review

Transgenic rabbits as models for atherosclerosis research

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Abstract Several characteristics of the rabbit make it an excellent model for the study of lipoprotein metabolism and atherosclerosis. New Zealand White (NZW) rabbits have low plasma total cholesterol concentrations, high cholesteryl ester transfer protein activity, low hepatic lipase (HL) activity, and lack an analogue of human apolipoprotein (apo) A-II, providing a unique system in which to assess the effects of human transgenes on plasma lipoproteins and atherosclerosis susceptibility. Additionally, rabbit models of human lipoprotein disorders, such as the Watanabe Heritable Hyperlipidemic (WHHL) and St. Thomas' Hospital strains, models of familial hypercholesterolemia and familial combined hyperlipidemia, respectively, allow for the assessment of candidate genes for potential use in the treatment of dyslipoproteinemic patients. To date, transgenes for human apo(a), apoA-I, apoB, apoE_2, apoE_3, HL, and lecithin:cholesterol acyltransferase (LCAT), as well as for rabbit apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1), have been expressed in NZW rabbits, whereas only those for human apoA-I and LCAT have been introduced into the WHHL background. All of these transgenes have been shown to have significant effects on plasma lipoprotein concentrations. In both NZW and WHHL rabbits, human apoA-I expression was associated with a significant reduction in the extent of aortic atherosclerosis, which was similarly the case for LCAT in rabbits having at least one functional LDL receptor allele. Conversely, expression of apoE_2 in NZW rabbits caused increased susceptibility to atherosclerosis. These studies provide new insights into the mechanisms responsible for the development of atherosclerosis, emphasizing the strength of the rabbit model in cardiovascular disease research.—Brousseau, M. E., and J. M. Hoeg. **Transgenic rabbits as models for atherosclerosis research. *J. Lipid Res.* 1999.** 40: **365–375.**

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Several characteristics of the rabbit make it an excellent model for assessment of the effects of human transgenes on lipoprotein metabolism and atherosclerosis susceptibility: *1*) rabbit apolipoprotein (apo) B-containing lipoproteins are similar to those of humans in their chemical composition and apoprotein content (1); *2*) rabbit liver does not edit apoB mRNA and, thus, produces apoB-100-containing very low density lipoproteins (VLDL) as does the human (2); *3*) cholesteryl ester transfer protein (CETP), which plays a central role in the atherosclerotic process, is abundant in both human and rabbit plasma (3, 4); and *4*) last, rabbits are very susceptible to diet-induced atherosclerosis (5, 6). Conversely, the rabbit differs from the most widely used transgenic model, the mouse, in each of these respects. In the mouse, *1*) apoB mRNA is edited extensively by the liver, generating apoB-48-containing lipoproteins (2); *2*) CETP is absent (4); and *3*) high density lipoproteins (HDL) are predominant in the plasma, confering resistance to diet-induced atherosclerosis (7). Additionally, the rabbit lacks an analogue of human apoA-II (1, 8) and is relatively deficient in hepatic lipase (HL) (9), neither of which is the case for the mouse. The HL present in the rabbit is bound to the vascular endothelium, as it is in the human, whereas the majority of HL in the mouse circulates in the plasma. Thus, the rabbit provides a unique system in which to study the effects of transgenes relevant to human atherosclerosis. Moreover, rabbit models of human lipoprotein disorders, such as the Watanabe Heritable Hyperlipidemic (WHHL) and St. Thomas' Hospital strains, models of familial hypercholesterolemia (FH) and familial combined hyperlipidemia, respectively, allow for the evaluation of candidate genes for potential use in the treatment of dyslipoproteinemic patients (10–13). This review summarizes each study in

Abbreviations: apo, apolipoprotein; APOBEC-1, apoB mRNA-editing enzyme catalytic polypeptide 1; CETP, cholesteryl ester transfer protein; FCR, fractional catabolic rate; FH, familial hypercholesterolemia; h, human; HDL, high density lipoprotein; HL, hepatic lipase; HLP, hyperlipoproteinemia; IDL, intermediate density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; Lp[a], lipoprotein(a); NZW, New Zealand White; PR, production rate; r, rabbit; VLDL, very low density lipoprotein; WHHL, Watanabe Heritable Hyperlipidemic.

