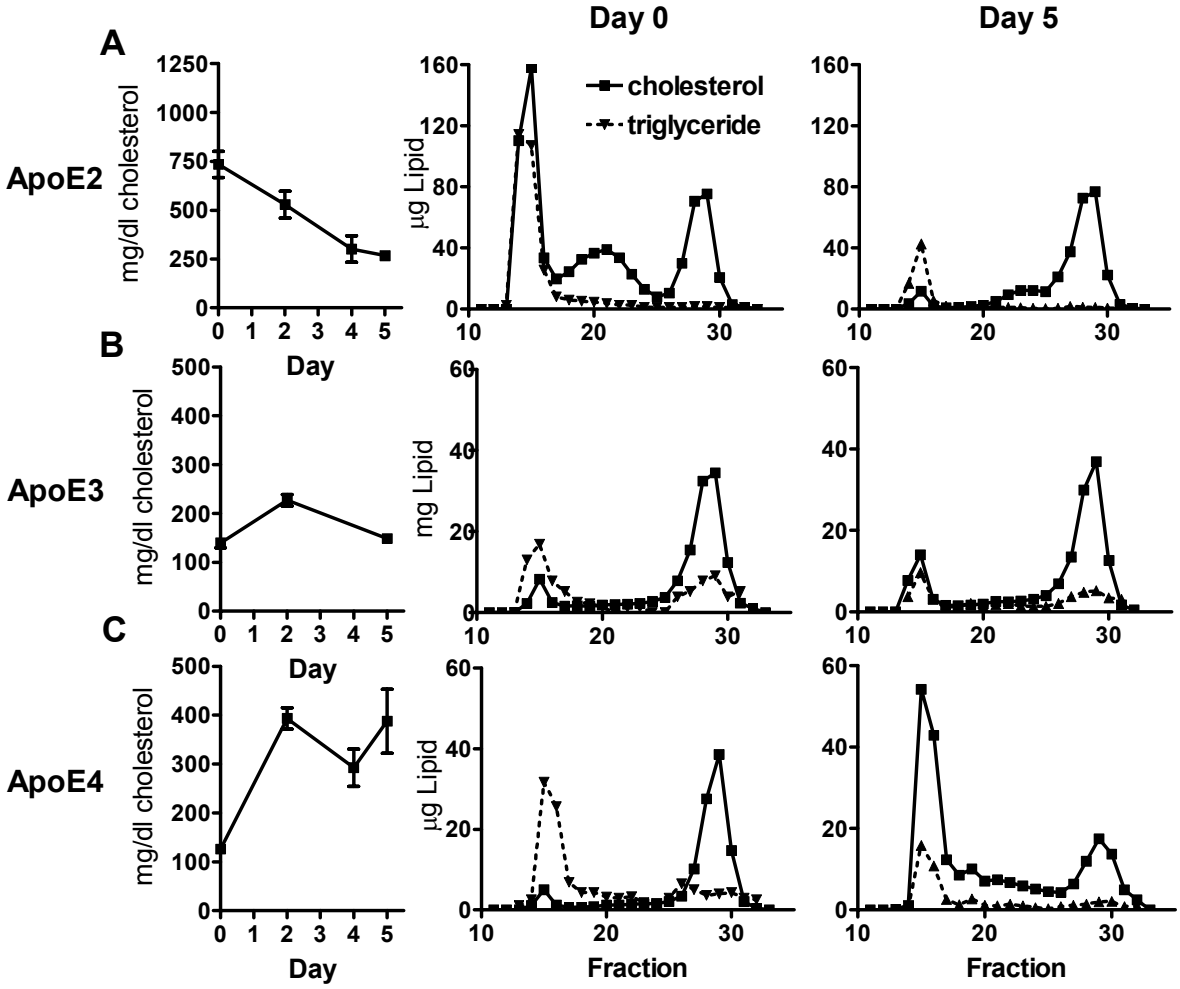
Statistical analysis. The significance of differences between means was calculated by using one-way ANOVA (JMP software; SAS Inc).

Results

Overexpression of LDLR in the liver of mice with human apoE. To determine the contribution of hepatic LDLR expression to plasma lipids, we injected Ad-LDLR carrying cDNA for the human LDLR (Herz and Gerard 1993) into mice expressing human apoE isoforms fed a HFW diet. *Apoe*$^{2/2}$ mice had high plasma cholesterol levels at baseline (733±68 mg/dl, *n*=7) but they decreased to 267±18 mg/dl by day 5 after Ad-LDLR injection. FPLC analyses of plasma showed substantial reductions in their VLDL and LDL fractions and increases in their HDL compared to the base line (Figure 5.1A). *Apoe*$^{3/3}$ mice showed little change in total cholesterol (140±10 mg/dl to 149±9 mg/dl at day 5, *n*=8) or in lipoprotein distribution (Figure 5.1B). In contrast, liver directed over-expression of LDLR in *Apoe*$^{4/4}$ mice increased total cholesterol from 126±9 mg/dl to 388±66 mg/dl (*n*=10) by day 5. Cholesterol, but not triglycerides, were markedly increased mainly in the VLDL/IDL fractions, while HDL cholesterol was decreased (Figure 5.1C). The plasma lipid/lipoprotein profiles induced by the Ad-LDLR in the *Apoe*$^{2/2}$, *Apoe*$^{3/3}$ and *Apoe*$^{4/4}$ mice are identical to those observed in *Apoe*$^{2/2}$*Ldlr*$^{h/+}$, *Apoe*$^{3/3}$*Ldlr*$^{h/+}$, and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice that express 2.5X normal levels of LDLR globally [37, 39]. Since gene expression from a recombinant adenoviral vector is mainly restricted to the liver [28, 91], we conclude that the interactions between apoE and the LDLR in the liver are responsible for the association between apoE isoforms and plasma lipid profiles, and that over-production of liver LDLR leads to an accumulation of cholesterol-rich, triglyceride-poor remnants in mice expressing human apoE4.

Figure 5.1. Adenovirus mediated overexpression of the human LDLR in mice with human apoE isoforms. A. Change in total cholesterol over 5 days after Ad-LDLR (left column). Distribution of lipids within different lipoprotein fractions, as assessed by FPLC of mice on HFW diet (center column) and 5 days after Ad-LDLR (right column) after Ad-LDLR infection. A. Mice with ApoE2 before (left), and after 5 days Ad-LDLR (center). B. Mice with ApoE3. C. Mice with ApoE4.

Figure 5.1


Day 0
Day 5
A
ApoE2
B
ApoE3
C
ApoE4
mg/dl cholesterol
µg Lipid
mg Lipid
cholesterol
triglyceride
Day
Fraction

Secretion of ApoE from liver and primary hepatocytes. An increased interaction between apoE4 and LDLR in the liver lead to the reduction of apoE associated with plasma lipoproteins [39]. Slot blot analysis of plasma and liver apoE from $Apoe^{2/2}$, $Apoe^{3/3}$, $Apoe^{4/4}$, and $Apoe^{4/4}Ldlr^{h/+}$ mice on NC diet confirmed that apoE affinity for the LDLR determines plasma apoE levels in mice. The higher affinity apoE4 in $Apoe^{4/4}$ mice had the lowest plasma concentration among the different isoforms and was further reduced by overexpression of the LDLR in $Apoe^{4/4}Ldlr^{h/+}$ mice (Figure 5.2A). ApoE isoform and LDLR also had modest affects on liver apoE concentration (Figure 5.2A). To determine the cellular metabolism of the different apoE isoforms, we isolated primary hepatocytes from mice expressing different apoE isoforms and compared the apoE protein by using Western blot analysis.

After culturing in DMEM without FBS for 24 hours, primary hepatocytes from $Apoe^{2/2}$ mice secreted more than 10 fold apoE protein into the medium compared to the cells from $Apoe^{4/4}$ mice and had double the ratio of medium apoE to cell-associated apoE (Figure 5.2B). After 24 hours in culture, ratios of medium apoE to cell-associated apoE in the $Apoe^{2/2}$ hepatocytes were significantly higher than those in the $Apoe^{3/3}$ or $Apoe^{4/4}$ mice (Figure 5.2B). These results indicate that the affinity of apoE isoform for the LDLR is an important factor for the secretion of apoE from the liver.

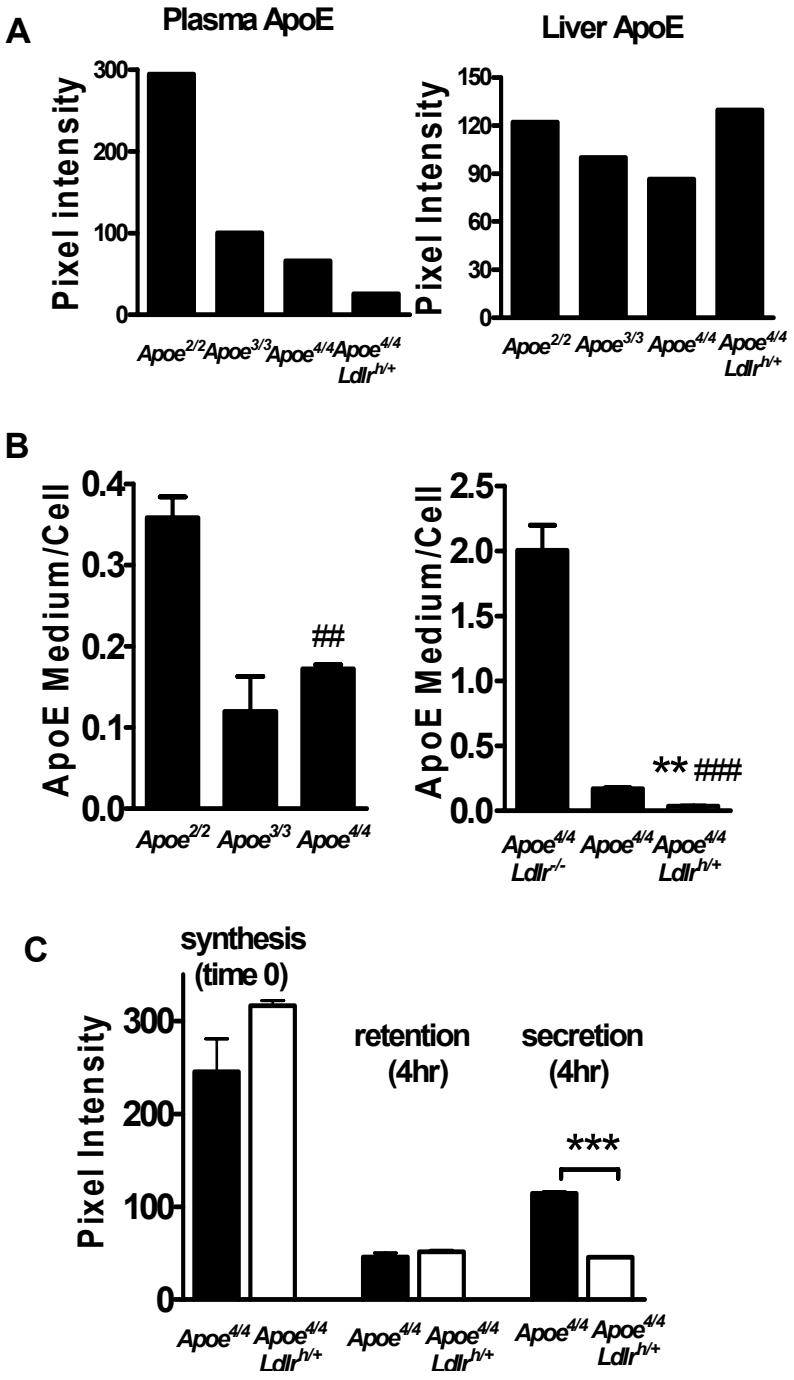
In addition, LDLR level affected the amount of apoE secreted from the cell in cultured primary hepatocytes and the higher the LDLR receptor expression levels were, the higher the cellular apoE increase, and lower the apoE in the medium (Figure 5.2B). Thus, the ratio of medium apoE to cell-associated apoE in the cultured $Apoe^{4/4}Ldlr^{h/+}$ hepatocytes was significantly lower than in $Apoe^{4/4}$ cells. In turn, the ratio in $Apoe^{4/4}Ldlr^{-/-}$ hepatocytes lacking LDLR expression was significantly higher than in $Apoe^{4/4}$ hepatocytes.

These data indicate that the relative amount of cell-associated apoE is a direct consequence of its affinity to the receptor and the levels of LDLR expression. The same relationships were also seen in liver slices that were isolated from mice with the human apoE isoforms and incubated in lipoprotein-free medium at 37^{o}C for 24 hrs (not shown).

Effects of LDLR on synthesis and degradation of apoE in primary hepatocytes. The ratio of extracellular to intracellular apoE amounts is likely determined by the uptake, but could also be due to changes in apoE synthesis or degradation. To determine how the LDLR expression levels affect production and degradation of apoE, we used a pulse chase system in the primary hepatocytes isolated from *Apoe*$^{4/4}$ and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice. The synthesis during a 30 min pulse was not significantly different between *Apoe*$^{4/4}$ and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ hepatocytes indicating that a 2.5X higher LDLR expression had no effect on the apoE production rate (Figure 5.2F). Although *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ cells had a slightly higher, though not significant, apoE at the start of the chase incubation compared to that in *Apoe*$^{4/4}$ cells, intracellular apoE after a 4hr chase was the same in hepatocytes of both genotypes. However, consistent with the observation described above, a higher LDLR expression led to a decreased amount of apoE secreted from hepatocytes, and apoE4 secreted from *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ cells into the medium was about 40% of the level secreted from *Apoe*$^{4/4}$ cells. The sum of the secreted and cell-retained amounts in *Apoe*$^{4/4}$ and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ cells was 65% and 31% respectively, of the initial synthesis amounts. The increased loss of labeled apoE in *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ cells suggests that an elevated LDLR also increases apoE degradation. This result is consistent with the finding that increased LDL receptor expression reduced the secretion and enhanced the degradation of apoE4 in peritoneal macrophages of mice by Lucic et al [40].

