[Frontiers in Bioscience 2, d298-308 , June 15, 97]

TRANSGENIC RABBIT MODELS FOR THE STUDY OF ATHEROSCLEROSIS

John M. Taylor[1] and Jianglin Fan

Gladstone Institute of Cardiovascular Disease, the Department of Physiology, and the Cardiovascular Research Institute, University of California, San Francisco, CA

TABLE OF CONTENTS

1. ABSTRACT

The rabbit rapidly develops severe hypercholesterolemia leading to premature atherosclerosis in response to dietary manipulation. Transgenic rabbit models with altered expression of specific genes will provide new approaches to understanding the underlying mechanisms that contribute to this disease. Recently established lines of transgenic rabbits that overexpress hepatic lipase and apolipoprotein E are yielding fresh insights into the functions of these proteins and their role in lesion development.

2. INTRODUCTION

The finding that the cholesterol component in diets of meat, milk, and eggs resulted in atherosclerotic lesions in rabbits was made more than eighty years ago (reviewed in refs. (1, 2)), and it established the rabbit as the first experimental model for the study of this disease. In the decades following this observation, investigators demonstrated that laboratory chow supplemented with less than 2% cholesterol reproduced the effect, rapidly leading to plasma cholesterol concentrations that can exceed 2,000 mg/dl. This response could be enhanced by including up to 10% extra fat in the diet, with saturated fats increasing both circulating cholesterol levels and the extent of lesion development (summarized in ref. (3)).

Received 5/19/97 Accepted 5/26/97

[1] To whom correspondence should be addressed at:Gladstone Institute of Cardiovascular Disease, PO Box 419100, San Francisco, CA 94141-9100 Tel:(415)826-7500 Fax:(415) 285-5632 E-mail: john_taylor@ quickmail.ucsf.edu

The major consequence of hypercholesterolemia in the rabbit is the rapid development of atherosclerosis (1). One of the earliest events in lesion development is a focal increase in arterial concentrations of low density lipoproteins (LDL) (4). After less than two weeks on the cholesterol diet, subendothelial deposits of extracellular lipid and cytosolic lipid droplets within vascular smooth muscle cells appear (5). After just one month, fatty streaks are detected in the aorta which progress quickly to raised lesions containing macrophage-derived foam cells. During the next three to six months, these sites progress to more complex fibrous plaques that accumulate both intracellular and extracellular lipid deposits (6, 7). The plaques become advanced atheromatous lesions characterized by an extensive distribution of smooth muscle cell–derived foam cells, collagen fibers, necrotic debris, and cholesterol crystals (6, 7).

In the rabbit, lesions are distributed predominantly in the aortic arch and thoracic aorta, at the origins of intercostal arteries, and to a lesser extent in the abdominal aorta. The involvement of the intercostal ostia may be due to a higher vascular permeability of LDL and to turbulent blood flow patterns at these locations (8, 9). All lesion sites in the aorta are characterized by an increased binding of LDL to collagen and glycosaminoglycans in the vascular wall (8). The retention of lipoproteins by the extracellular matrix may facilitate their oxidative modification, thereby triggering local cellular responses that include the release of cytokines, the recruitment of lymphocytes and monocytes, expression of cell adhesion receptors, the retention of macrophages, and the migration and proliferation of smooth muscle cells (summarized in ref. (10)). These changes have been viewed as a focal response of the artery wall to local

vascular injury (1, 10).

When fed to rabbits, experimental diets containing high amounts (1–2% or more) of cholesterol have resulted in lipid-laden, macrophage-enriched foamy lesions that were not typical of human atherosclerosis (1) and abundant lipid storage lesions in rabbit visceral organs (11). In humans, plaques are more abundant in the abdominal aorta, enriched in smooth muscle cells, and usually have a fibrous cap. These features led to the widely held view that rabbit atherosclerosis differed substantially from the corresponding human disease. However, recent studies that employed milder dietary regimens have demonstrated that the hypercholesterolemic rabbit can develop complex, advanced lesions that more closely resemble those found in humans (6, 7). Thus, the rabbit should continue to serve as an important model for the study of the underlying mechanisms that contribute to the development of atherosclerosis.

3. RABBIT MODELS FOR THE STUDY OF LIPID METABOLISM

The rabbit is a herbivore, and typical laboratory chow diets contain ~15% protein, 40–50% carbohydrate, 2% vegetable fat, and 15–25% fiber. On this type of diet, typical plasma cholesterol concentrations for the New Zealand White rabbit are in the range of ~30–65 mg/dl, with young animals (<3 kg body weight) usually found at the upper portion of this range. High density lipoproteins (HDL), the most abundant lipoprotein class in the normal rabbit (summarized in ref. (12)), transport more than half of the circulating cholesterol in fasting rabbit plasma (6, 13).

Supplementing the diet with cholesterol rapidly results in a marked increase in the production of cholesteryl ester–rich, β-migrating very low density lipoproteins (β-VLDL) by the liver and intestine. The VLDL become the major class of plasma lipoproteins (14-16). Subsequent clearance of the β-VLDL by the liver is reduced due to a downregulation of cell-surface lipoprotein receptors and the saturation of the remaining receptors (17). Two additional factors exacerbate the response of the rabbit: the relatively efficient absorption of dietary cholesterol and limited hepatic conversion of cholesterol to bile acids (18-20). The β-VLDL, including chylomicron remnants, that accumulate in the circulation are highly atherogenic. Plasma levels correlate closely with the extent of lesion development (21).

Hypercholesterolemia in the rabbit also can be induced by feeding diets that are high in animal protein (summarized in ref. (22)). In response to a casein-supplemented diet, there is a greater reabsorption of bile acids by the small intestine into circulation that leads to an increased uptake by the liver. The consequence is an inhibition in the conversion of cholesterol to bile acids by the liver due to dramatically decreased levels of mRNA encoding 7α-hydroxylase, which catalyzes the rate-limiting step in bile acid synthesis (23). The resultant elevation in liver cholesterol content leads to an increase in VLDL production, a decrease in lipoprotein receptor activity, and an accumulation of cholesteryl ester–rich VLDL and LDL in the plasma. This change in plasma lipoproteins leads to the development of advanced atherosclerotic lesions (6).

Important genetic variants of the New Zealand White rabbit have been identified that confirm the link between plasma cholesterol and atherosclerosis. An important model for human familial hypercholesterolemia, the Watanabe heritable hyperlipidemic (WHHL) rabbit, has defective LDL receptors. Thus, LDL accumulate in plasma to high levels (24), with homozygotes having plasma cholesterol levels in excess of 400 mg/dl (25). The WHHL rabbit develops complex fibrous lesions that are rich in foam cells (26). The St. Thomas Hospital strain of hyperlipidemic rabbits has increased levels of VLDL, intermediate density lipoproteins (IDL), and LDL due to an apparent overproduction of these lipoproteins, making this variant a potential model for familial combined hyperlipidemia (27). In these rabbits, the strongest predictor of aortic atherosclerosis is an elevated level of IDL.