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which a transgene relevant to human atherosclerosis has been overexpressed in the rabbit, with particular emphasis on lipoprotein profiles, metabolic consequences, and atherosclerosis susceptibility.

HUMAN APOLIPOPROTEIN TRANSGENES EXPRESSED IN THE NZW RABBIT

Apolipoprotein A-I

In 1993, Perevozchikov (14) described the successful production of New Zealand White (NZW) rabbits containing cDNA for human apoA-I. Three years later, Duverger and colleagues (15) reported the generation of transgenic NZW rabbits expressing human apoA-I in the liver. These rabbits were created by use of an 11 kb genomic human apoA-I construct containing a liver-specific promoter. The construct consisted of the entire apoA-I gene, as well as 3.5 kb downstream from the 3′ end and 5.5 kb of the 5′-flanking sequence (16). Northern blot analysis confirmed that human apoA-I mRNA was present only in the liver of transgenic rabbits, whereas rabbit apoA-I mRNA was detected only in the intestine. This is consistent with previous studies which have shown that the intestine is the sole site of apoA-I synthesis in the rabbit (17, 18). The five founder rabbits had plasma human apoA-I concentrations ranging from 8 to 100 mg/dL, with reduced concentrations of rabbit apoA-I relative to controls.

The effect of human apoA-I transgene expression on plasma lipids was determined in 3-month-old transgenic F1 progeny as shown in **Table 1**. The three lines described had human apoA-I concentrations ranging from 30 to 175 mg/dL and also had reductions in endogenous apoA-I levels. Plasma total cholesterol concentrations were elevated in human apoA-I transgenic rabbits relative to littermate controls due to significant increases in HDL cholesterol concentrations, with no differences noted in low density lipoprotein (LDL) cholesterol concentrations. Further characterization of HDL isolated from transgenic rabbits indicated that it had the same size distribution as that of nontransgenic animals and was of similar composition, with the exception that human, rather than rabbit, apoA-I was its principal protein component. However, the distribution of human apoA-I among HDL subspecies was different than that of rabbit apoA-I in that it was preferentially associated with pre-β-migrating HDL. An examination of cellular cholesterol efflux from Fu5AH cells incubated with serum of human apoA-I transgenic rabbits demonstrated a strong correlation between the ability to promote efflux and the concentration of apoA-I in pre-β HDL.

The influence of increased hepatic apoA-I expression on atherosclerosis susceptibility in this model was also reported by Duverger et al. in 1996 (19), using the F1 progeny described in their initial report. This group had a mean human apoA-I concentration of 175 ± 46 mg/dl (15). Eight human apoA-I transgenic and 15 nontransgenic littermate rabbits were fed a cholesterol-rich diet (0.48 g cholesterol per 120 g of diet) for 14 weeks, during which time the amount of cholesterol in the diet of transgenics was reduced from 0.48 g to 0.36 g to produce plasma concentrations of proatherogenic apoB-containing lipoproteins that were comparable to those of controls. Throughout the 14-week period, levels of nonHDL cholesterol remained similar between the two groups and accounted for 95% of total plasma cholesterol. However, the level of HDL cholesterol (mg/dl) in the human apoA-I transgenic group (68 ± 11) was almost twice that of the control group (37 ± 3). By week 2 of the diet, human apoA-I concentrations had decreased by more than 30% from baseline in transgenics, while rabbit apoA-I levels increased 2-fold. This trend continued throughout the course of the diet such that the levels of human and rabbit apoA-I were similar, at 60 mg/dl for each, by week 14. This level of total apoA-I was 3-fold higher than that of 40 mg/dl for the control group.