Figure 5.2. ApoE isoform and LDLR modulate apoE secretion from Liver and primary hepatocytes. A. Pooled (n=3) plasma (left graph) and liver (right graph) apoE in *Apoe*$^{2/2}$, *Apoe*$^{3/3}$, *Apoe*$^{4/4}$, and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice on NC diet by slot blot. B. Ratio of apoE in the medium or cell associated after over night culture (24hrs) in *Apoe*$^{2/2}$, *Apoe*$^{3/3}$, *Apoe*$^{4/4}$ (left) and *Apoe*$^{4/4}$*Ldlr*$^{-/-}$, *Apoe*$^{4/4}$, *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ (right) primary hepatocytes. C. Pulse chase analysis of *Apoe*$^{4/4}$ and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ hepatocytes. Cells were labeled for 30 min (synthesis), washed and incubated 4hrs. Labeled apoE intracellular (retention) and extracellular (secretion) was determined. Values shown are means of the duplicate measurements. * # $p \leq 0.05$, **, ## $p \leq 0.005$, ***, ### $p \leq 0.0005$

Figure 5.2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Pixel intensity
300
200
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Apoe2/2 Apoe3/3 Apoe4/4 Apoe4/4 Ldlrh/+
Liver ApoE
Pixel Intensity
150
120
90
60
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Apoe2/2 Apoe3/3 Apoe4/4 Apoe4/4 Ldlrh/+
B
ApoE Medium/Cell
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Apoe2/2 Apoe3/3 Apoe4/4
ApoE Medium/Cell
2.5
2.0
1.5
1.0
0.5
0.0
** ###
Apoe4/4 Ldlr-/- Apoe4/4 Apoe4/4 Ldlrh/+
C
synthesis (time 0)
retention (4hr)
secretion (4hr)
Pixel Intensity
300
200
100
0
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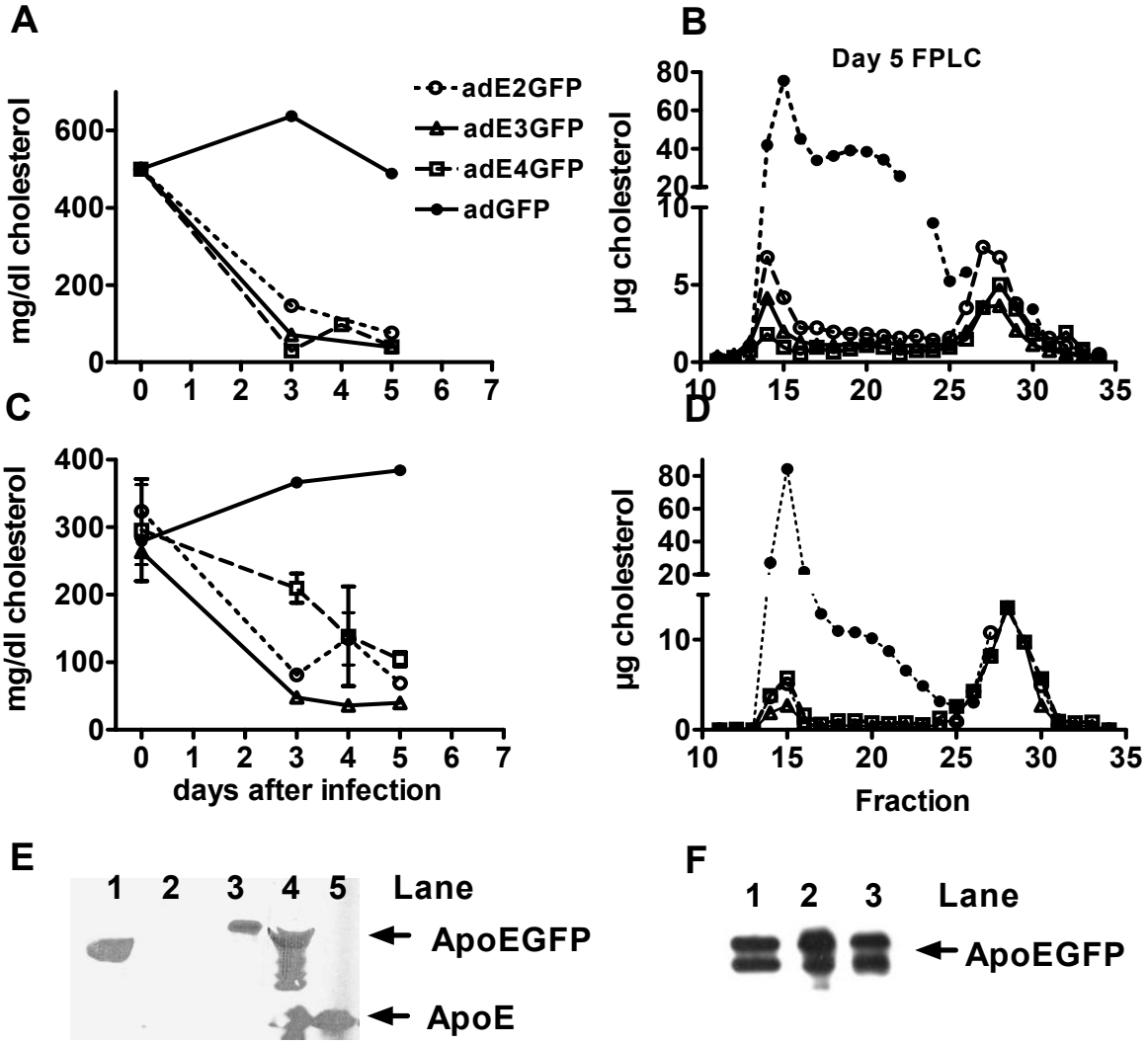
Apoe4/4 Apoe4/4 Ldlrh/+ Apoe4/4 Apoe4/4 Ldlrh/+ Apoe4/4 Apoe4/4 Ldlrh/+

Effect of Ad-apoE-GFP on plasma lipid levels. In order to track hepatic localization of apoE isoforms, we chose to express apoE-GFP fusion proteins using recombinant adenovirus in mice expressing different levels of LDLR. The apoE-GFP fusion proteins appear to function normally when mice were infected with Ad-apoE-GFP. All isoforms lowered cholesterol levels in *Apoe*$^{-/-}$ mice except that apoE2-GFP did not decrease as quickly or to the same levels as apoE3-GFP or apoE4-GFP (Figure 5.3A). However in *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice, lacking apoE but expressing high levels of LDLR, transfection with Ad-apoE2-GFP normalized cholesterol levels to approximately the same as apoE3-GFP and apoE4-GFP (Figure 5.3B). In contrast, decreases in the cholesterol levels of *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice injected with Ad-apoE4-GFP were somewhat slower to respond than those injected with Ad-apoE3-GFP, or Ad-apoE2-GFP (Figure 5.3C). Despite the delayed response, however, apoE4-GFP ultimately decreased cholesterol to similar levels as apoE3-GFP, or apoE2-GFP in *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice. Similarly, both Ad-apoE4-GFP or Ad-apoE3-GFP was able to ameliorate the hypercholesterolemic phenotype in *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice fed a HFW diet (not shown).

ApoE-GFP was detectable in the plasma of transfected mice and was associated with all lipoprotein subclasses by Western blotting (Figure 5.3 E, F). These data demonstrate that apoE-GFP fusion proteins are functional and appear to retain isoform-specific characteristics. Since apoE4-GFP can lower cholesterols when over-expressed by adenoviral vector in the liver of *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice, adverse effects of LDLR must be dependent on relative ratios between apoE4 and LDLR.

Figure 5.3. Ad-apoE-GFP decreases plasma lipids in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice. A. Cholesterol levels in *Apoe*$^{-/-}$ mice transfected with Ad-apoE2-GFP, Ad-apoE3-GFP, Ad-apoE4-GFP, or Ad-GFP over a 5 day span. B. Day 5 distribution of lipids within different lipoprotein fractions, as assessed by FPLC. C. *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice with Ad-apoE2-GFP, Ad-apoE3-GFP, Ad-apoE4-GFP 5 day Cholesterol time course. D. Day 5 distribution of lipids within different lipoprotein fractions, as assessed by FPLC. E. SDSPAGE of plasma isolated from *Apoe*$^{-/-}$ mice transfected with Ad-apoE3-GFP (lane1), without transfection (lane 2), VLDL isolated from Ad-apoE3-GFP transfected *Apoe*$^{-/-}$ mice (lane 3), Wt B6 transfected with Ad-apoE3-GFP (lane 4), and Wt plasma apoE (lane 5). F. Ad-apoE3-GFP associated with VLDL (fraction 15), LDL (fraction 23), and HDL (fraction 28).

Figure 5.3


A
mg/dl cholesterol
adE2GFP
adE3GFP
adE4GFP
adGFP
B
Day 5 FPLC
µg cholesterol
C
mg/dl cholesterol
days after infection
D
µg cholesterol
Fraction
E
Lane
ApoEGFP
ApoE
F
Lane
ApoEGFP

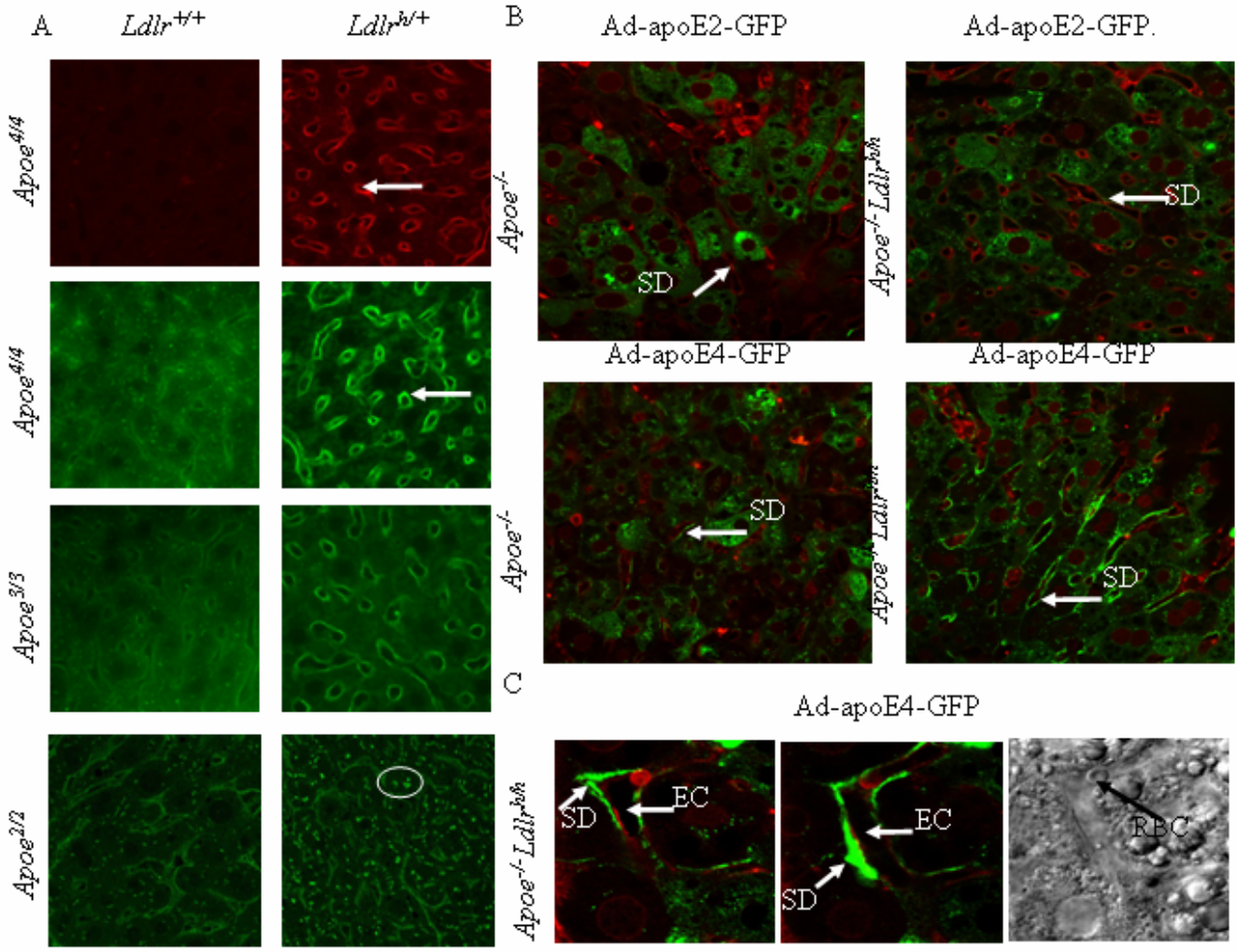
Localization of apoE in the liver. The microenvironment of the liver is unique and plasma lipoproteins bind receptors on the hepatic surface in the space of Disse, the space formed between hepatocytes and fenestrated endothelial cells in the liver sinusoids. Immunostaining with an antibody against human LDLR of the liver sections of mice carrying the *Ldlr*h* allele illustrated the sinusoidal localization of the human LDLR (Figure 5.4A top row). ApoE4 co-localized with LDLR in the $Apoe^{4/4}Ldlr^{h/+}$ liver as highlighted by the very intense staining of sinusoids with antibody against human apoE. Thus, overproduction of LDLR forces apoE4 to accumulate in the space of Disse. In contrast, apoE4 staining in the $Apoe^{4/4}Ldlr^{+/+}$ liver was diffuse and also present in the cytoplasm in a punctate pattern (arrow in Figure). Very similar staining patterns were observed in the livers from $Apoe^{3/3}Ldlr^{h/+}$ and $Apoe^{3/3}$ mice, except that the sinusoidal staining relative to cytoplasmic staining was less intense in the $Apoe^{3/3}Ldlr^{h/+}$ liver than in the $Apoe^{4/4}Ldlr^{h/+}$ liver. This suggests that the LDLR levels influence sinusoidal localization of apoE4 and, to a lesser extent, of apoE3. $Apoe^{2/2}$ and $Apoe^{2/2}Ldlr^{h/+}$ mice both had low sinusoidal staining and more intra-hepatic punctate staining than other apoE isoforms (Figure 4A bottom row) and $Apoe^{2/2}Ldlr^{h/+}$ mice had more intra-cellular staining than $Apoe^{2/2}$ mice.

To compare the hepatic microenvironment localization of apoE2 and apoE4 and the effect of increased LDLR in more detail, we injected Ad-apoE2-GFP and Ad-apoE4-GFP into $Apoe^{-/-}$ and $Apoe^{-/-}Ldlr^{h/h}$ mice which are deficient of apoE. ApoE localization was analyzed under confocal microscopy in the liver sections stained with the TRITC-labeled lectin to demarcate liver sinusoids, endothelial cells, and the space of Disse [92]. Both apoE4-GFP and apoE2-GFP were visible as bright green hepatocytes with perinuclear concentration in the cytoplasm of 70-80% of the infected liver (Figure 5.4B). However, substantially more apoE4-

GFP was also present in the space of Disse than apoE2-GFP (SD in Figure 5.4B). The sub-endothelial accumulation of apoE4-GFP was more pronounced in the liver of the *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice injected with Ad-apoE4-GFP (Figure 5.4B, second column). In contrast, injection of these mice with Ad-apoE2-GFP showed little accumulation of apoE2-GFP on the hepatocyte surface despite the increased LDLR and efficient reduction of plasma cholesterol (Figure 5.4B). Inspection under higher magnification reveals that GFP and TRITC signals were not overlapping, suggesting that the accumulation of apoE4-GFP is sub-endothelial in the space of Disse, between hepatocytes and endothelial cells (Figure 5.4C). These data demonstrate that the accumulation of apoE on the hepatocyte surface in the space of Disse is dependent on its affinity to the LDLR and the expression levels of LDLR.

Figure 5.4. Localization of apoE2, apoE3, and apoE4 in mice overexpressing the LDLR. A. Immunodetection of LDLR (red) in *Apoe*$^{4/4}$, and *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ in liver using an anti-huLDLR IgG (top row). Second row shows apoE (green) in apoE isoform mice (left) with elevated LDLR (right). *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ staining for anti-apoE showed similar sinusoidal pattern (second row) as anti-huLDLR (arrows). *Apoe*$^{3/3}$*Ldlr*$^{h/+}$ also had increased sinusoidal apoE compared to *Apoe*$^{3/3}$ (3rd row) but less than *Apoe*$^{4/4}$*Ldlr*$^{h/+}$. *Apoe*$^{2/2}$*Ldlr*$^{h/+}$ had increased intracellular apoE (circled) (last row) B. Ad-apoE2GFP (top panels) and Ad-apoE4GFP (bottom panels) were injected into *Apoe*$^{-/-}$ (left panels) and *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ (right panels) mice were sacrificed after 5 days. 100 μm sections were stained with TRITC labeled lectin (red) to show structure and examined confocally C. (1500x) AdapoE4-GFP in to *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice. (SD) space of Disse, (EC) endothelial cell, (RBC) red blood cell.

Figure 5.4.