At the opposite end of the spectrum, a partially inbred line of cholesterol-resistant rabbits has been established that does not readily develop atherosclerosis (28). When they are fed a cholesterol-supplemented diet, the levels of total plasma cholesterol, VLDL, and LDL remain essentially unchanged. These effects may be the result of an enhanced production and secretion of bile salts due to elevated levels of 7α-hydroxylase mRNA (29). However, the findings that both fibroblasts and hepatocytes from the resistant animals have increased rates of cholesterol synthesis, decreased rates of cholesterol esterification, and an increased uptake of LDL indicate that the basis for cholesterol resistance may be more complex (30).

4. RABBIT BREEDING

The most common breeds of rabbits used for research are descended from the European rabbit (Order Lagomorpha, Family Leporidae, Subfamily Leporinae, Genus *Oryctolagus*, Species *cuniculus*) (31). The European rabbit is the only domesticated species of this animal, but there are more than 50 established breeds. Uniform stocks of several breeds have been developed for laboratory research, including the albino New Zealand White (5–6 kg adult body weight) and the agouti Dutch Belted (~2.5 kg adult body weight). Homozygous inbred rabbits have reduced reproductive efficiency. Many partially inbred substrains having desired genotypes are maintained by various suppliers. Important variants like the WHHL rabbits are maintained as partially inbred closed colonies, with sublines developing distinct phenotypic characteristics (32).

The domestic rabbit may be ready for mating activity by ~4.5 months of age. The rabbit ovulates in

response to mating activity, and cyclic estrous behavior can occur with 5–6 day intervals. The gestation period is ~31 days. A few days prior to parturition, the pregnant mother will build a nest with her own body hair and straw if it is available. A nesting box and a quiet room with restricted access facilitate this activity. The typical litter size for New Zealand White rabbits is 8–10 pups. Litters larger than 10 pups do not thrive.

Successful breeding of rabbits requires careful attention to animal husbandry (summarized in ref. (33)). Rabbits are highly susceptible to respiratory diseases, caused mainly by *Pasteurella multocida* and *Bordetella bronchiseptica*. These pathogens are endemic in most colonies. Another major problem for rabbits is coccidiosis in the liver or intestine caused by various protozoa, the most widespread being *Eimeria* and *Encephalitozoa.* Minimizing the occurrence of these diseases and maintaining a tightly regulated housing environment are crucial elements of a successful transgenic rabbit colony. These conditions generally can be met by housing specific pathogen-free rabbits in a restricted barrier facility with close veterinary monitoring.

5. GENERATING TRANSGENIC RABBITS

The first report on the application of transgenic technology to the rabbit was in 1985 by Hammer *et al.* (34, 35). A construct of the human growth hormone gene directed by the mouse metallothionein promoter was used to investigate transgenic methods in different species by examining construct expression in fetuses and neonates. The transgene was integrated into the rabbit genome with an efficiency of 13%, about half of that observed with the mouse genome. Transgene mRNA was expressed in 25% of the rabbit founders, and one live founder exhibited human growth hormone in serum. In 1988, Knight *et al.* (36) described the characteristics of three transgenic founders expressing the *c-myc* oncogene directed by the rabbit immunoglobulin heavy chain enhancer region. These animals were the first reported transgenic rabbits in which transgene expression was characterized. The use of transgenic rabbit blood as a source of transgenic human α-1 antitrypsin was described by Massoud *et al.* in 1990 (37), and a mouse monoclonal antibody gene was used to generate two transgenic rabbits by Weidle *et al.* in 1991 (38). In 1993, Peng *et al.* (39) described three interesting transgenic rabbits that were generated with rabbit papillomavirus DNA, in which the transgenic founders developed extensive squamous carcinomas of the skin at an early age.

Fan *et al.* (13) were the first to apply this technology to the study of lipid metabolism, and they have established several transgenic rabbit models for the study of atherosclerosis. In this protocol (Fig.1), pathogen-free New Zealand White females are superovulated by an intramuscular administration of follicle-stimulating hormone to enhance the yield of ova, followed 4 days later by intravenous delivery of chorionic gonadotropin. The animals are mated to fertile males, and a typical yield of 20–30 zygotes is flushed from the oviducts 19 hours later. The large male pronucleus of the rabbit zygote is microinjected with a solution of DNA, and then 10–20 injected zygotes are implanted into each oviduct of a recipient female that has been mated previously with a vasectomized male. Following gestation, founder pups are identified by screening DNA using standard techniques when the animals are about one month of age. The microinjection techniques employed in this study were similar to those used for generating transgenic mice with just a few notable differences: 1) rabbit zygotes have about twice the diameter as mouse zygotes but with similar pronuclear sizes; 2) rabbit zygotes have a thick zona pellucida; and 3) rabbit pronuclei appear to be more sensitive to DNA purity.

6. HEPATIC LIPASE TRANSGENIC RABBITS

The rabbit is naturally deficient in hepatic lipase, having about 10% the activity found in other animals such as the rat (40). Hepatic lipase occupies a central role in plasma lipoprotein metabolism, with probable functions (Fig. 2) in the conversion of IDL to LDL, the remodeling of large triglyceride-rich HDL2 to smaller dense HDL3 (reviewed in ref. (41)), the transfer of HDL cholesterol to the liver (42, 43), and the clearance of chylomicron and VLDL remnants (44). Thus, the overexpression of hepatic lipase in the rabbit offered a unique opportunity to understand the mechanisms by which this enzyme modulates plasma lipid metabolism.

A total of nine transgenic founders were generated initially using a human hepatic lipase cDNA construct that directed expression only to the liver. Protein expression was detected subsequently in six of the founders (13). There was no hepatic lipase found normally in plasma, but the intravenous administration of heparin released substantial activity into the circulation of the transgenic animals, ranging from 7 to 83-fold above nontransgenic control values. The amount of human hepatic lipase corresponded approximately to the transgene copy number, and the enzymatic activity was comparable to that reported for post-heparin human plasma. Immunocytochemical studies at the ultrastructural level have demonstrated that the transgenic hepatic lipase is concentrated at cell surfaces in the space of Disse in the liver, consistent with its metabolic action (45).