TABLE 1. Effects of human apolipoprotein transgenes on plasma lipid concentrations in transgenic rabbits

Animal	Reference	Range of Plasma Human Apolipoprotein Concentration	Chow Diet				High Cholesterol Diet			
			TC	LDL-C	HDL-C	TG	TC	LDL-C	HDL-C	TG
		mg/dl								
New Zealand White										
hApoA-I	Duverger (15, 19)	30–175	↑	↔	↑	↔	↑	↔	↑	↔
hApoB-100	Fan (23)	5–100	↑	↑	↓	↑	ND	ND	ND	ND
$hApoE_2$	Huang (29)	30–70	↑[a]	ND[b]	↓♂,↑♀	↑[a]	ND	ND	ND	ND
$hApoE_3$	Fan (31)	6-low								
	Taylor (36)	13-med[c] 26-high	↑	↑	↔	↓	↑	↑	ND	ND
Watanabe Heritable Hyperlipidemic										
hApoA-I	Hoeg (37)	2–59[d]	↑	↔	↑	↔	ND	ND	ND	ND
hApoA-I	Emmanuel (38)	101 ± 6	↑	↔	↑	↔	ND	ND	ND	ND

Data from F1 generation. Changes are expressed relative to nontransgenic control values; ND, not determined.

[a] Levels were significantly increased in both male and female $hApoE_2$ transgenic rabbits; however, the increases in males were much greater than those in female transgenic rabbits.

[b] Data provided were for LDL + HDL_1, not for LDL alone.

[c] This line was used for further study.

[d] Unpublished data.

TABLE 2. Effects of human apolipoprotein and lipid enzyme transgenes on atherosclerosis in rabbits

Animal	Reference	Extent of Atherosclerosis	Comments
New Zealand White			
hApoA-I	Duverger (19)	↓	In order to produce similar levels of nonHDL-C, hApoA-I transgenic and control rabbits were fed 120 g of diet per day supplemented with 0.36 g and 0.48 g of cholesterol, respectively. At 14 wks, the control group had 30 ± 8% of the thoracic aorta surface covered by lesions vs. 15 ± 12% in the transgenic group ($P < 0.003$).
$hApoE_2$	Huang (29)	↑	♂ and ♀ $hApoE_2$ transgenic rabbits had significantly more atherosclerosis in both the aortic arch (24% ♂, 10% ♀) and abdominal aorta (10% ♂, 5% ♀) relative to nontransgenic controls, who had no stained lesions. ♂ $hApoE_2$ transgenic rabbits had more extensive atherosclerosis than ♀ transgenic rabbits, but the differences were not statistically significant.
hHL	Fan (41)	?	Cholesterol supplementation in the diet of control rabbits was reduced to yield an average plasma cholesterol level similar to that observed in hHL transgenic rabbits that were fed a 0.3% cholesterol diet. After 10 wks, about 8% of the aortic surface was covered with lesions in both the control and transgenic groups.
	Taylor (36)		
hLCAT	Hoeg (47)	↓	After 17 wks on a 0.3% cholesterol diet, hLCAT transgenic rabbits had significantly less aortic atherosclerosis relative to nontransgenic controls as assessed by both quantitative planimetry (−86%, $P < 0.003$) and quantitative immunohistochemistry (−93%, $P < 0.009$).
Watanabe Heritable Hyperlipidemic			
hApoA-I	Emmanuel (38)	↓	In hApoA-I transgenic rabbits, 47 ± 4% of the surface of the thoracic aorta was covered by lesions vs. 73 ± 12% in nontransgenic controls.
hLCAT	Hoeg (50)		
LDLr+/−		?	Neither transgenic nor control LDLr+/− rabbits had significant spontaneous atherosclerosis.
LDLr−/−		↔	Both transgenic and control LDLr−/− rabbits had 84 ± 3% of the aortic surface covered with plaque.

Studies in NZW rabbits assessed diet-induced atherosclerosis, with the exception of the $hApoE_2$ study in which spontaneous atherosclerosis was determined. All studies involving WHHL rabbits assessed spontaneous atherosclerosis, after a period of 1 to 2 years. Changes are expressed relative to nontransgenic controls.