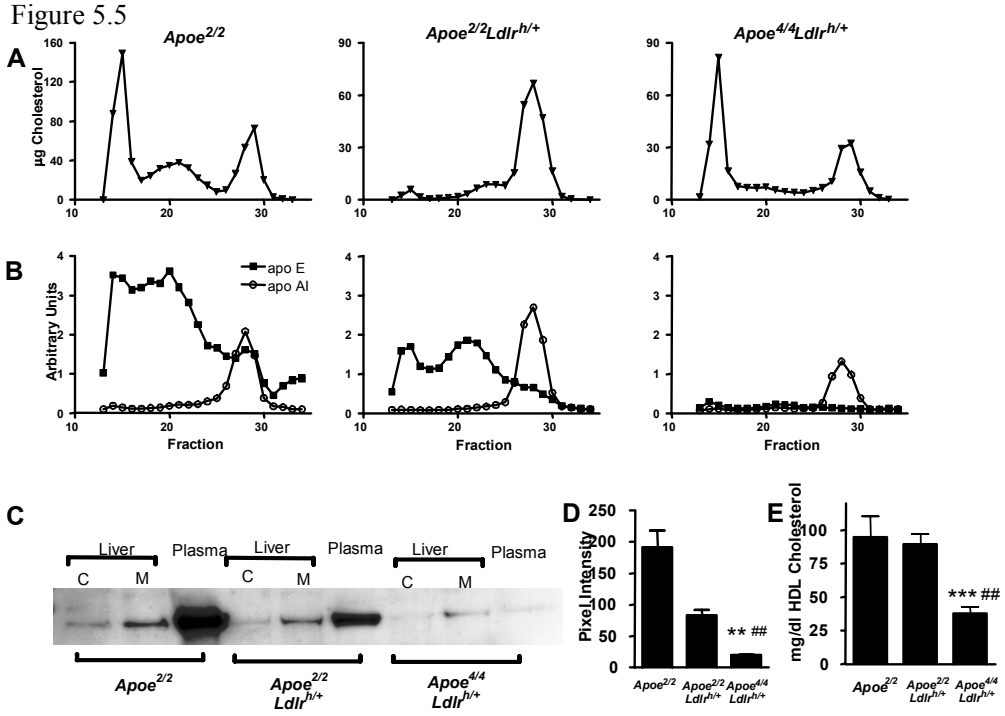

A
Ldlr+/+
Ldlr-/-
B
Ad-apoE2-GFP
Ad-apoE2-GFP.
Apoe4/4
Apoe-/-
Apoe-/-Ldlr-/-
SD
Ad-apoE4-GFP
Apoe4/4
Apoe3/3
C
Apoe2/2
EC
RBC

Sinusoidal localization of apoE and remnant uptake. VLDL remnants are normally converted to LDL and cleared by apoB100-binding to LDLR, or directly cleared by apoE-binding to LDLR and LRP. The striking differences between apoE2 and apoE4 in their cellular localizations of $Apoe^{2/2}Ldlr^{h/+}$ and $Apoe^{4/4}Ldlr^{h/+}$ livers raises a question regarding the physiological relevance of apoE accumulated on the sinusoidal surface in the remnant clearance. $Apoe^{2/2}$, $Apoe^{2/2}Ldlr^{h/+}$, and $Apoe^{4/4}Ldlr^{h/+}$ mice represent phenotypic extremes in lipoprotein profiles and atherosclerosis risk (Figure 5.5A) [37, 39]. $Apoe^{4/4}Ldlr^{h/+}$ mice have plasma apoE levels 30% of $Apoe^{4/4}$ mice, while apoE in the livers of $Apoe^{4/4}Ldlr^{h/+}$ mice were slightly higher than in the livers of $Apoe^{4/4}$ mice (Figure 5.2A). $Apoe^{4/4}Ldlr^{h/+}$ had 10-fold lower plasma apoE than $Apoe^{2/2}Ldlr^{h/+}$ mice (Figure 5.5D). The reduced plasma apoE in $Apoe^{4/4}Ldlr^{h/+}$ was associated with a reduced HDL compared to both $Apoe^{2/2}Ldlr^{h/+}$and $Apoe^{2/2}$ mice (38.0 ± 4.8 n=4, 89.5 ± 7.2 n=5, 94.6 ± 15.5 n=4 respectively, Figure 5.5 E). $Apoe^{2/2}$, $Apoe^{2/2}Ldlr^{h/+}$ and $Apoe^{4/4}Ldlr^{h/+}$ mice have markedly different accumulation of apoE in sinusoids as described above. Despite little VLDL and LDL, $Apoe^{2/2}Ldlr^{h/+}$ mice have substantially more apoE in their larger lipoprotein fractions than $Apoe^{4/4}Ldlr^{h/+}$ mice. $Apoe^{2/2}Ldlr^{h/+}$ mice also have more plasma apoA1 than $Apoe^{4/4}Ldlr^{h/+}$ mice (Figure 5.5B). To examine how these different phenotypes affect plasma VLDL clearance and liver uptake we injected them with DiI labeled $Apoe^{-/-}$ VLDL. Decay of plasma DiI-labeled $Apoe^{-/-}$VLDL particles after injection into these mice showed that the $Apoe^{2/2}$ mice had the slowest clearance of $Apoe^{-/-}$ VLDL (Figure 5.6A, left panel). While the decay in the $Apoe^{2/2}Ldlr^{h/+}$ and $Apoe^{4/4}Ldlr^{h/+}$ mice were not different at 10 min after injection, the disappearance of plasma VLDL during the next 10 min was significantly more in the $Apoe^{2/2}Ldlr^{h/+}$ mice than in $Apoe^{4/4}Ldlr^{h/+}$mice. At 20 min post injection, DiI-VLDL were barely detectable in the liver of $Apoe^{2/2}$ mice (Figure 5.6A, second panel), but were avidly internalized

in the *Apoe*$^{2/2}$*Ldlr*$^{h/+}$ liver (Figure 5.6A third panel). Most of the signal was intracellular (circled) and surface-bound DiI-VLDL was negligible (arrows SD). In marked contrast, DiI-signal was strongly associated with the hepatic surface in *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ liver (arrow SD), while intracellular DiI-VLDL was minimum (circled in Figure 5.6A, right panel). Thus while apoE2, which is elevated in the plasma and minimally associated with the hepatocyte surface, can facilitate internalization of apoE-deficient VLDL remnants in the presence of increased LDLR. In contrast higher LDLR affinity apoE4 that is accumulated on the hepatocyte surface appears to be less efficient in internalizing the remnant particles.

Figure 5.5. LDLR expression and apoE isoform modulate liver apoE secretion and HDL levels. A. Distribution of lipids within different lipoprotein fractions, as assessed by FPLC of mice on HFW diet. B. ApoE and apoA1 ELISA on plasma FPLC fractions on mice fed HFW diet. Densitometry of n=3 separate plasma samples. C. Western blot for apoE liver cytosol (C) and membrane (M) fractions and plasma D. Densitometry of n=3 separate plasma samples. E. Plasma HDL cholesterol on mice fed HFW diet.

Figure 5.5


A
Apoe2/2
Apoe2/2Ldlrh/+
Apoe4/4Ldlrh/+
µg Cholesterol
B
apo E
apo AI
Arbitrary Units
Fraction
C
Liver
Plasma
C
M
Apoe2/2
Apoe2/2 Ldlrh/+
Apoe4/4 Ldlrh/+
D
Pixel Intensity
** ##
E
mg/dl HDL Cholesterol
*** ##

We next sought whether the surface bound apoE4 can associate with VLDL lacking apoE and facilitate lipoprotein internalization, and injected DiI labeled *Apoe*$^{-/-}$ VLDL into *Apoe*$^{-/-}$ *Ldlr*$^{h/h}$ mice that were infected with Ad-apoE4-GFP 5 days earlier. When livers were examined 10 min after the injection for the distribution of apoE4-GFP, strong GFP signal was again on the hepatic cell surface (Figure 5.6B left panel). Strong DiI signals demarcated microvessels as well as punctated within the cytoplasm of hepatocytes (Figure 5.6B middle panel). Most of the DiI-labeled VLDL was co-localized with apoE4-GFP in both cell surface and in cytoplasm, but there remained significant amount of cell surface apoE4 that was not associated with DiI (Fig 5.6B right panel). This indicates that the cell surface apoE4 is minimally contributing to the internalization of exogenous lipoproteins.

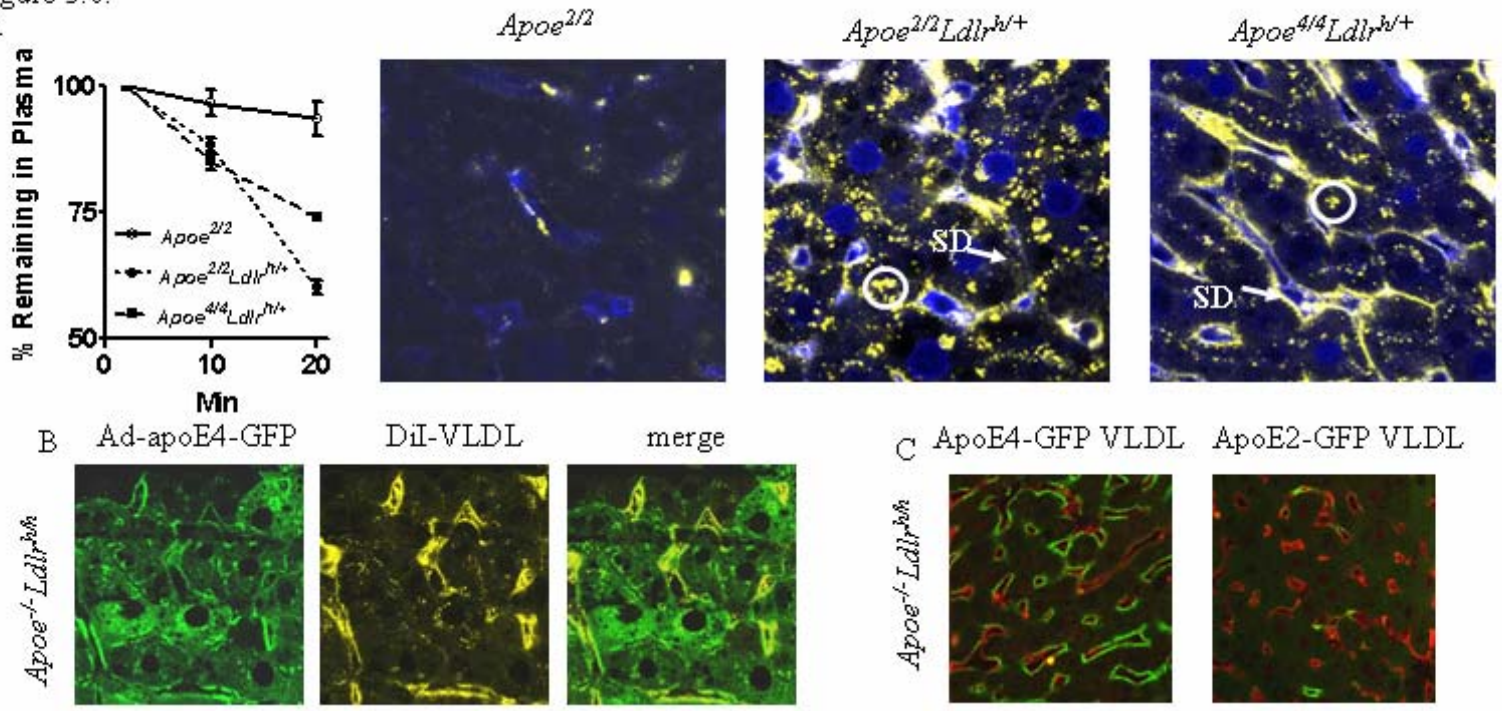
To examine how VLDL, with apoE2 or apoE4 already present on their surface, would localize in the liver, we isolated VLDL fractions from *Apoe*$^{-/-}$ mice 5 days after transfection with Ad-apoE2-GFP or Ad-apoE4-GFP and introduced them into *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice through tail veins. When livers were examined under confocal microscopy 5 minutes after the injection, apoE4-GFP was already localized on hepatocyte surfaces (Figure 5.6C right). This suggests that lipoproteins that aquire apoE4 during the circulation can accumulate in the space of Disse very efficiently. In contrast, very little apoE2-GFP was localized on the hepatocyte surface, but there were a few cells with some GFP signals (Figure 5.6C).

Taken together, apoE4 containing VLDL avidly binds and accumulates in the space of Disse. While apoE4 on the surface of hepatocytes trapped by the LDLR may play roles in sequestering remnant particles to cell surface, they appear to have only a limited capacity to participate in the internalization of remnant lipoproteins. That hepatic surface bound apoE

accumulation inhibits clearance is also supported by the absence of apoE2 on the hepatocyte surface and its association with better VLDL internalization.

Figure 5.6. Effects of ApoE isoform and LDLR levels on localization and clearance of *Apoe*$^{-/-}$ VLDL and apoE-enriched VLDL. A. DiI-labeled *Apoe*$^{-/-}$ VLDL injected into *Apoe*$^{2/2}$, *Apoe*$^{2/2}$*Ldlr*$^{h/+}$, *and Apoe*$^{4/4}$*Ldlr*$^{h/+}$ mice. (first panel) Plasma DiI was measured at 2, 10, and 20 minutes. 2 min was taken as 100%. The liver was removed and analyzed by fluorescence microscopy for DiI-VLDL (*yellow*) distribution. TRITC labeled lectin (blue) shows structure. B. DiL VLDL (250 µg of protein) from *Apoe*$^{-/-}$ mice was injected into the tail vein of Ad-apoE4-GFP infected *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice, and localizes to the SD, and overlaps with apoE4-GFP in the SD. C. VLDL isolated from *Apoe*$^{-/-}$ mice transfected to express Ad-apoE4-GFP or Ad-apoE2-GFP was injected into tail veins of untransfected *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice. 5 min after injection apoE4-GFP (left panel) was visible subendothelially in the space of Disse (SD). ApoE2-GFP (right panel).

Figure 5.6.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Apoe2/2Ldlrh/+
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Apoe2/2Ldlrh/+
Apoe4/4Ldlrh/+
SD
SD
B
Ad-apoE4-GFP
DiI-VLDL
merge
Apoe-/-Ldlrhh
C
ApoE4-GFP VLDL
ApoE2-GFP VLDL
Apoe-/-Ldlrhh

Discussion

The higher binding affinity of the apoE4 isoform to the LDLR than apoE2 or apoE3 has been implicated in leading to increased plasma LDL cholesterol and atherosclerosis in individuals carrying apoE4 [2, 3]. In this study, we used Adenovirus mediated LDLR and apoE-GFP expression in mouse models to investigate the effects of LDLR expression on the localization of the different apoE isoforms in the liver. Our data show that over-expression of LDLR in the liver of mice with apoE4 elevates plasma cholesterol levels while reducing it in mice with apoE2. ApoE4 co-localizes with LDLR on the sinusoidal surface of the liver, and increased LDLR expression enhances this accumulation. In contrast, apoE2 is minimally present on the sinusoidal surface of the liver even when LDLR expression is elevated.

The liver is the main source of plasma apoE, although low levels of extra-hepatic apoE are sufficient for normalization of hyperlipidemia, since *Apoe*$^{-/-}$ mice that were transferred with wild type bone marrows have near normal plasma cholesterol levels [93]. Remnant lipoproteins are cleared in the liver mainly by LDLR and by LRP, but the uptake by these receptors require the remnants to acquire and be enriched with apoE proteins [47, 94-96]. Liver-derived and localized apoE facilitates the receptor-mediated internalization of remnants in the liver [96, 97]. Linton et al demonstrated, using bone marrow transfer of apoE marrow to *Apoe*$^{-/-}$*Ldlr*$^{-/-}$ mice, that intense immunoreactivity for extrahepatic apoE was present on the cell surface and in the space of Disse but the pattern of punctuated staining was not detected within the cytoplasm indicating that no uptake of apoE-containing lipoproteins was occurring [93]. The authors concluded that whereas the LDLR efficiently clears remnant lipoproteins irrespective of the site of origin of apoE, endocytosis by the chylomicron remnant receptor (LRP) is absolutely dependent on hepatic expression of apoE. In contrast, Yu, et al. showed that the chylomicron

remnant clearance by the apoE-deficient liver depends on the amount of apoE associated with the remnant particles and hepatic apoE is not a pre-requisite for clearance, although the addition of hepatic apoE can accelerate their uptake [98]. Previously we showed in mice that all human apoE-isoforms are less effective ligands to non-LDLR mediated clearance of TG-rich lipoproteins than mouse apoE, and that VLDL remnants with very high cholesterol and apoE accumulates in the absence of LDLR [69]. Dependence of remnant clearance on LDLR are likely to be exaggerated in mice with human apoE isoforms.