The hepatic lipase transgenic rabbits had reductions in plasma lipid levels compared to normal animals, with total cholesterol decreased up to 40% and total triglycerides decreased by 60%. An 80% average decrease in HDL cholesterol accounted for most of the total decrease in plasma cholesterol. The changes in plasma lipid levels were similar in both male and female transgenic rabbits.

To examine the effects of transgene expression on individual lipoprotein classes, different density fractions were isolated by sequentially adjusting plasma to various densities with potassium bromide, then floating

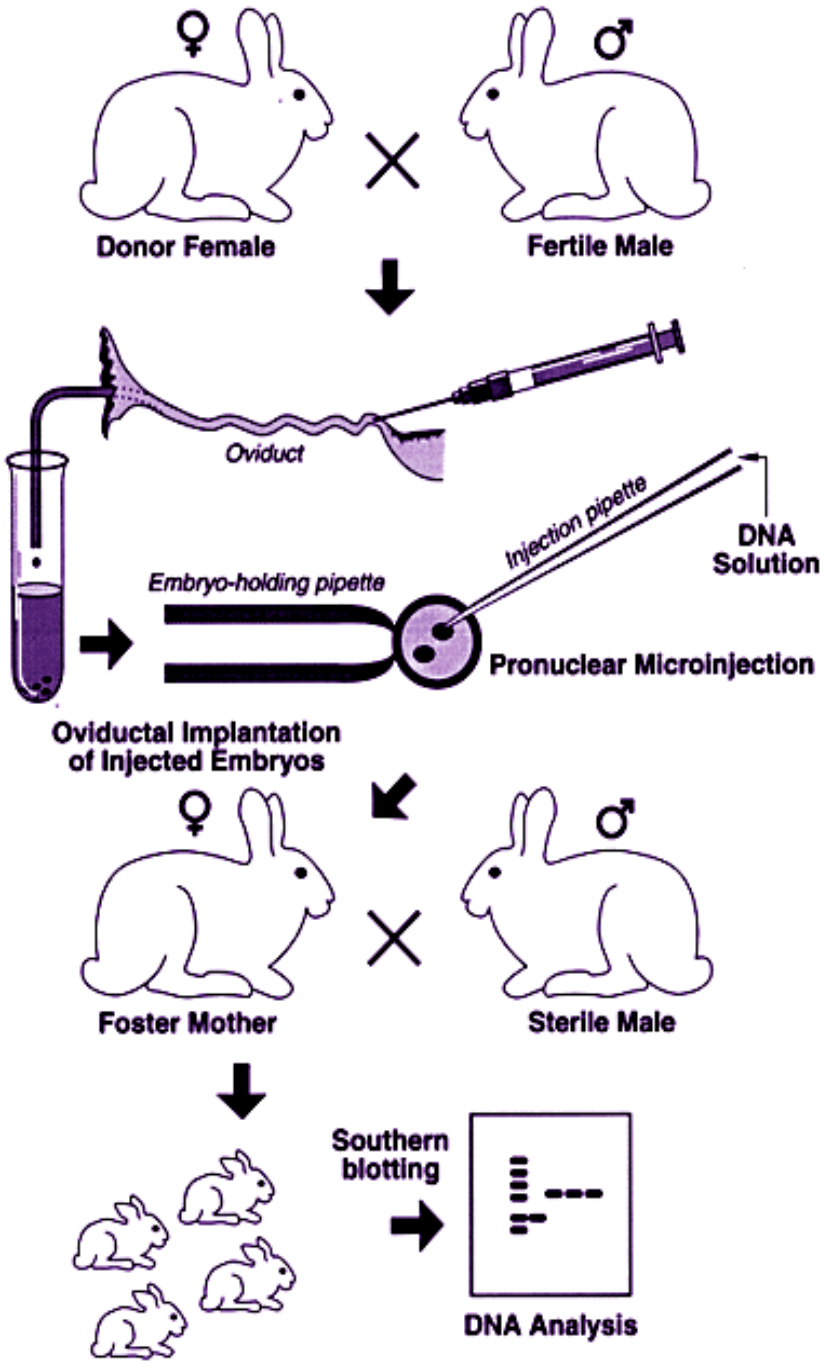

Figure 1. Production of transgenic rabbits. Specific pathogen-free New Zealand White donor females at five months of age are superovulated by the injection of 400 units of pregnant mare serum followed four days later by 150 units of human chorionic gonadotropin (13). The donors are mated to fertile males; single-cell embryos are flushed from the oviducts 19 h later. A DNA solution (~5 mg/ml) of the construct of interest is microinjected into the male pronucleus of the embryo while it is immobilized by a holding pipette under a gentle vacuum. Injected embryos are implanted through the fimbrial end of the oviduct (10–20 embryos per oviduct) of a pseudopregnant recipient that had been mated with a vasectomized male 24 hours earlier. Founder pups are identified one month after birth by DNA screening using Southern blot analysis or polymerase chain reaction.