Morphological examination of the thoracic aortas of transgenic and control rabbits revealed that the control group had 30 ± 8% of the aortic surface covered by lesions versus 15 ± 12% for the human apoA-I transgenic group (**Table 2**). The amounts of total, free, and esterified cholesterol were all significantly reduced in the aortas of transgenic, relative to control, rabbits, with values in transgenics that were 47%, 64%, and 38% of those observed for controls, respectively. The results of cellular cholesterol efflux studies indicated that the preceding was due, in part, to enhanced reverse cholesterol transport in human apoA-I transgenic animals. Incubation of serum from transgenic rabbits with Fu5AH cells promoted efflux to a greater extent (+24%) than did control serum. These data suggest that the protective effect of the human apoA-I transgene must have been related to its influence on HDL levels and reverse cholesterol transport, as there were no differences in the concentrations of apoB-containing lipoproteins between the two groups.

To further investigate the mechanism by which the apoA-I transgene may protect against atherosclerosis, Lebuffe and colleagues (20) tested the hypothesis that human apoA-I might influence the biological activity of the endothelium-derived relaxing factor, nitric oxide. This was based on the knowledge that oral l-arginine, the substrate for nitric oxide synthase, has been associated with a marked reduction in aortic and coronary atherosclerosis when supplemented in the diet of hypercholesterolemic rabbits (21, 22). In vitro endothelium-dependent receptor-dependent vascular relaxation and endothelium-dependent receptor-independent vascular relaxation were assessed in human apoA-I transgenic and control rabbits. Endothelium-independent vascular relaxation was also investigated with sodium nitroprusside. The maximal endothelium-dependent receptor-dependent relaxation elicited by acetylcholine was significantly attenuated in the group transgenic for human apoA-I (27 ± 10%) relative to the control group (69 ± 3%), as was endothelium-dependent receptor-independent relaxation. In contrast, endothelium-independent relaxation did not differ between the two groups, and only maximal endothelium-dependent receptor-dependent vascular relaxation was negatively correlated with total apoA-I concentration. The mechanism responsible for the impairment of endothelium-dependent vascular relaxation in human apoA-I transgenic rabbits was not defined. It is possible that apoA-I may have directly affected nitric oxide synthesis or, alternatively, may have induced endothelium-dependent endothelin secretion.

Apolipoprotein B

The development of rabbits transgenic for human apoB-100 was described by Fan et al. in 1995 (23). In this study, an 80 kb fragment that spanned the entire apoB gene, including 17.5 kb and 19 kb of 5′- and 3′-flanking sequences, respectively, was used for microinjection into NZW rabbit zygotes (24). Four founder animals, with plasma

human apoB-100 concentrations ranging from <12–94 mg/dl, were generated, one of which was bred to yield a line of transgenic rabbits for further study (Table 1). The plasma of fasting transgenic rabbits contained human apoB-100 but no human apoB-48, consistent with the absence of apoB mRNA editing in rabbit liver (2).

Analysis of plasma lipid concentrations in human apoB-100 transgenic rabbits revealed that total plasma cholesterol and triglyceride levels were 2- to 3-fold higher than those observed in age-, sex-, and diet-matched nontransgenic littermates, whereas those of HDL cholesterol were markedly reduced. Nearly all of the cholesterol and human apoB-100 in the plasma of transgenics was in the LDL fraction, with a striking enrichment also noted in LDL triglyceride content. LDL size, estimated by gradient polyacrylamide gel electrophoresis, was significantly reduced in transgenics (≈240Å) relative to controls (≈275Å). Transgenic rabbit LDL were further found to contain large amounts of apolipoproteins C-III and E, suggesting that hepatic overproduction of apoB-100 may have led to the assembly of nascent triglyceride-rich particles in these animals that had the size and density of LDL rather than of VLDL. The triglycerides in these smaller, denser nascent lipoproteins would likely have been less accessible to lipoprotein lipase, contributing to the increased plasma triglycerides observed in the human apoB-100 transgenic rabbits. Atherosclerosis susceptibility was not determined in this study.