We earlier observed that mice globally expressing 2-3 times the normal *Ldlr* gene exhibit hypercholesterolemia when fed a HFW diet only when they are also carrying the human apoE allele [39]. We hypothesized that a high-affinity interaction between LDLR and apoE4 in the liver limits the transfer of apoE protein to the TG-rich lipoproteins, limiting their clearance. Over-expression of the LDLR by adenoviral vector raised plasma cholesterol levels only in the $Apoe^{4/4}$ mice, confirming our first prediction based on this hypothesis and demonstrating that the interaction between increased LDLR and apoE4 in the liver is sufficient to cause the hypercholesterolemia. Furthermore, adenoviral over-expression of apoE4-GFP fusion protein was able to overcome this phenotype in HFW fed $Apoe^{4/4}Ldlr^{h/+}$ mice, suggesting that a ratio between apoE4 and LDLR in the liver is an important factor for this phenotype. Neither Ad-LDLR nor Ad-apoE4-GFP expression is physiological as both vectors use a CMV promoter which does not respond to physiological gene regulation. However, since a 2-3 fold decrease in the apoE4/LDLR ratio in $Apoe^{4/4}Ldlr^{h/+}$ mice compared to $Apoe^{4/4}$ mice causes a dramatic phenotypic change [39], a small difference in the basal levels of these two proteins between humans and mice may be sufficient to cause the association between apoE4 and the small increase in plasma cholesterol seen in humans.

The paradoxical association of apoE4 with elevated plasma cholesterol in humans may be due to its high LDLR affinity and a reduced ability to exchange onto and clear TRLs and remnants. In cell culture binding or in-vivo clearance, when apoE is already present on the particle, apoE4 containing particles show enhanced binding and clearance compared to apoE3 and apoE2 [14, 19, 99]. In contrast, apoE deficient-VLDL binding and clearance is reduced when cells or animals express apoE4 compared to apoE3 [14]. Here we show that apoE2, with the lowest LDLR affinity, clears VLDL faster than apoE4 when they are expressed by the liver in-vivo and apoE-deficient particles are used. Thus the clearance of exogenous apoE deficient VLDL is faster when the recipient animal expresses apoE with low LDLR affinity and higher LDLR level. Newly secreted chylomicron and VLDL that have yet to acquire apoE, combined with the reduced plasma apoE level associated with apoE4, increases the plasma retention time of these lipoproteins. This is consistent with the higher post prandial lipemia in apoE4 humans [68] as well as $Apoe^{4/4}Ldlr^{h/+}$ mice on HFW diet [39]. In contrast, the lower LDLR affinity of apoE2 increases its plasma concentration and increases VLDL clearance (Figure 5.7A).

Previous studies have demonstrated the presence of apoE immunoreactivity along the sinusoidal front of hepatocytes together with some punctated cytoplasmic staining in the liver of wild type rat and mouse [100, 101]. Electron microscopic examinations by Hamilton et al further demonstrated that the immuno-gold labeling for apoE clustered on hepatocytic microvilli projecting into the space of Disse[100]. In addition, endosomes near the sinusoidal front and multivesicular bodies in the Golgi/biliary area were labeled intensely with apoE. Confirming these observations, we found intense apoE immunoreactivities demarcating the space of Disse and as punctated perinuclear staining in the liver of both $Apoe^{3/3}$ and $Apoe^{4/4}$ mice. Importantly, a higher expression of LDLR in the $Apoe^{3/3}Ldlr^{h/+}$ and $Apoe^{4/4}Ldlr^{h/+}$ animals enhanced the

intensity of sinusoidal immunostaining relative to the cytoplasmic staining, and appeared to do so more in the liver of mice with apoE4 than with apoE3. We also found intense fluorescence on the hepatocyte surface of *Apoe*$^{-/-}$*Ldlr*$^{+/+}$ mice that received Ad-apoE4-GFP, but the levels in the livers of mice that received Ad-apoE2-GFP showed minimal localization of apoE2 in the space of Disse. Injecting Ad-apoE2-GFP to *Apoe*$^{-/-}$*Ldlr*$^{h/h}$ mice did not increase the apoE2-GFP on the hepatocyte surface. Thus these results clearly demonstrate that a majority of apoE4 but not apoE2 proteins co-localizes with LDLR on the hepatocyte surface, and that the accumulation depends on both affinity to LDLR and on the levels of LDLR.

Neither immunostaining nor Ad-apoE4-GFP expression allows us to dissociate whether apoE accumulating on the hepatocyte surface is newly synthesized by the hepatocyte or originated from lipoproteins in circulation. However, we note that not all the cells demarcated by intense sinusoidal apoE4-GFP signals have strong intracellular GFP signals, suggesting that a substantial part of the apoE4-GFP may be derived from apoE4-GFP synthesized by other infected hepatocytes. This is also consistent with the apoE4-GFP accumulation in the space of Disse of *Apoe*$^{-/-}$ liver shortly after injection of VLDL particles enriched with apoE4-GFP, while no such accumulation of apoE2-GFP was seen (Figure 5.5C). Multiple experiments both in vivo and in vitro have shown that a substantial amount of apoE internalized with TG-rich lipoproteins by the liver are recycled back to the cell-surface and re-secreted [52, 79, 102]. HDL appears to stimulate this process, serving as an acceptor for recycled apoE in hepatocytes [24, 47, 48]. For example, Heeren et al. demonstrated that cell-surface binding and internalization of TG-rich lipoprotein-derived apoE4 are increased compared with apoE3 in HuH7 hepatoma cells [79]. They also showed that HDL-induced recycling of apoE4 is reduced in these cells compared to recycling of apoE3. These experiments were carried out with VLDL enriched with human apoE3

or apoE4 in vitro and human hepatoma cells which synthesize endogenous apoE, while our experiments used genetic models with the VLDL remnants enriched with apoE isoforms in vivo, and recipient livers were either apoE-deficient or making the same isoform. Nevertheless, impaired recycling measured by the reduced re-secretion of apoE4 into medium from the cultured cells relative to that of apoE3 observed by Heeren et al is consistent with the enhanced cellular localization of apoE4 we observed in vivo. Additionally, our experiments showed that the localization of apoE4 on the hepatocyte surface is dependent of LDLR levels and that apoE4 accumulated to this location had enhanced binding but a limited capacity to internalize remnant lipoproteins.

Our results are consistent with and support our "apoE4 trapping by LDLR" as a mechanism to explain the hypercholesterolemia associated with apoE4. Retention of apoE4 at the hepatic surface by LDLR reduces its availability in the plasma to bind to and mediate remnant internalization. We do not know the form of apoE4 interacting with LDLR on the hepatocyte surface at present, but they are unlikely to be free proteins considering the report by Ruiz *et al.* that both LDLR and LRP prefer lipid bound forms of apoE [41]. HDL, which can modulate apoE recycling, and is a source of plasma apoE, is a likely regulator of this process [47, 94, 103-105]. HDL as an apoE carrier may be necessary for timely apoE exchange to other lipoproteins such as TRLs, allowing nascent lipoproteins to enter the plasma without immediate apoE-mediated reuptake and also to remove them before they become slowly cleared remnant LDL. Modulation of HDL levels by LDLR is apoE isoform dependent; increased LDLR expression leads to increased HDL-cholesterol in mice with apoE2 and decreases HDL levels in mice with apoE4 [37, 39]. Elevated HDL associated with apoE2 could enrich apoE-poor VLDL and promote their clearance. In contrast, reduced HDL and apoE4 trapped in the space of Disse,

directs apoE-poor VLDL to remnant conversion rather than internalization. The decreased apoE content on TRLs can increase conversion to remnants by LPL [22, 106-108]. Further studies are necessary to elucidate the form of apoE and potential role of HDL in the isoform-specific interactions between LDLR and apoE and whether other apolipoproteins are involved in these interactions.

In conclusion, our study in mice with apoE isoform and elevated LDLR revealed that there may be a critical range of LDLR levels for proper ratios of cholesterol carried by atherogenic non-HDL lipoproteins and by HDL particles (Figure 5.7B). The optimum range of LDLR levels is different for each apoE isoform and proportional to each isoform's LDLR affinity. For instance, all apoE isoforms mice have elevated VLDL and LDL when the LDLR is absent [69]. With wildtype levels of LDLR, both $Apoe^{4/4}$ and $Apoe^{3/3}$ mice have high HDL and low non-HDL cholesterol [14]. However, $ApoE^{2/2}$ mice have high non-HDL /HDL ratios [34]. When LDLR levels are elevated, HDL cholesterol levels dramatically decrease in both $Apoe^{4/4}$ $Ldlr^{h/+}$ and $Apoe^{3/3}Ldlr^{h/+}$ mice. Since non-HDL cholesterol are elevated only in $Apoe^{4/4}$ $Ldlr^{h/+}$ mice their non-HDL /HDL ratios are critically high, while $Apoe^{3/3}Ldlr^{h/+}$ mice have nonHDL /HDL ratios that are modestly increased. $Apoe^{2/2}Ldlr^{h/+}$ mice have reductions in their VLDL and elevated HDL levels and thus have low non-HDL /HDL. If we apply the same relationships to phenotypes associated with apoE isoforms in humans, one may predict that LDLR activity in humans are in the upper ranges of Figure 5.7B where apoE2 is beneficial and apoE4 is somewhat detrimental. In the lower range of LDLR activities, the decreased affinity of apoE2 may cause apoE2-rich TRLs to accumulate, similar to the 5% of apoE2 homozygous humans who develop type III hyperlipidemia characterized by elevated TG. Careful titration of apoE and LDLR levels

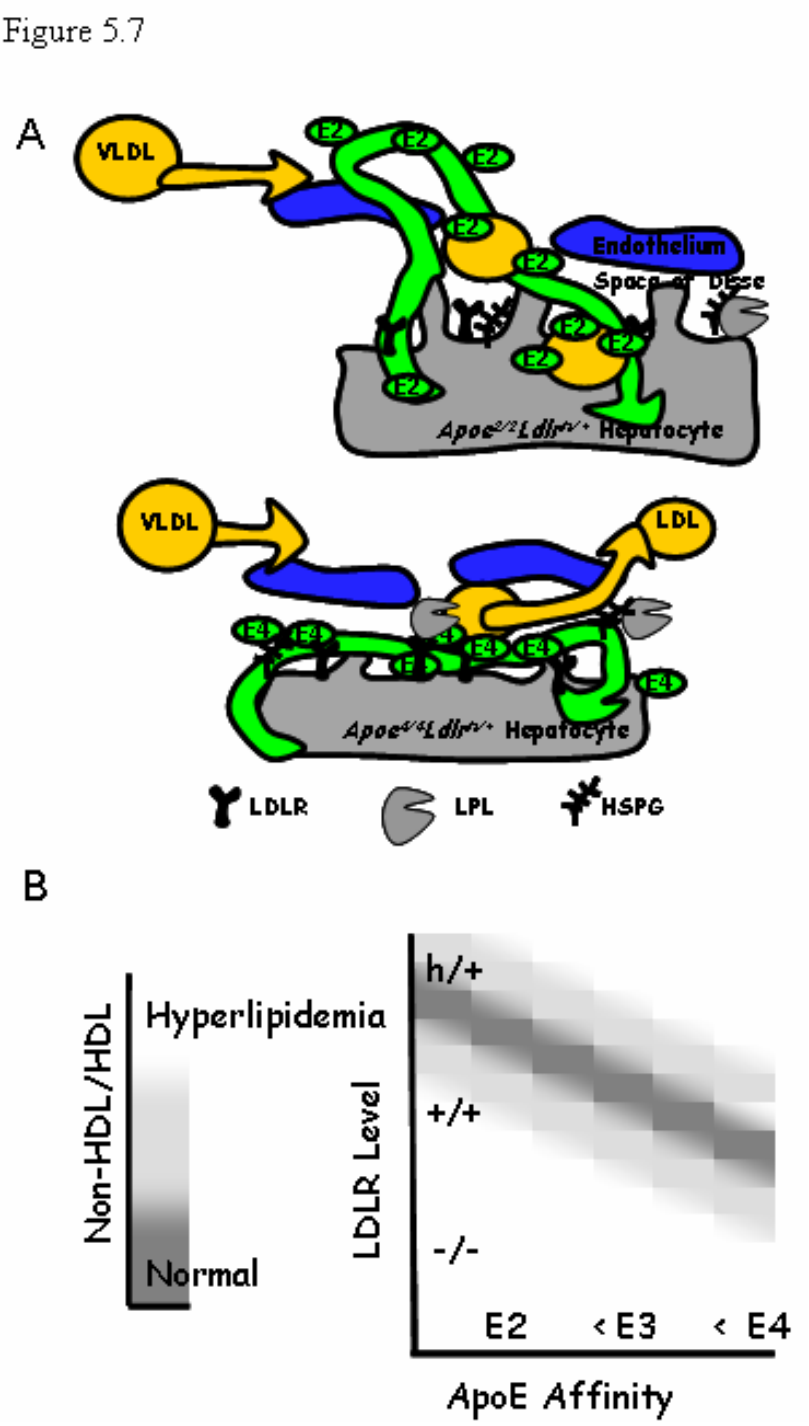
may shed light on the relevance of this mechanism to the well-established associations between the risk for coronary heart disease and apoE isoforms.

Figure 5.7. Hypothetical mechanism to explain apoE LDLR affinity affect on VLDL metabolism.

A.Top diagram shows apoE2 metabolism of apoE-poor VLDL. The lower LDLR affinity of apoE2 increases plasma apoE level (green arrow). This increases the enrichment of apoE2 onto VLDL which facilitates LDLR and HSPG mediated uptake. A. Lower diagram shows apoE4 metabolism of apoE-poor VLDL. High LDLR affinity of apoE4 keeps it bound to the hepatic surface, which decreases VLDL enrichment. VLDL at the hepatic surface are not internalized and subsequently converted to remnants and LDL possibly by LPL.

B. Interaction between LDLR level and apoE affinity and resulting atherosclerosis risk (non-HDL cholesterol /HDL cholesterol) in mice. Legend on the left: darker shaded area shows a decreased non-HDL/HDL. Upper unshaded areas reflect elevated non-HDL/HDL and hyperlipidemia. Panel on the right shows non-HDL/HDL ratio (by amount of shading) in mice, determined by their LDLR level (y-axis) and apoE affinity (x-axis). Shaded areas reflect a good balance between LDLR level and apoE isoform. Unshaded area is a poor non-HDL/HDL and hyperlipidemia. Similar non-HDL/HDL levels occur at different levels of LDLR for each apoE isoform reflected by the change in position of the shaded region.