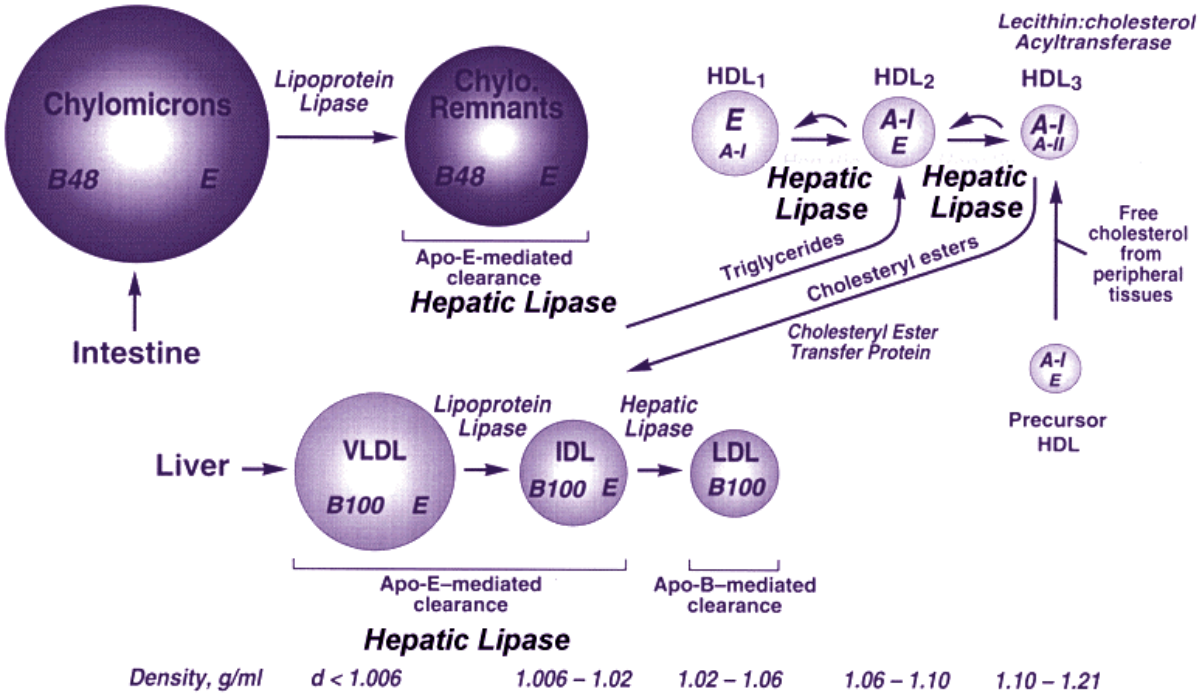

Figure 2. Metabolic pathways of lipoproteins. The chylomicron, VLDL, and HDL pathways for plasma lipoprotein metabolism are shown with major sites of action for principal enzymes and factors. The separation densities of individual classes of lipoprotein fractions are indicated, as well as the major apolipoprotein components of each lipoprotein class. Abbreviations are explained in the text.

them by ultracentrifugation. Each fraction was resolved further by agarose gel electrophoresis. Then, neutral lipid in lipoproteins was detected by staining the gels with Fat Red 7B, and their protein components were identified by blotting the gels to membrane filters and reacting the filters with specific antibodies to rabbit apolipoproteins.

The detailed analysis of plasma lipoproteins confirmed that the overexpression of hepatic lipase in the transgenic rabbit reduced the plasma content of HDL. All major classes of HDL decreased in quantity. These decreases were reflected in a substantial reduction in the content of apoA-I, a major characteristic component of HDL. The reduction in HDL1 and HDL2 levels is consistent with a role for hepatic lipase in HDL remodeling, whereby large, triglyceride-rich HDL are converted to smaller dense HDL by lipolytic activity. However, the decrease in HDL3 suggests that the action of hepatic lipase on this class of lipoproteins is an important function. By its location on liver cell surfaces, hepatic lipase may act on the surface of HDL through its phospholipase activity, altering the lipid environment to favor cholesterol transfer to cell membranes. Thus, hepatic lipase may facilitate the ability of HDL to deliver cholesterol directly to the liver and contribute to the function of HDL in reverse cholesterol transport. The overall reduction in HDL levels may reflect an increased catabolism, an increased clearance from plasma, or a decreased rate of production; but metabolic studies are required to determine the key mechanism.

The amount and distribution of apoB-containing lipoproteins also were affected by hepatic lipase expression. There was a reduction in IDL levels that was accompanied by a slight increase in the content of small LDL. These findings are consistent with roles for hepatic lipase in the conversion of IDL to LDL and in the increased uptake of remnant lipoproteins by the liver. However, additional studies are needed to determine if the changes in apoB-containing lipoproteins in transgenic rabbit plasma reflect increased clearance or increased catabolism. Thus, changes in the distribution of plasma lipoproteins were observed in the transgenic rabbit that could have opposing effects on the development of atherosclerosis: a decrease in atherogenic IDL, and a decrease in lipoproteins (HDL) that are thought to be protective against lesion development.

To determine the susceptibility of the hepatic lipase transgenic rabbit to atherosclerosis, a study of the effects of transgene expression on the response of the rabbit to dietary cholesterol was undertaken (J. Taylor *et al*., manuscript in preparation). Both transgenic and normal control rabbits, beginning at three months of age, were maintained on a diet supplemented with 0.3% cholesterol and 3% soybean oil. In the normal rabbit, plasma cholesterol levels increased from a baseline of about 50 mg/dl to reach peak levels of about 1200–1500 mg/dl by 4–5 weeks, after which there was no further increase. ApoB-containing particles, migrating as β-VLDL, were the most abundant class of lipoproteins as expected. In the cholesterol-fed hepatic lipase transgenic

rabbits, plasma cholesterol levels were raised only to about one-third the level of the control rabbits. Relatively large, apoE-deficient VLDL were the major lipoproteins found in the transgenic rabbits. There was also a striking reduction in the content of IDL and LDL compared to the controls, consistent with the increased expression of hepatic lipase in the transgenic rabbits.

The development of atherosclerosis in hepatic lipase transgenic rabbits was examined after a period of 10 weeks on the 0.3% cholesterol-supplemented diet. A comparison was made to normal rabbits that had been maintained on this diet, but with the lipid content varied to match plasma cholesterol levels of the normal controls with that of the transgenic animals. Aortas were harvested, trimmed of external fat, spread out, and stained with Sudan IV to detect lipid deposits. The area of the aortic surface that was covered with lesion was quantified by digitized image analysis, and lesion sections were examined to determine thickness, involvement of the artery wall, and pathology. As expected, most of the lesion involvement was in the aortic arch with additional lesions found at intercostal artery ostia and little involvement in the abdominal region. In both types of animals, about 8% of the aortic surface was covered with thick, raised lesions. However, the lesions in the ascending aorta and the aortic arch of the transgenic rabbits were significantly thicker than in the normal rabbits. Further studies will be needed to determine the basis for this remarkable difference in the atherosclerotic response of the vascular wall in the hepatic lipase transgenic rabbit to dietary cholesterol.

7. APOLIPOPROTEIN E TRANSGENIC RABBITS

Apolipoprotein E (M_r = 35,000) participates in diverse metabolic processes (summarized in ref. (46)). As a component of various lipoprotein classes, apoE occupies a central role in cholesterol metabolism (Fig. 1), mediating the clearance of chylomicron and VLDL remnants from plasma via cell-surface receptors, facilitating the delivery and incorporation of cholesterol into HDL, mediating the redistribution of lipid between cells and tissues, and acting as a co-factor for hepatic lipase (47). To determine the contributions that these mechanisms make to the development of atherosclerosis, transgenic rabbits that overexpress human apoE (the E3 phenotype) were generated (J. Fan *et al*., manuscript submitted). A 14-kb genomic fragment was used that contains the intact human apoE gene ligated to its hepatic control region (construct LE1 in ref. (48)). Three transgenic lines were established: the two highest expressers had human apoE levels of 12–13 mg/dl in circulation with little apparent effect on endogenous rabbit apoE levels. Compared to normal controls, total plasma triglycerides were decreased by nearly 50% in the transgenic animals, with all of this decrease found in the VLDL fraction. Unexpectedly, total plasma cholesterol was increased by up to twofold, with the additional cholesterol found in LDL and in HDL1.