Figure 5.7


A
VLDL
E2
Endothelium
Space of Disse
Apoe2/2Ldlr+/+ Hepatocyte
VLDL
LDL
E4
Apoe4/4Ldlr+/+ Hepatocyte
LDLR
LPL
HSPG
B
Non-HDL/HDL
Hyperlipidemia
Normal
LDLR Level
h/+
+/+
-/-
E2
< E3
< E4
ApoE Affinity

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Chapter VI

Genetics of Atherosclerosis in Murine Models

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Abstract

The pathology of atherosclerotic lesions that develop in mouse models of atherosclerosis, such as those lacking apolipoprotein E or lacking the low density lipoprotein receptor, is very similar to that seen in human patients. Consequently, genetic approaches to studying atherosclerosis in these mouse models have produced a wealth of information relevant to the genetic factors and pathways that modify the early stages of atherosclerosis in humans. Despite these advances, the later stages of atherosclerosis in humans, including spontaneous plaque rupture and hemorrhage, have not been observed reliably in current mouse models. Increasing sophistication and use of genetic manipulations, however, has produced significant advances in modeling these processes. The use of genetic tools to examine the physiology, pathology, and cell biology of atherosclerosis will enhance elucidation of the pathogenesis of the disease and lead to the development of novel therapeutic strategies.

Introduction

The development of atherosclerosis is largely influenced by genetic factors. It is also modified by a host of non-genetic factors including diet, social habits such as smoking and exercise, and pre-existing diseases such as diabetes and hypertension, though arguably even these factors have genetic components. Thus multiple genetic risk factors exist, and atherosclerosis susceptibility is likely to be determined by unfavorable combinations of small variations in the function or quantity of proteins involved in multiple pathways.

The mouse is a uniquely suited model system for studies of complex diseases. Its overall biology is closely comparable to that of humans and the characteristics of many pathologic conditions are quite similar in both species. The existence of numerous well-characterized and recently sequenced inbred mouse strains provides a rich resource for genetic analysis. Various genetic techniques, like transgenesis and gene targeting, are now common laboratory procedures for generating specific mutations in mice. Lastly, not only can the effects of genes be studied in mice in controlled environments, but also gene-gene and gene-environment interactions can be assessed relatively easily. In this review we discuss genetic models of atherosclerosis and the recent use of the mouse as a genetic tool to identify the genes and pathways involved in various stages of atherogenesis. The use of these genetic models, tools, and techniques will ultimately identify novel targets for the development of therapeutics to treat and hopefully prevent the development of atherosclerosis.

I. Genetic Models of Hyperlipidemia and Atherosclerosis

Atherosclerosis is the leading cause of death in Western society. Elevated concentrations of low-density lipoproteins (LDL) and reduced concentrations of high density lipoproteins (HDL), are both clearly associated with increased risk of atherosclerosis in humans and are significantly influenced by the genetic makeup of the individual [109, 110].

The high prevalence of atherosclerosis among humans contrasts with the fact that none of the common laboratory mouse strains develop atherosclerotic plaques. This is true even when the mice are maintained on diets (high fat Western type, HFW; 21 % fat, 0.15% cholesterol) similar to those consumed by humans in Western societies. This difference in susceptibility is mainly attributed to the different plasma lipid profiles of the two species. For example, the average plasma cholesterol concentration of wild type mice on a normal chow (NC) diet (4.5% fat, 0.022% cholesterol) is approximately 80 mg/dL. Most of this cholesterol is carried by HDL particles. Mice have very low concentrations of LDL and other atherogenic lipoproteins such as remnants of intestinal-derived chylomicrons and liver-derived very low-density lipoproteins (VLDL). This profile is in marked contrast to humans in which most of the plasma cholesterol (200 mg/dL) is associated with apolipoprotein B100 (apoB)-containing LDL particles. These differences are often attributed to variations in lipoprotein metabolism between mice and humans. For example, mice lack cholesterol ester transfer protein (CETP) activity that transfers cholesterol esters from anti-atherogenic HDL to atherogenic LDL and VLDL in humans [31]. In addition, in mice approximately 70% of liver apoB mRNA undergoes post transcriptional editing, resulting in production of apoB48-containing VLDL by the liver. In contrast, humans do not have hepatic apoB mRNA editing activity and secrete only the LDL precursor, apoB100-containing VLDL [29]. However, while the mouse plasma lipoprotein profile is less atherogenic

than the profile in humans, the pathways for cholesterol transport and metabolism in the two species are sufficiently similar to suggest that inducing disturbances in plasma lipoprotein metabolism should trigger atherogenesis in mice. Indeed, some inbred strains like C57BL/6 are susceptible to diet-induced hyperlipidemia and develop fatty streak lesions when fed an atherogenic diet (15.5% fat, 1.25% cholesterol) containing 0.5% sodium cholate [111].

The first genetic mouse models of atherosclerosis were generated by creating mice lacking functional alleles for either apolipoprotein E (apoE) [75, 112] or the LDL receptor (LDLR) [113]. ApoE plays a central role in lipoprotein metabolism and is required for the efficient receptor-mediated clearance of plasma chylomicrons and VLDL-remnants by the liver [16]. Lack of apoE in mice results in accumulation of cholesterol-rich remnant particles and elevation of plasma cholesterol concentrations to about 400 mg/dL, even when fed a NC diet. The apoE-deficient mice (*Apoe*$^{-/-}$) spontaneously develop aortic atherosclerotic plaques similar to those seen in humans [75, 112]. The LDLR is crucial for the removal of apoE- and apoB-containing lipoprotein particles from the circulation. The *Ldlr*$^{-/-}$ mice, like humans deficient for LDLR function, have increased plasma LDL cholesterol, although they demonstrate less overt vascular disease than do the *Apoe*$^{-/-}$ mice, and develop plaques more slowly on NC diet [113]. Feeding *Ldlr*$^{-/-}$ mice a HFW diet significantly accelerates atherosclerosis. These genetically well-defined hyperlipidemic *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mouse models quickly became the primary models for the study of atherosclerosis due to similarities to the human disease and the reasonable rate of disease progression.

Many mutant mice with altered lipoprotein profiles that develop varying degrees of atherosclerosis have since been described, and some of them are listed in Table 1. For instance, transgenic mice over-expressing a human *Apob* gene (HuApoB) in the liver have elevated levels

of circulating LDL and thus have lipoprotein profiles more similar to those of humans [114]. These mice develop severe atherosclerosis when fed an atherogenic high fat diet [115]. Interestingly, transgenic mice over-expressing mouse *Apoa-II* are also susceptible to diet-induced atherosclerosis [116]. Although the role of apoA-II in HDL function is not clear, it appears that the altered ratio of apoA-I to apoA-II in HDL impairs the anti-atherogenic properties of HDL. In addition to increased triglycerides and VLDL, the *Apoa-II* transgenic mice also exhibit traits associated with human insulin resistance syndrome [117].

Genetic manipulation in mice to create models of specific human genetic conditions has also been used to provide insight into the mechanisms of the corresponding human condition. While complete lack of apoE is extremely rare in humans, some apoE protein variants are associated with premature atherosclerosis [16]. Type III hyperlipoproteinemia is a condition characterized by elevated plasma cholesterol and triglycerides and an increased risk of premature atherosclerosis. Both dominant and recessive inherited forms of Type III hyperlipoproteinemia are known in humans. The dominant inherited form is caused by defective apoE proteins, such as apoE3-Leiden or apoE (Arg112, Cys142), and is associated with a plasma accumulation of VLDL [78]. Transgenic mice carrying these variant apoE genes are susceptible to diet-induced atherosclerosis [118, 119]. The recessive form of Type III hyperlipoproteinemia occurs in approximately 5% of humans who are homozygous for *APOE*2*, one of the three common alleles of *APOE* in humans [23]. The apoE2 isoform binds with lower affinity to the LDLR than do the other two common isoforms, apoE3 and apoE4 [13-15]. Because not all *APOE*2* homozygotes develop hyperlipoproteinemia, other factors are thought to be necessary for manifestation of this condition in humans [23]. Yet in targeted mice all the homozygous *Apoe*$^{2/2}$ mice that express the human apoE2 isoform in place of mouse apoE exhibit Type III

hyperlipoproteinemia regardless of their gender, age, or sex [34]. Additionally, all *Apoe*$^{2/2}$ mice develop atherosclerosis that is significantly accelerated by a Western style high fat diet (HFW), while mice with apoE3 and apoE4 do not [14, 33, 34]. *Apoe*$^{2/2}$ mice may reveal the causative factor for Type III hyperlipoproteinemia because the precise species difference necessary to precipitate the phenotype in some humans is apparently already present in these mice [34].

The *Ldlr*$^{-/-}$ mouse is a model of human familial hypercholesterolemia (FH) in which mutations in the *Ldlr* gene are the major cause of the autosomal dominant form of the disease [120]. In addition, gain of function mutations in the *Pcsk9* gene coding for pro-protein convertase subtilisin/kexin type 9 cause some rare autosomal dominant cases [121]. PCSK9 mediates LDLR degradation and therefore its activity is a major determinant of plasma LDL concentrations in humans [122]. Mice over-expressing PCSK9 in the liver show accelerated degradation of LDLR, and consequently have markedly increased plasma LDL concentrations [123, 124]. Autosomal recessive hypercholesterolemia (ARH) was shown to be caused by loss-of-function mutations in the *Arh* gene that encodes an adaptor protein that is important for the internalization of LDLR [125]. Although plaque development has not been reported, both the transgenic mouse overexpressing human PCSK9 and the mouse lacking ARH are likely to be excellent models of atherosclerosis since they show plasma lipoprotein profiles similar to the *Ldlr*$^{-/-}$ mice.

In aggregate the data from these studies suggest that once mice have plasma total cholesterol concentrations of over 200 mg/dL, and the cholesterol is primarily in non-HDL particles, they will eventually develop atherosclerosis. However, the data provide no compelling evidence that any particular model is more ideal than others for studying the overall process of atherogenesis. The predominance of studies employing *Apoe*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice

in recent years likely reflects the relative ease of using mice with single gene defects and the fact that these models were the first to be made available. However, it is important to bear in mind the specific mutations or deletions underlying the dyslipidemia in individual models, as certain models may be uniquely suited for the study of particular pathways that contribute to the atherosclerotic process.

Table 6.1. Mouse Models of Hyperlipidemia and Atherosclerosis

Models	Mutation	Diet[a]	Notes	reference
C57BL/6	Wild type inbred	HFC	Relatively more susceptible to diet-induced atherosclerosis than other common inbred strains of laboratory mice.	[111]
Apoe$^{-/-}$	Homozygous KO	NC	Elevated cholesterol-rich remnants, and spontaneous atherosclerosis	[75, 112]
Apoe$^{+/-}$	Heterozygous KO	HFC	Severe atherosclerosis only when fed high fat diet.	[126, 127]
Ldlr$^{-/-}$	Homozygous KO	HFW	Elevated LDL-cholesterol and severe atherosclerosis on high fat diet.	[113]
CETP tg	Transgene	HFC	Accelerated diet-induced atherosclerosis.	[128]
HuApoB tg	Transgene	HFC	Elevated LDL cholesterol and severe diet-induced atherosclerosis	[114]
ApoAII tg	Transgene	HFC	High plasma triglyceride levels. Increased diet-induced atherosclerosis.	[116]
ApoE3Leiden, ApoE (Arg112,Cys142) tg	Transgene	HFW	Type III hyperlipoproteinemia and severe atherosclerosis	[118, 119]
Apoe$^{2/2}$	Gene replacement	HFW	Type III hyperlipoproteinemia and severe atherosclerosis in homozyotes.	[34]
Apoe$^{4/4}$ *Ldlr*$^{h/+}$	Gene replacement	HFW	Accumulation of CH-rich, TG poor remnants and severe atherosclerosis only when mice are on high fat diet.	[39]
Srb1$^{-/-}$, *Apoe*$^{-/-}$	Double KO	NC	Very severe atherosclerosis and death at 5 weeks of MI. Treatment with probucol prolongs the life span to 40 weeks	[129]
sg/sg (*ROR*$\alpha^{-/-}$)	Natural mutant	HFC	Staggerer mouse has a mutation in nuclear receptor RORα. hypoalphaproteinemia, neurodegeneration	[130]
KOR-SHL	Natural mutant	NC	Hyperlipidemic and develop severe xanthomas, Spontaneous deletion of the *Apoe* gene.	[131]

a: Diets necessary to induce atherosclerosis; NC; regular mouse chow generally containing 4.5% (w/w) fat and 0.02% (w/w) cholesterol, HFW; Western type high fat diet containing 21.5% (w/w) fat and 0.15% cholesterol; or HFC, atherogenic diet with 15.5% (w/w) fat, 1.25% (w/w) cholesterol and 0.5% (w/w) sodium cholate.

II. Quantitative Trait Loci Mapping to Identify Atherosclerosis-Susceptible Genes

The atherosclerotic process is altered by interactions among products of genes from throughout the genome, many of which have likely not yet been discovered. Mapping the location of significant quantitative trait loci (QTL) for atherosclerosis in mice appears particularly promising due to the number of inbred strains available and because the mouse genome is relatively well annotated with markers. To this end the Jackson Laboratory, using 43 inbred mouse strains, employed large scale, comprehensive phenotyping efforts to find among these strains differences in a number of metabolic and disease parameters [132]. The large number of strains was chosen specifically to mimic human genetic diversity. Mice were fed a high fat, high cholesterol, cholate-containing diet; and body weight, percent body fat, percent lean mass, bone mineral density, total cholesterol, HDL, triglycerides, glucose, leptin, and insulin were measured. There was a large range in the measured parameters, and certain sets of characteristics assorted in a strain-dependent manner. Notably, CAST/EiJ, CBA/J, and MSM/Ms strains were identified as mimics of the human metabolic syndrome, for which a mouse model is currently lacking [132]. Results from this study and many other smaller phenotyping efforts are available from the Mouse Phenome Project's database at www.jax.org/phenome. In parallel, a large-scale effort to develop a sizeable group of recombinant inbred strains useful for mapping complex disease QTLs, including atherosclerosis, is on-going [133].

In addition to wild type inbred mouse strains, the *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice on several different genetic backgrounds have been generated and intercrossed to map genetic loci that modify atherosclerosis characteristics or susceptibility [134, 135]. A large number of QTL maps have been created from these crosses. They have identified multiple chromosomal loci

for new QTLs as well as QTLs that span or confirm previous reports. Used individually, or combined with human QTL maps, they promise to accelerate atherosclerosis-related gene and therapeutic target discovery. As mouse genome sequencing efforts expand and marker density increases, the ability to pinpoint causative genetic variations for these QTLs will continue to improve.

While it is often difficult to definitively identify a causative gene for atherosclerosis susceptibility based on QTL mapping studies, some genes have been identified in recent years. For example, the gene coding for A20, a regulator of nuclear factor-kappa B (NFκB), on chromosome 10 was identified using crosses between susceptible (C57BL/6J *Apoe*$^{-/-}$) and atherosclerosis resistant (FVB/N *Apoe*$^{-/-}$) mice [136]. Interestingly, despite C57BL/6J *Apoe*$^{-/-}$ being the susceptible strain in this cross, the C57BL/6J-A20 allele conferred resistance to atherosclerosis. A20, also called tumor necrosis factor α induced protein 3 (TNFαip3), terminates activation of NFκB following its stimulation by various agents including TNFα. A Glu at position 627 in C57BL/6J-A20 introduces a putative casein kinase 2 phosphorylation site making it a less effective terminator of TNFα-stimulated NFκB activation than the FVB/N-A20 with Ala at 627. Recently, common A20 polymorphisms in humans have been shown to result in significant differences in A20 expression that are associated with the susceptibility to coronary artery disease in people with type 2 diabetes [137]. In this case, the QTL mapping of A20 in mice led to the detection of a set of polymorphisms in humans that are likely to play a role in human atherosclerosis susceptibility.

A second example of gene identification through QTL mapping is the determination of the gene underlying *Ath1*. The QTL designated *Ath1* was originally mapped on

chromosome 1 in mice fed an atherogenic diet [138]. The C57BL/6J strain was atherosclerosis susceptible and C3H/HeJ and BALB/cJ strains were resistant [139]. Earlier studies suggested the *Prdx6* gene, coding for peroxiredoxin 6, a thiol-specific antioxidant protein, as a likely candidate for the causative gene in the *Ath1* QTL. Elevated *Prdx6* expression levels were associated with both resistant strains and with smaller lesions within strains. Additionally, the most susceptible strain, C57BL/6J, had an Ala124Asp substitution in Prdx6 [139]. However, subsequent studies of mice overexpressing *Prdx6* showed no protection against atherosclerotic plaque development [140]. Eventually, the powerful combination of mouse genetics and human association studies helped to find a convincing candidate for *Ath1*, and led to the identification of *Tnfsf4* by Wang et al. [141]. *Tnfsf4,* coding for Ox40 ligand (Ox40L), is expressed in the aorta and the level of its expression was significantly higher in the atherosclerosis susceptible C57BL/6J strain than in the resistant C3H/HeJ strain. Furthermore, mice overexpressing the Ox40L developed more atherosclerosis than wild type mice, and mice lacking Ox40L were protected from atherosclerosis. In support of the potential atherogenic role of Ox40L, a mouse atherosclerosis QTL was found on chromosome 4 near the gene coding for Ox40, the receptor for Ox40L [135]. In addition, a human mutation association study showed that mutations in Ox40L are predictive of myocardial infarction risk [141]. Together this evidence suggests that *Tnfsf4* is likely to be the gene underlying *Ath1*. The Ox40L-Ox40 pathway is known to control lymphocyte proliferation and survival. Finally, monoclonal antibody inhibition of Ox40L-Ox40 signaling effectively decreased atherosclerotic lesions more than 50% in *Ldlr*$^{-/-}$ mice [142]. The *Ath1* story is a lesson in perseverance and demonstrates the power of utilizing human association studies in conjunction with mouse

genetics to identify the gene responsible for a QTL as well as test potential targets for anti-atherosclerosis therapy.

A third example used QTL mapping to identify 5-Lipoxigenase (5LO) as an atherosclerosis susceptibility gene located on chromosome 6. 5LO is the rate-limiting enzyme in leukotriene synthesis and is expressed primarily in leukocytes, including monocytes and macrophages. Leukotrienes are potent pro-inflammatory lipid mediators and 5LO could play a role in atherosclerosis by regulating these pathways [143]. The QTL was mapped in a C57BL/6J x CAST/Ei F2 population [144]. Two conserved 5LO amino acid changes were associated with reduced 5LO expression in the congenic C57BL/6J mice carrying the CAST atherosclerosis 5LO allele [143]. In addition, the 5LO hemizygous *Ldlr*$^{-/-}$ mouse model showed significantly smaller atherosclerotic lesions. However, 5LO is not completely responsible for this QTL. Creation of reciprocal subcongenic lines (in this case a C57BL/6J mouse with just the locus of interest derived from CAST/Ei and visa versa) showed that two other loci within the same QTL, named *Ath37* and *Ath38*, play a significant role in the effect of this QTL [145]. Although rare to find three sizeable effect QTLs so close together, it is not uncommon for the large intervals found in most QTLs to subdivide into multiple responsible loci. While gratifying to find one responsible locus for each QTL, the likelihood of multiple genes being responsible is significant when the QTL is large.

Causal relationships between the majority of the atherosclerosis QTLs and the responsible strain-dependent genetic variants for atherosclerosis susceptibility remain to be proven. Nevertheless, with the completion of the human and multiple mouse genome sequences, as well as genome sequencing of other species, comparative genetics will

facilitate genome-wide mapping approaches and the identification of new genes and pathways.

III. Testing Candidate Genes by Intercross of Genetically Altered Mice

A highly effective approach to test the contribution of a candidate gene to atherosclerosis development in mouse models is to cross *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice with mice carrying the candidate mutation. The contributions of many such tested loci to atherosclerosis development in mouse models have been extensively reviewed [146-154]. The majority of the mutations tested in this manner have been loss of function mutations, which are rare in humans. In addition, because plaque rupture and subsequent thrombi are very rare in *Apoe*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice, current evaluations of genetic modifiers in mice focus mainly on the development of plaques and their progression to complex lesions.

Mouse models have confirmed the importance of inflammatory processes in atherogenesis and helped identify potential therapeutic targets. Inflammatory processes are crucial in all stages of atherosclerosis [72]. Initiation of the fatty streak is thought to involve monocyte recruitment, rolling, adherence, transendothelial migration and activation. Absence of monocyte chemoattractant protein-1 (MCP1) decreases lesion size in *Ldlr*$^{-/-}$ mice by at least 20% [44, 155]; absence of its receptor, chemokine receptor 2 (CCR2), limits lesion formation and development in *Apoe*$^{-/-}$ mice [156, 157]. Since plaques still develop in the *Ccr2*$^{-/-}$ *Apoe*$^{-/-}$ mice and continue to mature with time, the MCP1-CCR2 system cannot be the only pathway that is important for the recruitment of monocytes during the initiation of an atheroma. Other chemokines, cell adhesion molecules and various integrins are also involved in the recruitment and migration of monocytes.

Fatal vascular events in humans are rarely the result of lumen occlusion by the developing plaque. Instead they result from plaque rupture that leads to formation of occlusive thrombi and subsequent tissue ischemia. Plaque rupture and thrombosis followed

by infarction and heart failure is rarely seen in mice. However, evidence of unstable plaques such as low collagen content and thinning and disruption of the fibrous cap have been observed in several models [158-160]. In addition, studies demonstrating intraplaque hemorrhage without thrombosis in advanced lesions in the brachiocephalic artery of *Apoe*$^{-/-}$ mice show that lesions in this model progress to the late unstable stages seen in human plaque development [161]. Indeed, plaques in high fat fed *Apoe*$^{-/-}$ mice often show evidence of healed or buried plaque ruptures [161-163]. Ruptures in mice may often heal without the incidence of pathologic thrombosis [163]. Lack of consistent pathologic thrombosis and infarction in murine atherosclerosis models may be due to species and strain differences in circulation and coagulation.

At least one mouse model has shown signs of plaque rupture with resulting myocardial infarction and heart failure. Severe atherosclerosis accompanied by heart disease and early death was observed in *Apoe*$^{-/-}$ mice that also carried a homozygous deletion of the high-density lipoprotein receptor SR-BI (scavenger receptor class B, type I) [129]. The *Scarb1*$^{-/-}$*Apoe*$^{-/-}$ mice develop coronary artery occlusions with complex fibrin-containing lesions. The dramatically accelerated occlusive atherosclerotic disease observed in these mice may be caused by an alteration in their lipoprotein metabolism leading to decreased biliary cholesterol, increased plasma cholesterol and decreased reverse cholesterol transport [129, 164]. Treatment with probucol, a drug with antioxidant and cholesterol-lowering effects, prevented early coronary heart disease and death in the *Scarb1*$^{-/-}$*Apoe*$^{-/-}$ mice, and extended their life span from 6 weeks to an average of 36 weeks [129, 164]. Although it is not clear if thrombosis contributes to their myocardial infarctions, the *Scarb1*$^{-/-}$*Apoe*$^{-/-}$ mouse is currently the only model that demonstrates early atherosclerotic death. Interestingly, they

combine mutations from two fundamental pathways in atherosclerosis development-hyperlipidemia and monocyte cholesterol handling.

Interpretation of the data obtained from intercrosses of mutants generated by genetic engineering must be undertaken only with careful consideration of the genetic background, age, gender and diet of the mice, as these factors can significantly influence the experimental results. When the effects of a specific mutation itself are small and/or they interact with other yet-to-be-identified factors, the results may vary from one experiment to another due to other influences. For example, the role of inducible nitric oxide synthase (*Nos2*) on atherogenesis has been investigated by four different groups. Two groups reported a significant reduction of plaque development in the *Nos2*$^{-/-}$*Apoe*$^{-/-}$ mice [165, 166] while two others reported no change [167, 168]. Diet, age, and genetic background of the mice used by the four groups differed slightly and likely influenced the outcome. Effects can also be gender specific, as reported by Whitman *et al.,* who found that interferon-gamma (IFN-γ) deficiency had no effect on lesion size in female *Apoe*$^{-/-}$ mice, but profoundly decreased it in males [169]. In contrast, Matsui *et al.* observed that lack of osteopontin, a non-collagenous adhesive protein, decreased atherosclerosis in female *Apoe*$^{-/-}$ mice but had no effect in males [170]. In a cross of *Ldlr*$^{-/-}$ mC57BL/6J x fFVB and mFVB x fC57BL/6J mice, both lineage specific and sex-specific effects were apparent suggesting that genetic imprinting and hormone mediated effects are likely both important [171]. The mechanisms underlying the sex-limited effects of these factors are not known, but constitute an important area for future studies.

IV. Tissue Specific and Temporal Gene Modification

Tissues of both the vessel wall and the bone marrow contribute to the atherosclerotic process. The involvement of certain cell types at different stages of plaque development suggests that certain genetic pathways, and therefore the function of certain genes, are temporally relevant. Mouse models with tissue-specific or temporal gene expression can test the contribution of certain cell types to plaque progression, or their function at different stages of atherosclerosis development, respectively. Dissecting the spatial and temporal functions of the molecules, cells, and pathways involved in atherogenesis will be critical for understanding the disease process and for developing new therapeutic strategies.

Bone marrow transfer (BMT) protocols are widely used to investigate macrophage-dependent pathways that contribute to the atherosclerotic process. Reconstitution of lethally irradiated atherosclerosis-prone mice, such as *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice, with bone marrow cells from mice carrying additional mutations have allowed investigators to separate the function of gene products in the bone marrow derived cells from their function in the tissues of the recipient animals. For example, proteins involved in lipid metabolism such as LDLR have established functions in the liver as well as macrophages. BMT allows their tissue-specific roles to be examined in-vivo. Macrophage LDLR binds LDL and VLDL/chylomicron remnants, and the resulting lipid uptake can induce foam cell formation in culture [85, 172]. BMT studies have shown that wild type mice that received wild type bone marrow cells develop larger diet-induced plaques compared to the mice that received *Ldlr* $^{-/-}$ bone marrow cells [57, 85]. However, macrophage expression of the LDLR did not significantly increase atherosclerosis in the hyperlipidemic setting when *Ldlr* $^{-/-}$ recipient mice were given wild type bone marrow cells [86, 173].

We recently showed that LDLR expression affects atherosclerosis development but in an apoE isoform dependent manner. Thus in *Apoe*$^{4/4}$ *Ldlr*$^{-/-}$ mice, the replacement of native bone marrow cells with those expressing LDLR increased atherosclerotic lesions in a dose dependent manner [38]. In contrast, atherosclerosis was not altered in *Apoe*$^{3/3}$ *Ldlr*$^{-/-}$ mice that express the human apoE3 isoform, when the level of macrophage LDLR expression was varied. Results of these BMT experiments suggest that the interaction of macrophage LDLR expression with *Apoe* genotype may contribute to the association in humans between the apoE4 isoform and an increased cardiovascular risk.

Genetic models that employ BMT are also useful for examining the atherogenic role of a gene product in hematopoietic cells when mice lacking the product fail to thrive. For example, mice lacking cyclooxygenase 2 (COX2) die before weaning age [174]. Burleigh *et al.* were able to generate *Ldlr*$^{-/-}$ mice lacking macrophage COX2 by transplanting fetal liver cells from the COX2 deficient pups into *Ldlr*$^{-/-}$ adults [174, 175]. The *Ldlr*$^{-/-}$ mice that received the COX2-deficient bone marrow cells had significantly smaller atherosclerotic lesions than control mice receiving wild type bone marrow cells. The investigators concluded that *Cox2* expression in macrophages is pro-atherogenic, and provided further confirmation of the anti-atherosclerotic effects of COX2 inhibition.

Tissue-specific gene modification is a useful technique for determining the role of a gene product in certain tissues, or when global modification is embryonic lethal. The Cre/loxP system can be used to delete or activate a gene of choice in a tissue-specific manner. An excellent example in a mouse model of atherosclerosis is the use of this system to achieve smooth muscle cell-restricted deletion of the LDL receptor-related protein 1 (LRP1). Lack of LRP1 is embryonic lethal in mice. Therefore, Boucher *et al.* used the

Cre/loxP system to generate viable *Ldlr*$^{-/-}$ mice that lack LRP1 only in vascular SMCs [176]. They found that absence of LRP1 in SMCs resulted in the thickening of vessel walls that normally occurs as a consequence of SMC proliferation. Feeding a Western-type high fat diet to these mice increased their atherosclerosis burden as much as 10 fold compared to *Ldlr*$^{-/-}$ mice with intact LRP1 in their SMCs. In addition, a significant aneurysm-related dilatation of the aorta was observed in association with large plaques. The authors further demonstrated that LRP1 forms a complex with a receptor for the platelet-derived growth factor (PDGF) and controls activation of the receptor. Thus, models of tissue-specific gene deletion helped show that LRP1 plays a pivotal role in protecting the vascular wall and preventing atherosclerosis by suppressing SMC proliferation.

Combining tissue-specific deletion of a gene with BMT allows researchers to ask sophisticated questions about the mechanisms of atherogenesis. Transcription factors, such as peroxisome proliferator gamma (PPARγ), often have distinct roles in different cell types. Deletion of the *Pparγ* gene in mice is lethal in the developing embryos [177-179]. Babaev *et al.* generated mice with macrophage-specific deletion of *Pparγ* using the Cre/loxP system. Using BMT, the authors were able to demonstrate that the *Ldlr*$^{-/-}$ recipients of bone marrow with macrophage - PPARγ $^{-/-}$ cells had significantly larger atherosclerotic lesions than mice receiving bone marrow with wild type macrophage cells. The authors concluded that *Pparγ* expression in macrophages is anti-atherogenic [180]. The pleiotropic nature of molecules such as PPARγ and their prospect as therapeutic targets underscores the importance of detailing their tissue specific functions.

While some genes have important tissue-specific functions, other genes may be important at different stages of the disease process. Advanced human plaques have thick

fibrous caps and high collagen content. Degradation and remodeling of the extracellular matrix in these advanced lesions likely determines their vulnerability to rupture. Macrophages are believed to regulate plaque stability through their release of matrix metalloproteinases (MMP) [181-183]. Gough et al. transferred bone marrow cells that overexpress an auto-activating form of MMP-9 into older apoE-deficient mice with existing atherosclerosis. Overexpression of MMP-9 led to plaques with disrupted fibrous caps, increased fibrin deposits and intraplaque hemorrhage in the brachiocephalic artery and the aortic arch [184].

Modeling the temporal effect of gene expression has also been investigated using drug-inducible changes of gene expression, such as by doxycycline, that can reversibly control transgene expression [185, 186]. Environmentally produced reactive oxygen species (ROS) have been implicated in atherosclerosis; however, the contribution of metabolically produced ROS from oxidative phosphorylation in mitochondria is not clear. Uncoupling protein 1 (UCP1) expression lowers the efficiency of mitochondrial ATP production by increasing mitochondrial matrix proton permeability. Bernal-Mizrachi *et al.* created transgenic mice with doxycycline-inducible expression of UCP1 in SMCs and crossed them with *Apoe*$^{-/-}$ mice [187]. UCP1 expression measurably increased the metabolic production of ROS in the aorta and lowered nitric oxide levels, which led to an elevated blood pressure and increased atherosclerosis, confirming that metabolically-produced ROS can modulate the risk of atherosclerosis.