To understand these dramatic effects, the apoB-containing lipoproteins were characterized further. Negative-staining electron microscopy showed VLDL having a median particle diameter of 26 nm in both normal and transgenic animals, but the number of particles larger than 32 nm in diameter was decreased by 75% in the transgenic rabbits. Thus, large VLDL were preferentially decreased as a consequence of apoE overexpression. Studies of LDL receptor–binding competition using cultured human fibroblasts showed that VLDL from transgenic rabbits were about threefold more effective than VLDL from normal rabbits in competing with control LDL for fibroblast receptors. This difference was most likely due to an increase in the content of apoE on the transgenic VLDL. In contrast, LDL from the transgenic rabbit were equivalent to LDL from the normal rabbit in their receptor–binding activity, suggesting that the transgenic LDL were not defective in their receptor-binding capacity. However, radioactively labeled human LDL were cleared more slowly from transgenic rabbit plasma than from normal control rabbit plasma.

Taken together, these studies suggested that the greater content of apoE on the VLDL could make them more effective competitors than LDL for receptors, leading to an accumulation of LDL in plasma. This possibility is supported by the higher affinity of apoE-containing lipoproteins for the LDL receptor than apoB-containing lipoproteins (summarized in ref. (46)), which would favor an increased uptake of apoE-rich particles compared to apoB-only LDL. Alternatively, the accumulation of LDL in apoE transgenic animals might be due to a reduction in LDL receptor activity as a consequence of an increased cholesterol uptake into the liver by VLDL. To distinguish between these two mechanisms, a monoclonal antibody that binds to the LDL receptor was administered intravenously to the rabbits: the antibody was cleared from the plasma of both transgenic and control rabbits at the same rate. Thus, the level of LDL in plasma appeared to be influenced by the content of apoE on large VLDL, giving them a competitive advantage for LDL receptor binding and subsequent clearance.

A study of the response to dietary cholesterol of the apoE transgenic rabbit in comparison to normal control rabbits was initiated according to the protocol described above. Overexpression of apoE limited the increase in plasma cholesterol to about one-third of the level found in the controls. Only low levels of VLDL were present in these transgenic rabbits, and the small IDL/large LDL fraction constituted the dominant class of lipoproteins in these animals. Thus, the lipoprotein profile in the cholesterol-fed apoE transgenic rabbit was almost the inverse of that found in the cholesterol-fed hepatic lipase transgenic rabbit, consistent with the complementary nature of their functions. The proximal aorta of the apoE transgenic rabbit aorta had only thin fatty streaks with little increase in intimal thickening, but about 14% of the vascular surface had lesion involvement

significantly greater than controls. Thus, it appears that the predominance of atherogenic IDL might facilitate the initiation of an atherosclerotic process and the increased level of circulating apoE in the transgenic rabbit minimizes the deposition of lipid in the artery wall. While these studies are provocative, a study of the effects of transgene expression on HDL will be necessary to understand the response to dietary cholesterol in both apoE and hepatic lipase transgenic rabbit models.

Transgenic rabbits have been generated that express the human apoE2 phenotypic variants (Y. Huang *et al*., manuscript submitted). These animals have substantial elevations in plasma cholesterol that are reflected in an increase in β-VLDL with a markedly different plasma lipoprotein profile. These animals are likely to be excellent models for the disorder type III hyperlipoproteinemia.

8. APOLIPOPROTEIN B TRANSGENIC RABBITS

An 85-kb human genomic fragment that spanned the complete human apoB gene was used to generate transgenic rabbits (49). Prior analysis of the expression of this fragment in transgenic mice showed that it was expressed at high levels only in the liver and not in any other tissue (50). Examination of the transgenic human apoB from rabbit plasma by gel electrophoresis and reaction with a panel of monoclonal antibodies demonstrated the presence of only the apoB100 form, which was indistinguishable from normal human apoB100. This finding confirmed that the transgene was probably expressed only in the liver of the rabbits, since the apoB100 mRNA would have been processed normally by the intestine to the B48 form. The concentrations of human apoB in three transgenic rabbit founders were 5, 40, and 100 mg/dl, but only the high expresser was characterized.

The apoB transgenic rabbits had threefold elevations in plasma cholesterol and triglycerides, compared to nontransgenic controls. In the transgenic rabbits, ~75% of the triglycerides and ~90% of the cholesterol were found in LDL. Compared to control LDL, the triglyceride-enriched transgenic LDL were smaller and more dense, and they were enriched in apoE and apoC-III. In transgenic rabbits, the HDL cholesterol also was significantly reduced, consistent with a reduction in the content of apoA-I in the HDL fractions. This lipoprotein profile suggests that the overexpression of apoB100 may have resulted in the direct production of LDL-sized particles, although this possibility remains to be tested. The lack of apoB-mRNA editing in the rabbit liver makes these animals particularly attractive for the study of LDL metabolism.

9. APOLIPOPROTEIN B mRNA-EDITING PROTEIN (APOBEC-1) TRANSGENIC RABBITS

A cDNA encoding rabbit APOBEC-1 in a liver-specific expression vector was used to generate transgenic rabbits (51). The corresponding protein is an editing factor for apoB100 mRNA that deaminates a specific cytidine at residue 6666 to yield uridine: editing changes a glutamine codon (CAA) to a translation stop codon (UAA) resulting in the B48 form of the apolipoprotein. The editing factor is found in the intestine but not in the liver of the nontransgenic rabbit, making this animal species a superb model to investigate the role of apoB editing in lipoprotein metabolism. The transgenic rabbits had significant decreases in VLDL, IDL, and LDL accompanied by an increase in HDL. The most striking effect of liver-specific APOBEC-1 expression was the induction of hepatocellular carcinoma and liver displasia in the transgenic rabbits. These observations demonstrated that other mRNAs besides apoB100 can be edited and that aberrant editing of hepatic mRNAs can alter cell growth and contribute to tumorigenesis (52).

10. APOLIPOPROTEIN A-I TRANSGENIC RABBITS

Human apoA-I transgenic rabbits were generated with an 11-kb genomic fragment that directed expression specifically to the liver (53, 54). Several independent lines were established having plasma apoA-I levels ranging up to 175 mg/dl. The production of the corresponding endogenous rabbit apoA-I was depressed by the expression of human apoA-I, which became the major protein component of the transgenic rabbit HDL. The high-expresser apoA-I transgenic rabbits had elevated levels of HDL cholesterol with concomitant higher rates of cholesterol efflux compared to nontransgenic controls. There also was a substantial amount of pre-β-migrating apoA-I-containing lipoproteins with HDL properties in the high-expresser transgenic rabbit. These particles may have formed as a result of a relative deficiency in apoA-II, which is normally a major component of several subclasses of HDL, or they might play a role as HDL precursors.