V. Humanizing the Mouse Systems.

In some mouse models of atherosclerosis the phenotype does not precisely model all aspects of the human disease, even when genetic defects identical to those occurring in humans are introduced into the mouse genome. Humans are genetically diverse, while most studies in mice utilize a genetically uniform population. In certain cases, altered atherosclerotic susceptibility between humans and genetic mouse models may be due to differences in the concentration, activity, or pattern of expression of a particular homologous protein. Novel techniques have been used to create genetic alterations in mice that can reveal and begin to overcome some of these difficulties. As human therapeutics become increasingly tailored to certain mutations and protein isoforms, mouse models that contain the human target, or replicate the human physiological system, will allow us to better determine their mechanism of action, efficacy, and safety.

We tested whether mice can faithfully recapitulate a component of human lipid metabolism by replacing the mouse *Apoe* gene with the three human alleles [14, 33]. In humans the *APOE* alleles are strongly associated with both the plasma concentration of LDL-cholesterol and the risk of atherosclerosis, (in the order *APOE*2 < APOE* 3 < APOE* 4*) [3]. This association between apoE-isoforms and plasma lipoprotein profiles is one of the best predictors of cardiovascular disease in humans [2, 3, 188]. However the mechanisms underlying this association are still not clear since apoE4 binds to the receptor with a slightly higher affinity than apoE3 while apoE2 binds to the receptor with much less affinity than apoE3 or apoE4 [15]. A widely held explanation for this observation is that the high affinity of apoE4 for the LDLR leads to an increase in apoE-mediated cholesterol uptake and subsequent down-regulation of the *Ldlr* gene. Low LDLR levels in humans with apoE4

could account for their elevated plasma LDL and increased atherosclerosis. Conversely, the low affinity of apoE2 is thought to lead to up-regulation of *Ldlr* and decreased plasma LDL [3, 19].

Unexpectedly, mice expressing human apoE2, E3 or E4, in place of mouse apoE, recapitulate the human relationship, but only when the mice also express a high level of the human LDLR [37, 39]. Thus, in contrast to the current hypothesis, an increased expression of the LDL receptor in the mice with human apoE4 caused a marked accumulation of cholesterol-rich, apoE-poor remnants in plasma and severe atherosclerosis. We hypothesize that the LDLR can trap apoE4, and reduce its availability for transfer onto lipoproteins, thus delaying their clearance, which increases the plasma concentration of slowly-cleared apoE-poor remnants. The overall consequence is that increased LDLR expression is pro-atherogenic in mice with apoE4 and anti-atherogenic in mice with apoE2.

In humans who carry the *APOE*4* allele, the phenotypic effect is significant but not as pronounced as we observe in genetic mouse models. Nevertheless, the adverse effect of the apoE4-LDLR interaction in remnant clearance observed in mice can explain, at least in part, the increased risk of atherosclerosis in humans associated with the *APOE*4* genotype. Our explanation is also consistent with observations that humans carrying *APOE*4* have prolonged post-prandial lipemia [2, 64, 68]. This work suggests that humans and mice differ in their relative use of apoE and LDLR in lipoprotein metabolism. It also provides an example that species differences should not be simply dismissed, because they can reveal new insight into old problems.

These studies demonstrate that genetic models of atherosclerosis that humanize the mouse can potentially enhance our understanding of the human disease process. This

approach may be particularly important when studying therapeutic targets to overcome species-specific differences in the function of particular gene products. For instance, investigating therapeutic monoclonal antibodies (Mab) in mouse disease models is complicated because the target protein may differ from the human homolog. Recently mice with humanized VEGF-A, generated by gene-targeting, were used for testing the efficacy of Mab directed against VEGF [106]. In contrast to the cell culture promise of anti-angiogenesis, anti-VEGF Mab had little effect on tumourogenesis in the in-vivo model, but did cause kidney damage. This model also shows the potential utility of genetic mouse models in testing toxicity and efficacy in the early stages of drug development.

Differences between the human and mouse immune systems result in other challenges to using genetic mouse models for disease study and drug discovery. The role of the immune system in atherogenesis is clearly established, but the divergence of genes in the two species has resulted in differences both in the innate and adaptive immune systems. For example, mouse A20-binding inhibitor of NFκB-3 (ABIN-3), induced selectively by the anti-inflammatory cytokine IL-10, is incapable of inhibiting murine NFκB while human ABIN-3 protein does inhibit human NFκB [189]. One solution would be to replace divergent mouse factors with their human counterparts. For example, replacement of the mouse immune system by transferring human hematopoietic stem cells into immunodeficient NOD/SCID mice can be used to model human immune responses in mice [190].

Transgenic mice carrying a large fragment of human genomic DNA often yield more human-like temporal and spatial expression of a transgene when compared with the endogenous mouse gene. To this end, techniques have been developed to replace a large segment of the mouse genome with the syntenic human region [191]. Finally, although not

yet practical for general use, transmission of a whole human chromosome to a mouse has been demonstrated in a transspecies aneuploid mouse line carrying an almost complete human chromosome 21 that models aspects of human Down syndrome [192].

VI. Identifying targets in atherosclerotic disease using systems biology approach.

Large scale genome analyses and genetic manipulations using transgenic expression, knockout, and gene replacement technologies have led successfully to a recent explosion in our understanding of the in-vivo molecular and cellular mechanisms of atherosclerosis. Assessing the contribution of a single target and grouping genes and their products according to their functional pathophysiology, however, is somewhat tedious. Scientists are beginning to look beyond the analysis of single genes, proteins, or lipids to study the function of these individual constituents as components of a larger integrated biologic system. Systems biology has the potential to delineate the components of a given system, define the interaction among those components, and thereby help explain the behavior and function of the system. Recent publications detail the systems biology approach to studying atherosclerosis [193-195].

Ideally, the study of a complex disease process using systems biology would incorporate relevant data and information about the genome, transcriptome, proteome, metabolome, and phenome. To date, integrating these diverse data sets and other methodology challenges has limited the systems biology approach to the examination of interactions among a smaller subset of these data sets. Nevertheless, the power of this approach to studying a complex disease such as atherosclerosis is evident. For example, King et al. performed comprehensive micro-array gene expression analysis of human coronary artery atherosclerotic lesions and then developed pathway generation and network analysis tools to integrate their expression profiles with previously known information [196]. This approach allowed the authors to establish a network of interacting genes relevant to the disease process. It also identified “nexus” genes that are pivotal components of the network and therefore are interesting targets both for understanding the disease process and for pharmaceutical development. Similar array and quantitative PCR-

based approaches were used with human samples to construct pathways with known and novel components that are important to the response of endothelial cells to oxidized phospholipids and to the process of in-stent restenosis, again identifying interesting therapeutic targets [197, 198]

Investigators are beginning to apply systems biology techniques to mouse genetic models of atherosclerosis. For example, de Roos et al. used a proteomics approach in *Apoe*$^{-/-}$ mice to examine the pattern of differentially expressed hepatic proteins in response to dietary isomers of conjugated linoleic acid [199]. They identified isoform-specific regulation of certain proteins and metabolic pathways as well as isoform-dependent linkages to pathways that contribute to insulin resistance and the inflammatory response. Similarly, Mayr et al. compared wild-type and *Apoe*$^{-/-}$ aortic tissue using proteomics combined with metabolomics to describe a set of atherosclerosis-related alterations in proteins and metabolites [200]. The results identified components of pathways in energy metabolism, oxidative stress, inflammation, and the immune response that contribute to atherogenesis.

Genetic mouse models of atherosclerosis are also yielding gene expression profiles associated with this disease. A recent study used the F2 mice from an intercross of sensitive and resistant strains and compared the gene expression profile of bone marrow-derived macrophages from animals with large or small lesions [201]. The researchers used the data to identify transcription factor binding sites, within promoter elements, that were differentially associated with either large or small atherosclerotic lesions. In combination with QTL data previously generated, they used the gene expression data to propose two candidate gene products, Williams-Bueren syndrome protein (Wbscr) and adaptor protein with pleckstrin and SH2 domains (Aps), as possible modifiers of lesion size.

In another study, gene expression data was collected from mouse strains with varying susceptibility to atherosclerosis, on a normal or high fat diet, and at defined points along the course of the disease [202]. Appropriate tools were developed and applied to confirm the involvement of known genes and to identify novel genes as well as molecular and biologic pathways involved at various stages of the atherosclerotic process. They combined these data with the expression data derived from the study of coronary artery disease in humans to identify subsets of differentially expressed genes particularly relevant to describing disease progression and severity and distinguishing between native and in-stent atherosclerotic processes [197].

These examples highlight the potential of the systems biology approach to identify novel targets and pathways that contribute to de novo atherosclerosis and related pathologic processes. This approach will help to define the complex relationships among, and regulation of, the functional components of atherosclerosis and other complex diseases. A single study can generate numerous possible targets to explore. As methodology improves, these studies are becoming more feasible and informative, and they have great potential to unravel complex disease processes. They also provide us with the challenge of validating each of the identified components as legitimate and feasible targets for therapeutic manipulation. In large part, target validation in the study of atherosclerosis currently utilizes genetically manipulated mouse models including transgene expression and gene knockout. Thus, systems biology will help us to understand the genetics of atherosclerotic disease by identifying potential therapeutic targets. It will simultaneously heighten the need for genetic-based validation of potential therapeutic targets using genetic mouse models of atherosclerosis to study the disease process.

Conclusion

The etiology of atherosclerosis is complex. Its pathogenesis is influenced by a number of biologic systems including lipoprotein metabolism, inflammation, coagulation, and others. Genetic mouse models of atherosclerosis have allowed us to identify certain components of these systems and examine their contribution to the mechanisms of atherogenesis. Yet, other components of these systems remain to be discovered, as do their precise contributions to atherosclerosis. The number and nature of genetic tools to identify and examine these contributing factors is expanding and they are being used more frequently. Our understanding of the complex process of atherosclerosis will be enhanced if we integrate the knowledge derived from these different models. Systems biology approaches will help reveal the fundamental mechanisms of atherosclerosis, as well as model-specific mechanisms of atherosclerosis. As these mechanisms are better understood, therapeutic targets for modulation of atherogenesis will become evident and be validated. The genetic models of atherosclerosis can then also be used to test new therapeutic agents and therefore will lead to the development of effective treatments for atherosclerosis (Figure 6.1).

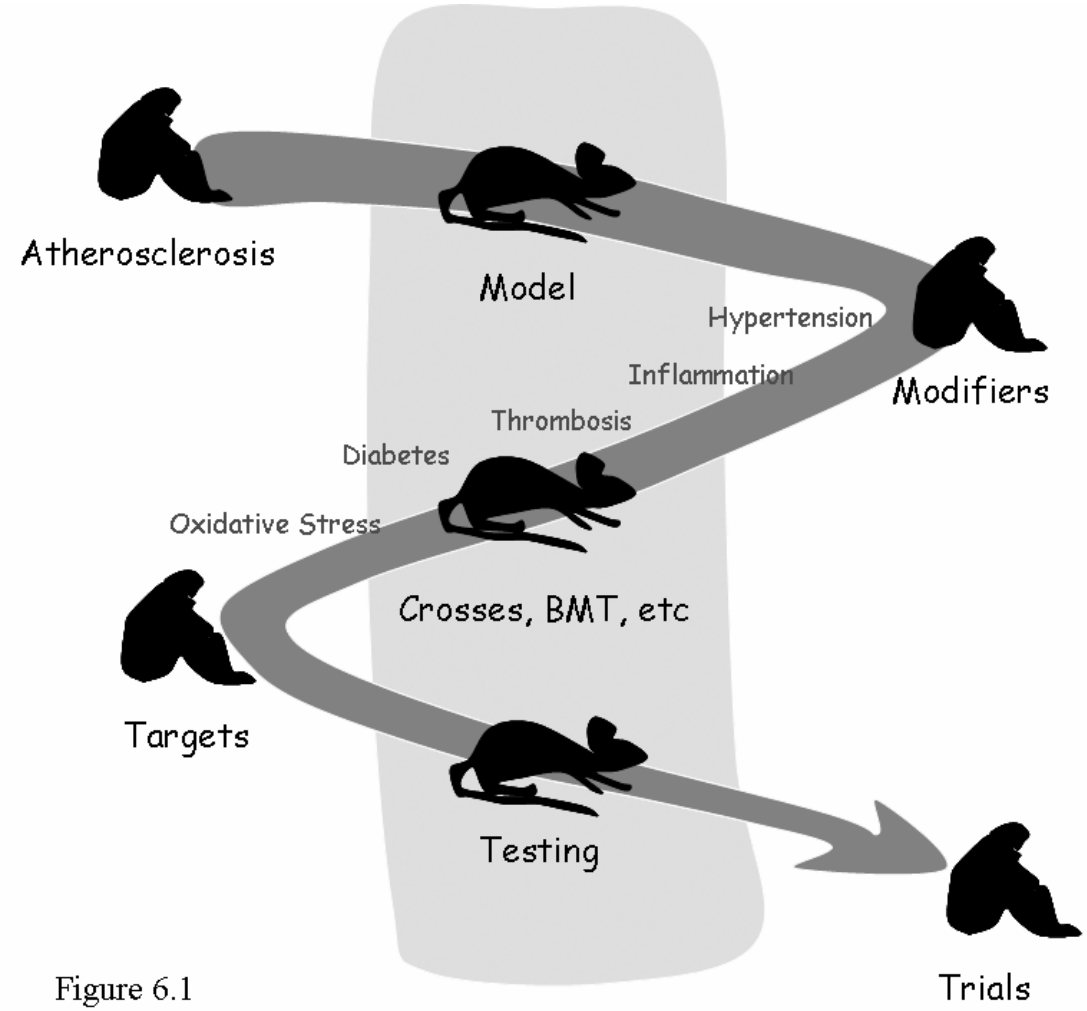

Atherosclerosis
Model
Hypertension
Inflammation
Modifiers
Thrombosis
Diabetes
Oxidative Stress
Crosses, BMT, etc
Targets
Testing
Trials

Figure 6.1

Chapter VII

Conclusion and future directions

Conclusion and future directions

The higher binding affinity of the apoE4 isoform to the LDLR than apoE2 or apoE3 has been implicated in leading to increased plasma LDL cholesterol and atherosclerosis in individuals carrying apoE4 [2, 3]. Combining mice targeted with human apoE crossed to either HuLDLR overexpressing or LDLR knock outs, along with adenovirus mediated LDLR and apoE-GFP expression, we investigated the effects of LDLR expression on the localization of the different apoE isoforms in the liver. Additionally, apoE isoforms have been shown to have seemingly lipid independent in-vitro affects on oxidation, SMC migration, and inflammation. Using a BMT system we determined that the apoE-LDLR interaction has affects on atherosclerosis independent of their affect on lipid levels in the plasma. Our results demonstrate, for the first time in animal models, that some of the risk associated with human apoE4 isoform may be due to its interaction with the LDLR in macrophages as well as with hepatic LDLR.

The liver is the main source of plasma apoE. Remnant lipoproteins are cleared in the liver mainly by LDLR and by LRP, but the uptake by these receptors require the remnants to acquire and be enriched with apoE proteins [47, 94-96]. Liver-derived and localized apoE facilitates the receptor-mediated internalization of remnants in the liver [96, 97]. Previously we showed in mice that all human apoE-isoforms are less effective ligands to non-LDLR mediated clearance of TG-rich lipoproteins than mouse apoE, and that VLDL remnants with very high cholesterol and apoE accumulates in the absence of LDLR [69]. Dependence of remnant clearance on LDLR is likely to be exaggerated in mice with human apoE isoforms.

We earlier observed that mice globally expressing 2-3 times the normal *Ldlr* gene exhibit hypercholesterolemia when fed a HFW diet only when they are also carrying the

human apoE allele [39]. We hypothesized that a high-affinity interaction between LDLR and apoE4 in the liver limits the transfer of apoE protein to the TG-rich lipoproteins, limiting their clearance.

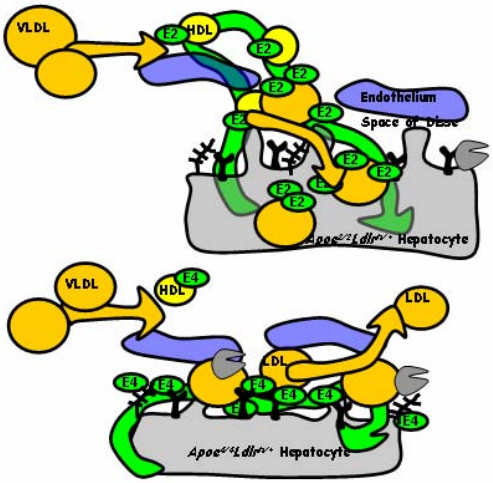
The paradoxical association of apoE4 with elevated plasma cholesterol in humans may be due to its high LDLR affinity and a reduced ability to exchange onto and clear TRLs and remnants. In cell culture binding or in-vivo clearance assays, when apoE is already present on the particle, apoE4 containing particles show enhanced binding and clearance compared to apoE3 and apoE2 [14, 19, 99]. Assaying apoE LDLR interaction this way may be misleading when used to interpret in vivo clearance mechanisms because it does not account for apoE isoform dependent differences in VLDL apoE-enrichment. Acquiring apoE is necessary for VLDL remnant clearance. For example apoE4, due to its high LDLR affinity, may stay stuck in or on cells and out of the plasma, leaving circulating VLDL apoE-poor. In fact , apoE deficient-VLDL binding and clearance is reduced when cells or animals express apoE4 compared to apoE3 [14]. We have shown that apoE2, with the lowest LDLR affinity, clears apoE deficient VLDL faster than apoE4 when they are expressed by the liver in-vivo and apoE-deficient particles are used. Thus the clearance of exogenous apoE deficient VLDL is faster when the recipient animal expresses apoE with low LDLR affinity along with a higher LDLR level. This may be more relevant to in vivo apoE-LDLR interaction because newly secreted chylomicron and VLDL have yet to acquire apoE. Combined with the reduced plasma apoE level associated with apoE4, this could increase the plasma retention time of these lipoproteins. Interestingly, this is consistent with the higher post prandial lipemia in apoE4 humans [68] as well as $Apoe^{4/4}Ldlr^{h/+}$ mice on HFW diet [39]. In contrast, the lower LDLR affinity of apoE2 increases its plasma concentration and HDL

levels and this circulating apoE2 has ample opportunity to exchange onto and increases VLDL clearance. For a visual depiction of the clearance of apoE deficient-VLDL in animals that synthesize apoE versus the clearance of apoE-containing VLDL see figure 7.1.

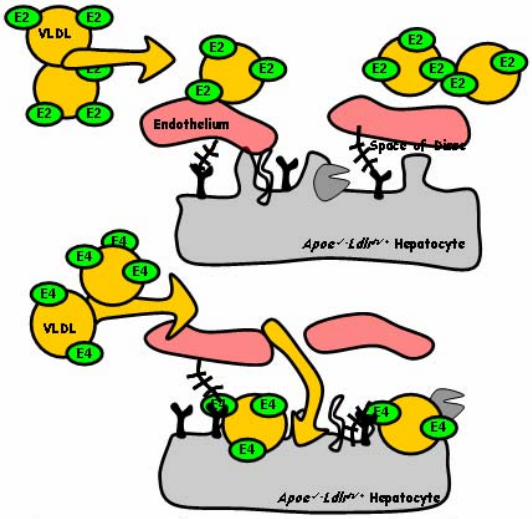
In summary, LDLR affinity determines whether plasma lipoproteins can acquire apoE. Acquisition of apoE determines lipoprotein clearance. Higher affinity apoE4 is not available to transfer to nascent VLDL in the plasma and consequently these VLDL accumulate.

Figure 7.1. Proposed mechanism to explain apoE effect on VLDL metabolism. Left Diagrams show apoE-poor VLDL clearance by apoE secreting hepatocytes. Right diagrams show apoE-enriched VLDL clearance by apoE deficient hepatocytes. Top left shows apoE2 metabolism of apoE-poor VLDL. The lower LDLR affinity of apoE2 increases plasma apoE level (green arrow) possibly via its elevated HDL level. This increases the enrichment of apoE2 onto VLDL which facilitates LDLR and HSPG mediated uptake. A. Lower diagram shows apoE4 metabolism of apoE-poor VLDL. High LDLR affinity of apoE4 keeps it bound to the hepatic surface, which decreases VLDL enrichment. VLDL at the hepatic surface are not internalized and subsequently converted to remnants and LDL possibly by LPL. When VLDL is isolated with, or enriched with apoE clearance of VLDL directly reflects the LDLR affinity of apoE. Right top low affinity apoE2 is poorly cleared. Right bottom, High affinity apoE4-enriched VLDL is rapidly cleared.

Proposed ApoE mediated VLDL Clearance Mechanisms



Clearance of VLDL without apoE by hepatocytes that secrete apoE



Clearance of VLDL enriched with apoE by hepatocytes that do not secrete apoE

Our results are consistent with and support our "apoE4 trapping by LDLR" as a mechanism to explain the hypercholesterolemia associated with apoE4. Retention of apoE4 at the hepatic surface by LDLR reduces its availability in the plasma to bind to and mediate remnant internalization. HDL, which can modulate apoE recycling, and is a source of plasma apoE, is a possible regulator of this process [47, 94, 103-105]. HDL as an apoE carrier may be necessary for timely apoE exchange to other lipoproteins such as TRLs, allowing nascent lipoproteins to enter the plasma without immediate apoE-mediated reuptake and also to remove them before they become slowly cleared remnant LDL. Modulation of HDL levels by LDLR is apoE isoform dependant; increased LDLR expression leads to increased HDL-cholesterol in mice with apoE2 and decreases HDL levels in mice with apoE4 [37, 39]. Elevated HDL with apoE2 could enrich apoE-poor VLDL and promote their clearance. While reduced HDL and apoE4 trapped in the space of Disse, directs apoE-poor VLDL to remnant conversion rather than internalization. Decreased apoE content on TRLs can increase conversion to remnants by LPL [22, 106-108]. It is interesting to also consider the inverse relationship between HDL and larger LDL and VLDL that exists in many mouse models and is typically observed in humans. Perhaps increased HDL serves as reservoir for apoproteins ligands and cofactors to transfer onto larger lipoproteins. Lack of apoE and other apoproteins on newly secreted VLDL and chylomicrons would ensure escape from the liver and allow lipid delivery to tissues. Increased interaction with elevated HDL containing transferable apoprotein ligands, such as apoE, could help to clear these larger lipoproteins. Further studies are necessary to elucidate the form of apoE and potential role of HDL in the isoform-specific interactions between LDLR and apoE and whether other apolipoproteins are involved in these interactions. High affinity ligands such as apoE4 may have difficulty

maintaining adequate plasma concentration of both apoE4 and HDL due to increased clearance. Experiments to determine the role of apoE containing HDL in determining VLDL and LDL levels can be addressed using the mice in this work. For instance, directly injecting apoE2 or apoE4 HDL into *apoE*$^{-/-}$ or *apoE*$^{-/-}$ *Ldlr*$^{h/h}$ mice to see if their HDLs can mediate a decrease in VLDL remnants that accumulate in the absence of apoE. Our hypothesis predicts that, if equal HDL or apoE is given, apoE4 HDL may assist remnant clearance better than apoE2. However, if volumes of HDL that are actually observed in *Apoe*$^{4/4}$*Ldlr*$^{h/+}$ and *Apoe*$^{2/2}$*Ldlr*$^{h/+}$ mice are injected, then the elevated apoE2 and HDL in *Apoe*$^{2/2}$*Ldlr*$^{h/+}$ mice could be expected to mediate better VLDL clearance.

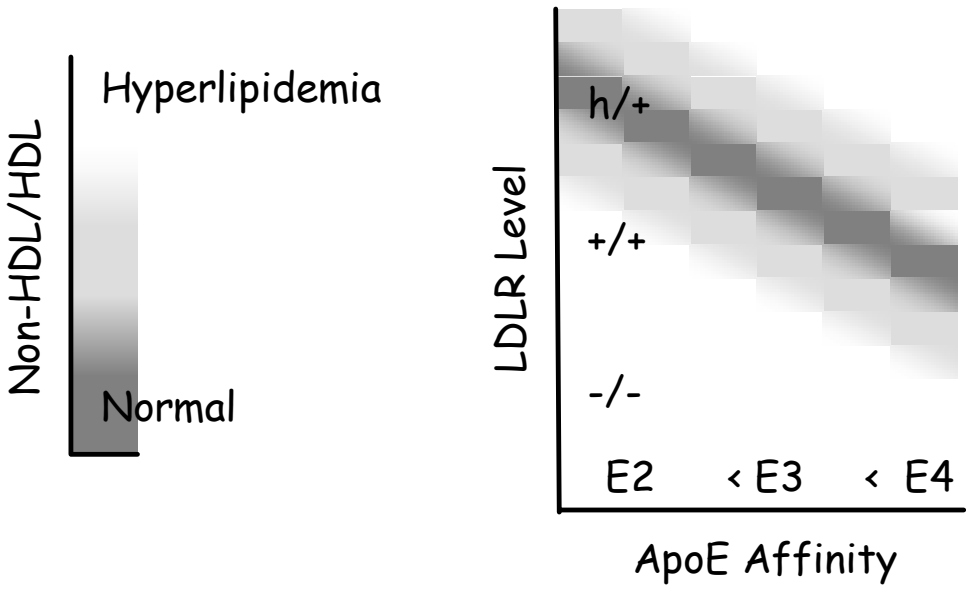
An important difference in our model and apoE4 possessing humans is that the mice do not accumulate LDL and instead have elevated VLDL remnants. This is often attributed to the editing of *apoB* in the mouse liver. There are several apoB mouse models that have either apoB100 only or transgenics that overexpress human apoB100. ApoB100 only mice without LDLR develop more LDL and atherosclerosis than those that still have apoB48 VLDL, likely due to shunting of apoE VLDL to LRP uptake. It is also likely that when crossed to our Apoe$^{4/4}$*Ldlr*$^{h/+}$ that increased apoB100 and LDL cholesterol will result. This would be more similar to their human counterparts and thus a better model to continue studying the apoE isoform risk and interaction with LDLR in hepatic localization.

Mice with apoE isoform and elevated LDLR revealed that there may be a critical range of LDLR levels for proper ratios of cholesterol carried by atherogenic non-HDL lipoproteins and by HDL particles. The optimum range of LDLR levels is different for each apoE isoform and proportional to each isoforms LDLR affinity. For instance, all apoE isoforms mice have elevated VLDL and LDL when the LDLR is absent [69]. With wildtype

levels of LDLR, both *Apoe*$^{4/4}$ and *Apoe*$^{3/3}$ mice have high HDL and low non-HDL cholesterol [14]. However, *ApoE*$^{2/2}$ mice have high non-HDL /HDL ratios [34]. When LDLR levels are elevated, HDL cholesterol levels dramatically decrease in both *Apoe*$^{4/4}$ *Ldlr*$^{h/+}$ and *Apoe*$^{3/3}$*Ldlr*$^{h/+}$ mice. Since non-HDL cholesterol is elevated only in *Apoe*$^{4/4}$ *Ldlr*$^{h/+}$ mice their non-HDL /HDL ratios are critically high, while *Apoe*$^{3/3}$*Ldlr*$^{h/+}$ mice have nonHDL /HDL ratios that are modestly increased. *Apoe*$^{2/2}$*Ldlr*$^{h/+}$ mice have reductions in their VLDL and elevated HDL levels and thus have low non-HDL /HDL. If we apply the same relationships to phenotypes associated with apoE isoforms in humans, one may predict that LDLR activity in humans are in the upper ranges of figure 7.2 where apoE2 is beneficial and apoE4 is somewhat detrimental. In the lower range of LDLR activities, the decreased affinity of apoE2 may cause apoE2-rich TRLs to accumulate, similar to the 5% of apoE2 homozygous humans who develop type III hyperlipidemia characterized by elevated TG.

Figure 7.2. Interaction between LDLR level and apoE affinity and resulting atherosclerosis risk (non-HDL cholesterol /HDL cholesterol) in mice. Legend on the left: darker shaded area shows a decreased non-HDL /HDL. Upper unshaded areas reflect elevated non-HDL/HDL and hyperlipidemia. Darker shading represents a beneficial interaction; low LDL, high HDL. For each apoE isoform there is a different non-HDL/HDL level for each LDLR level. Panel on the right shows non-HDL/HDL ratio (by amount of shading) in mice, determined by their LDLR level (y-axis) and apoE affinity (x-axis). Darker shaded areas reflect a good balance between LDLR level and apoE isoform that results in a good non-HDL/HDL. Unshaded area is a poor non-HDL/HDL and hyperlipidemia. For each apoE isoform as LDLR level goes up or down away from its critical range a poor non-HDL/HDL results. Similar non-HDL/HDL levels occur at different levels of LDLR for each apoE isoform reflected by the change in position of the shaded region. For example on an *Ldlr*$^{-/-}$ background all mice are hyperlipidemic regardless of apoE isoform (unshaded area across the bottom). As LDLR level is increased to wildtype mice with apoE3 and apoE4 have reductions in non-HDL (darker shading), while mice with apoE2 remain hyperlipidemic with a Type III phenotype (unshaded). With elevated LDLR mice with apoE4 are out of range and hyperlipidemic, while mice with apoE2 are now in range and have elevated HDL. Refer to lipoprotein profiles in figure 1.2 on page 22.

Figure 7.2


Hyperlipidemia
Non-HDL/HDL
Normal
LDLR Level
h/+
+/+
-/-
E2
< E3
< E4
ApoE Affinity

Careful titration of apoE and LDLR levels may shed light on the relevance of this mechanism to the well-established associations between the risk for coronary heart disease and Type III penetrance among apoE2 homozygotes as well as that of other apoE isoforms. Different doses of AdhuLDLR to induce varying LDLR expression levels combined with real-time PCR and western blotting for LDLR message and protein levels coordinated with lipid profiles could address this relationship and its predictions. Determination of this relationship could also answer the surprising prediction by this hypothesis that elevation of LDLR in $Apoe^{3/3}Ldlr^{h/+}$ mice would further increase non-HDL lipoproteins.

ApoE isoform mice that express varying levels of LDLR have generated interesting data that have led to novel hypothesis that appear to replicate and predict a consistent mechanism to explain the associated apoE isoform risks in humans. It is important to continue to improve this model by elevating LDL and better replicating the human profiles. However ultimately these hypothesis will need to be tested in humans. Human genetic variability and different environmental exposure often make these studies difficult to interpret and combined with sample availability almost impossible. Surrogate indicators at the DNA level for expression level compared to plasma phenotype and apoE genotype have been initiated by Mullem et al. [203]. Immunohistochemical detection of apoE in human liver samples could, if similar to our studies, support our predictions. In combination with plasma lipid, testing in human liver samples whether the pattern of elevated LDLR staining matches the prediction of a critical change for each isoform. This would undoubtedly require large numbers of samples to first aquire the correct genotypes as well as reduce the sample variability.

Finally, after these studies, if in agreement with our predictions, the targeting of therapeutics to apoE isoform effects would be warranted and interpretable. Our BMT experiments would suggest that the apoE4 isoform has a role in macrophage dependant atherosclerosis. While our hepatic studies suggest that elevated LDLR increases lipid levels. Together this would indicate that the response to treatment of atherosclerosis, in humans with apoE4, with anti-inflammatory therapy and less reliance on LDLR up-regulating, statin, treatment should be investigated.

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