The diet of the apoA-I transgenic rabbits was supplemented with 0.3–0.5% cholesterol for 14 weeks, which raised plasma cholesterol levels to 1,200 mg/dl (55). Compared to cholesterol-matched nontransgenic controls, the transgenic rabbits had a significant 50% decrease in atherosclerotic lesion areas in their aortas. These findings are in accordance with a protective role for apoA-I-rich HDL in atherosclerosis.

Hoeg *et al*. have generated three founders that express human apoA-I using zygotes derived from WHHL donors (56). In transgenic rabbits that express 2 mg/dl of human apoA-I, there was an increase in HDL cholesterol from 16 to 32 mg/dl. This effect is quite interesting considering that a relatively low level of transgenic apoA-I is being expressed in these animals. This model may be especially useful for assessing the effect of increased HDL levels on the development of atherosclerosis.

11. LECITHIN-CHOLESTEROL ACYL TRANSFERASE (LCAT) TRANSGENIC RABBITS

A human LCAT gene construct was used to generate transgenic rabbits that expressed the transgene at high levels in the liver and lower levels in the brain (57, 58). Three founders were identified, one of which had a high transgene integration copy number and a 16-fold increase in plasma levels of LCAT activity over nontransgenic control animals. The total and HDL cholesterol levels were increased fourfold and sevenfold, respectively, in the transgenic rabbits, indicating that LCAT activity plays a direct regulatory role in determining the HDL content of plasma. These changes were associated with an accumulation of cholesteryl ester–rich apoE-enriched HDL1 and a large reduction in VLDL content.

Supplementing the diet with 0.3% cholesterol for 17 weeks resulted in a 20% increase in HDL cholesterol (59). The non-HDL cholesterol in plasma was increased to ~500 mg/dl in nontransgenic controls but to only ~200 mg/dl in the LCAT transgenic rabbits. These changes were associated with a marked protection against atherosclerosis: the arterial surfaces of control rabbits had a 35% lesion area (~550 mg/dl total cholesterol), whereas the transgenic rabbits had only a 5% lesion area (~400 mg/dl cholesterol). These studies demonstrated the involvement of LCAT in the metabolism of both HDL and apoB-containing lipoproteins.

12. CONCLUSION

The rabbit is an important model for the study of the relationship between plasma cholesterol metabolism and atherosclerosis. To provide new experimental tools, transgenic rabbits have been generated in which individual genes involved in plasma lipoprotein metabolism have been overexpressed. In each case, alterations in plasma cholesterol and lipoprotein distribution were observed. Research on additional transgenic models is also underway that will extend these studies further, exploiting the unique advantages and characteristics of the rabbit. We can expect that these transgenic animals will bring new insights into the mechanisms that contribute to the development of atherosclerosis, expanding the power of the rabbit model for the study of cardiovascular disease.

13. ACKNOWLEDGMENT

We would like to express gratitude to Dr. Robert Mahley for his support and encouragement of our work on transgenic rabbits. We also are grateful for the contributions of Dr. Harshini deSilva in helping to establish the technology in our laboratory and to Dr. David Sanan, Dr. Fred Cornhill, Dale Newland, Kim Nguyen, Jennifer Murata, and Ed Herderick for their help on the analysis of lesions. We thank Ricky Quan and Jean Wang for help with rabbit surgery and procedures. Thanks also are extended to Drs. Tom Innerarity, André Bensadoun, Katherine Knight, and John Krieder for help and advice. We are grateful for the expert assistance of Drs. Joe Spinelli, Jan Wyrick, and Dan Lau and the staff of the SFGH/UCSF Animal Care Facility. Our research was supported in part by the J. David Gladstone Institutes and by NHLBI grant #HL 51588.

14. REFERENCES

1. Duff, G. L. Experimental cholesterol arteriosclerosis and its relationship to human arteriosclerosis. *Arch. Pathol.* 20, 81–123, 259–304 (1935)

2. Clarkson, T. B., Lehner, N. D. M., and Bullock, B. C. Specialized research applications: I. Arteriosclerosis research. In The Biology of the Laboratory Rabbit. (Weisbroth, S. H., Flatt, R. E., and Kraus, A. L., eds.) pp. 155–165, *Academic Press, San Diego* (1974)

3. Kritchevsky, D. Role of cholesterol vehicle in experimental atherosclerosis. *Am. J. Clin. Nutr*. 23, 1105–1110 (1970)

4. Schwenke, D. C., and Carew, T. E. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. I. Focal increases in arterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis* 9, 895–907 (1989)

5. Guyton, J. R., and Klemp, K. F. Early extracellular and cellular lipid deposits in aorta of cholesterol-fed rabbits. *Am. J. Pathol.* 141, 925–936 (1992)

6. Daley, S. J., Herderick, E. E., Cornhill, J. F., and Rogers, K. A. Cholesterol-fed and casein-fed rabbit models of atherosclerosis. Part 1: Differing lesion area and volume despite equal plasma cholesterol levels. *Arterioscler. Thromb.* 14, 95–104 (1994)

7. Daley, S. J., Klemp, K. F., Guyton, J. R., and Rogers, K. A. Cholesterol-fed and casein-fed rabbit models of atherosclerosis. Part 2: Differing morphological severity of atherogenesis despite matched plasma cholesterol levels. *Arterioscler. Thromb.* 14, 105–114 (1994)

8. Schwenke, D. C., and Carew, T. E. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. *Arteriosclerosis* 9, 908–918 (1989)

9. Herrmann, R. A., Malinauskas, R. A., and Truskey, G. A. Characterization of sites with elevated LDL permeability at intercostal, celiac, and iliac branches of the normal rabbit aorta. *Arterioscler. Thromb.* 14, 313–323 (1994)

10. Ross, R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* 362, 801–809 (1993)

11. Prior, J. T., Kurtz, D. M., and Ziegler, D. D. The

hypercholesteremic rabbit. An aid to understanding arteriosclerosis in man? *Arch. Pathol.* 71, 672–684 (1961)

12. Chapman, M. J. Animal lipoproteins: Chemistry, structure, and comparative aspects. *J. Lipid Res.* 21, 789–853 (1980)

13. Fan, J., Wang, J., Bensadoun, A., Lauer, S. J., Dang, Q., Mahley, R. W., and Taylor, J. M. Overexpression of hepatic lipase in transgenic rabbits leads to a marked reduction of plasma high density lipoproteins and intermediate density lipoproteins. *Proc. Natl. Acad. Sci. USA* 91, 8724–8728 (1994)

14. Shore, V. G., Shore, B., and Hart, R. G. Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesteremia. *Biochemistry* 13, 1579–1585 (1974)

15. Thompson, K. H., and Zilversmit, D. B. Plasma very low density lipoprotein (VLDL) in cholesterol-fed rabbits: Chylomicron remnants or liver lipoproteins? *J. Nutr.* 113, 2002–2010 (1983)

16. MacKinnon, A. M., Savage, J., Gibson, R. A., and Barter, P. J. Secretion of cholesteryl ester-enriched very low density lipoproteins by the liver of cholesterol-fed rabbits. *Atherosclerosis* 54, 145–155 (1985)

17. Kovanen, P. T., Brown, M. S., Basu, S. K., Bilheimer, D. W., and Goldstein, J. L. Saturation and suppression of hepatic lipoprotein receptors: A mechanism for the hypercholesterolemia of cholesterol-fed rabbits. *Proc. Natl. Acad. Sci. USA* 78, 1396–1400 (1981)

18. Dietschy, J. M., and Wilson, J. D. Regulation of cholesterol metabolism (first of three parts). *N. Engl. J. Med.* 282, 1128–1138 (1970)

19. Dietschy, J. M., and Wilson, J. D. Regulation of cholesterol metabolism (second of three parts). *N. Engl. J. Med.* 282, 1179–1183 (1970)

20. Dietschy, J. M., and Wilson, J. D. Regulation of cholesterol metabolism (third of three parts). *N. Engl. J. Med.* 282, 1241–1249 (1970)

21. Mahley, R. W. Atherogenic lipoproteins and coronary artery disease: Concepts derived from recent advances in cellular and molecular biology. *Circulation* 72, 943–948 (1985)

22. Beynen, A. C., Van der Meer, R., and West, C. E. Mechanism of casein-induced hypercholesterolemia: Primary and secondary features. *Atherosclerosis* 60, 291–293 (1986)

23. Krause, B. R., Pape, M. E., Kieft, K., Auerbach, B., Bisgaier, C. L., Homan, R., and Newton, R. S. ACAT inhibition decreases LDL cholesterol in rabbits fed a cholesterol-free diet. Marked changes in LDL cholesterol without changes in LDL receptor mRNA abundance. *Arterioscler. Thromb.* 14, 598–604 (1994)

24. Kita, T., Brown, M. S., Bilheimer, D. W., and Goldstein, J. L. Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. *Proc. Natl. Acad. Sci. USA* 79, 5693–5697 (1982)

25. Havel, R. J., Kita, T., Kotite, L., Kane, J. P., Hamilton, R. L., Goldstein, J. L., and Brown, M. S. Concentration and composition of lipoproteins in blood plasma of the WHHL rabbit. An animal model of human familial hypercholesterolemia. *Arteriosclerosis* 2, 467–474 (1982)

26. Buja, L. M., Kita, T., Goldstein, J. L., Watanabe, Y., and Brown, M. S. Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. *Arteriosclerosis* 3, 87–101 (1983)

27. Nordestgaard, B. G., Tybjærg-Hansen, A., and Lewis, B. Influx *in vivo* of low density, intermediate density, and very low density lipoproteins into aortic intimas of genetically hyperlipidemic rabbits. Roles of plasma concentration, extent of aortic lesion, and lipoprotein particle size as determinants. *Arterioscler. Thromb.* 12, 6–18 (1992)

28. Overturf, M. L., Smith, S. A., Hewett-Emmett, D., Loose-Mitchell, D. S., Soma, M. R., Gotto, A. M., Jr., and Morrisett, J. D. Development and partial metabolic characterization of a dietary cholesterol-resistant colony of rabbits. *J. Lipid Res.* 30, 263–273 (1989)

29. Poorman, J. A., Buck, R. A., Smith, S. A., Overturf, M. L., and Loose-Mitchell, D. S. Bile acid excretion and cholesterol 7α-hydroxylase expression in hypercholesterolemia-resistant rabbits. *J. Lipid Res.* 34, 1675–1685 (1993)

30. Soma, M. R., Morrisett, J. D., Gotto, A. M., Jr., Loose-Mitchell, D. S., Poorman, J. A., Smith, S. A., and Overturf, M. L. Cholesterol metabolism in fibroblasts from rabbits resistant to diet-induced hypercholesterolemia. *J. Lipid Res.* 31, 985–994 (1990)

31. Fox, R. R. Taxonomy and genetics. In The Biology of the Laboratory Rabbit. (Weisbroth, S. H., Flatt, R. E., and Kraus, A. L., eds.) *Academic Press, San Diego*, pp. 1–22 (1974)

32. Donnelly, T. M., Kelsey, S. F., Levine, D. M., and Parker, T. S. Control of variance in experimental studies of hyperlipidemia using the WHHL rabbit. *J. Lipid Res.* 32, 1089–1098 (1991)

33. Hagen, K. W. Colony husbandry. In The Biology of

the Laboratory Rabbit. (Weisbroth, S. H., Flatt, R. E., and Kraus, A. L., eds.) pp. 23–47, *Academic Press, San Diego.* (1974)

34. Hammer, R. E., Pursel, V. G., Rexroad, C. E., Jr., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D., and Brinster, R. L. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* 315, 680–683 (1985)

35. Hammer, R. E., Pursel, V. G., Rexroad, C. E., Jr., Wall, R. J., Bolt, D. J., Palmiter, R. D., and Brinster, R. L. Genetic engineering of mammalian embryos. *J. Anim. Sci.* 63, 269–278 (1986)

36. Knight, K. L., Spieker-Polet, H., Kazdin, D. S., and Oi, V. T. Transgenic rabbits with lymphocytic leukemia induced by the c-myc oncogene fused with the immunoglobulin heavy chain enhancer. *Proc. Natl. Acad. Sci. USA* 85, 3130–3134 (1988)

37. Massoud, M., Bischoff, R., Dalemans, W., Pointu, H., Attal, J., Schultz, H., Clesse, D., Stinnakre, M.-G., Pavirani, A., and Houdebine, L.-M. La production de protéines humaines dans le sang d'animaux transgéniques. (The production of human proteins in the blood of transgenic animals) (abstract and abridged version in English). *C. R. Acad. Sci. Paris III* 311, 275–280 (1990)

38. Weidle, U. H., Lenz, H., and Brem, G. Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs. *Gene* 98, 185–191 (1991)

39. Peng, X., Olson, R. O., Christian, C. B., Lang, C. M., and Kreider, J. W. Papillomas and carcinomas in transgenic rabbits carrying EJ-ras DNA and cottontail rabbit papillomavirus DNA. *J. Virol.* 67, 1698–1701 (1993)

40. Warren, R. J., Ebert, D. L., Mitchell, A., and Barter, P. J. Rabbit hepatic lipase cDNA sequence: Low activity is associated with low messenger RNA levels. *J. Lipid Res.* 32, 1333–1339 (1991)

41. Olivecrona, T., and Bengtsson-Olivecrona, G. Lipoprotein lipase and hepatic lipase. *Curr. Opin. Lipidol.* 1, 222–230 (1990)

42. Rothblat, G. H., Mahlberg, F. H., Johnson, W. J., and Phillips, M. C. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. *J. Lipid Res.* 33, 1091–1097. (1992)

43. Kadowaki, H., Patton, G. M., and Robins, S. J. metabolism of high density lipoprotein lipids by the rat liver: Evidence for participation of hepatic lipase in the uptake of cholesteryl ester. *J. Lipid Res.* 33, 1689–1698 (1992)

44. Ji, Z.-S., Lauer, S. J., Fazio, S., Bensadoun, A., Taylor, J. M., and Mahley, R. W. Enhanced binding and uptake of remnant lipoproteins by hepatic lipase-secreting hepatoma cells in culture. *J. Biol. Chem.* 269, 13429–13436 (1994)

45. Sanan, D. A., Fan, J., Bensadoun, A., and Taylor, J. M. Hepatic lipase is abundant on both hepatocyte and endothelial cell surfaces in the liver. *J. Lipid Res.* 38, 1002–1013.

46. Mahley, R. W. Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* 240, 622–630 (1988)

47. Thuren, T., Weisgraber, K. H., Sisson, P., and Waite, M. Role of apolipoprotein E in hepatic lipase catalyzed hydrolysis of phospholipid in high-density lipoproteins. *Biochemistry* 31, 2332–2338 (1992)

48. Simonet, W. S., Bucay, N., Lauer, S. J., and Taylor, J. M. A far-downstream hepatocyte-specific control region directs expression of the linked human apolipoprotein E and C-I genes in transgenic mice. *J. Biol. Chem.* 268, 8221–8229 (1993)

49. Fan, J., McCormick, S. P. A., Krauss, R. M., Taylor, S., Quan, R., Taylor, J. M., and Young, S. G. Overexpression of human apolipoprotein B-100 in transgenic rabbits results in increased levels of LDL and decreased levels of HDL. *Arterioscler. Thromb. Vasc. Biol.* 15, 1889–1899 (1995)

50. Linton, M. F., Farese, R. V., Jr., Chiesa, G., Grass, D. S., Chin, P., Hammer, R. E., Hobbs, H. H., and Young, S. G. Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein(a). *J. Clin. Invest.* 92, 3029–3037 (1993)

51. Yamanaka, S., Balestra, M. E., Ferrell, L. D., Fan, J., Arnold, K. S., Taylor, S., Taylor, J. M., and Innerarity, T. L. Apolipoprotein B mRNA-editing protein induces hepatocellular carcinoma and dysplasia in transgenic animals. *Proc. Natl. Acad. Sci. USA* 92, 8483–8487 (1995)

52. Yamanaka, S., Poksay, K. S., Arnold, K. S., and Innerarity, T. L. A novel translational repressor mRNA is edited extensively in livers containing tumors caused by the transgene expression of the apoB mRNA-editing enzyme. *Genes Dev.* 11, 321–333 (1997)

53. Duverger, N., Emmanuel, F., Viglietta, C., Tailleux, A., Benoit, P., Attenot, F., Cuiné, S., Fievet, C., Laine, B., Fruchart, J. C., Houbebine, L. M., and Denèfle, P. Human apolipoprotein A-I gene transfer in the rabbit: Incidence on atherogenesis. *Circulation* 90, I-240 (abstr.) (1994)

54. Duverger, N., Viglietta, C., Berthou, L., Emmanuel,

F., Tailleux, A., Parmentier-Nihoul, L., Laine, B., Fievet, C., Castro, G., Fruchart, J. C., Houbebine, L. M., and Denèfle, P. Transgenic rabbits expressing human apoliproprotein A-I in the liver. *Arterioscler. Thromb. Vasc. Biol.* 16, 1424–1429 (1996)

55. Duverger, N., Kruth, H., Emmanuel, F., Caillaud, J.-M., Viglietta, C., Castro, G., Tailleux, A., Fievet, C., Fruchart, J. C., Houdebine, L. M., and Denefle, P. Inhibition of atherosclerosis development in cholesterol-fed human apolipoprotein A-I–transgenic rabbits. *Circulation* 94, 713–717 (1996)

56. Hoeg, J. M., Vaisman, B. L., Demosky, S. J., Jr., Santamarina-Fojo, S., Brewer, H. B., Jr., Remaley, A. T., Hoyt, R. F., and Feldman, S. Development of transgenic Watanabe heritable hyperlipidemic rabbits expressing human apolipoprotein A-I. *Circulation* 88, I-2 (abstr.) (1993)

57. Hoeg, J. M., Vaisman, B. L., Demosky, S. J., Jr., Meyn, S. M., Talley, G. D., Hoyt, R. F., Jr., Feldman, S., Bérard, A. M., Sakai, N., Wood, D., Brousseau, M. E., Marcovina, S., Brewer, H. B., Jr., and Santamarina-Fojo, S., Lecithin: cholesterol acyltransferase overexpression generates hyperalpha-lipoproteinemia and a nonatherogenic lipoprotein pattern in transgenic rabbits. *J. Biol. Chem.* 271, 4396–4402 (1996)

58. Hoeg, J. M., Santamarina-Fojo, S.,Vaisman, B. L., Demosky, S.J., Jr., Hoyt, R. F., Jr., Feldman, S. H.,Talley, G., and Brewer, H.B., Jr. Overexpression of human lecithin-cholesterol acyl transferase in transgenic rabbits leads to hyperalphalipoproteinemia. *Atherosclerosis* 109, 11 (abstr.) (1994)

59. Hoeg, J. M., Santamarina-Fojo, S., Bérard, A. M., Cornhill, J. F., Herderick, E. E., Feldman, S. H., Haudenschild, C. C., Vaisman, B. L., Hoyt, R. F., Jr., Demosky, S. J., Jr., Kauffman, R. D., Hazel, C. M., Marcovina, S. M., and Brewer, H. B., Jr. Overexpression of lecithin:cholesterol acyltransferase in transgenic rabbits prevents diet-induced atherosclerosis. *Proc. Natl. Acad. Sci. USA* 93, 11448–11453 (1996)