Apolipoprotein B-100/apolipoprotein [a]

To investigate the in vivo assembly requirements for lipoprotein[a] (Lp[a]), rabbits containing human genomic transgenes for apoB, apo[a], or both were generated by Rouy et al. (25). ApoB transgenic rabbits were produced using a 90 kb phagemid containing the human apoB gene (26), while a yeast artificial chromosome (YAC) clone containing the human apo[a] gene was used to generate the apo[a] transgenic rabbits (27). Rabbits transgenic for both the apoB and apo[a] genes were generated by mating founder animals. Liver and testis were the major sites of expression for both the apoB and apo[a] transgenes, with small amounts of apo[a] mRNA also detected in the brain. The mean concentration of human apoB in the plasma of human apoB transgenic rabbits was 18 mg/dl, whereas that of apo[a] was 2.5 mg/dl in both apo[a]-only and apoB/apo[a] transgenic rabbits. The effects of these transgenes on the concentrations of plasma lipids and lipoproteins were not discussed in detail in this report.

The formation of Lp[a] in the plasma of transgenic rabbits was evaluated in this study by immunoblotting with antisera specific for human apo[a]. Under nonreducing conditions, plasma samples from both the apo[a]-only and the combined apoB/apo[a] transgenic rabbits demonstrated two bands, a higher molecular weight band corresponding to the apoB/apo[a] complex and a lower molecular weight band representing noncovalently bound apo[a]. In the human apoB/apo[a] transgenics, approximately 20% of the apo[a] consistently migrated as free apo[a], with the remaining 80% covalently bound to either human or rabbit apoB. Conversely, in the human apo[a]-only transgenic rabbits, the reverse distribution was observed, demonstrating the ability of rabbit apoB to form a covalent linkage with human apo[a]. Due to the ability of rabbit apoB, unlike murine apoB, to form a covalent linkage with human apo[a] (28), these investigators also performed a comparison of its amino acid sequence and found that the site of apo[a] attachment differed from that in human apoB. Future studies will evaluate atherosclerosis susceptibility in these rabbits but interpretation will be limited due to the fact that the plasma Lp[a] concentrations expressed in this model are about 10-fold lower than those deemed to put humans at risk for atherosclerosis.

Apolipoprotein E_2

Transgenic rabbits that produced and expressed high plasma concentrations of human apolipoprotein E_2 (Cys_{112}, Cys_{158}), an apoE variant associated with the human genetic disorder type III hyperlipoproteinemia (HLP), were described by Huang and colleagues in 1997 (29). The DNA construct used to generate these rabbits consisted of the complete human $apoE_2$ gene, along with 5 kb and 1.7 kb of its 5′- and 3′-flanking sequence, respectively, as well as a 3.8 kb downstream fragment containing the hepatic control region for this gene (30). Transgenic F1 progeny expressing high levels of $apoE_2$, 30–70 mg/dl, were generated from one male founder whose plasma $apoE_2$ concentration was 43 mg/dl. Nontransgenic rabbits expressed approximately 4 mg/dl of endogenous apoE.

The effect of $apoE_2$ expression on plasma lipid levels was assessed in both male and female transgenic rabbits and their nontransgenic littermates at 5 months of age (Table 1). Nontransgenic rabbits had minor gender differences in their lipid values (mg/dl), with females having slightly higher total (49 ± 8) and HDL (37 ± 7) cholesterol concentrations relative to males, 34 ± 5 and 22 ± 2, respectively. In contrast, marked gender differences were noted in the human $apoE_2$ transgenic animals. Male transgenic rabbits had 8- and 15-fold increases in plasma total cholesterol (289 ± 148) and triglyceride (697 ± 452) levels, respectively, as compared with nontransgenic males, whereas 3- and 6-fold elevations were noted in total cholesterol (140 ± 46) and triglycerides (174 ± 66) in female $apoE_2$ transgenics, respectively, relative to controls. The mean HDL cholesterol concentration in female transgenic rabbits (52 ± 8) was twice that of male transgenic rabbits (26 ± 5) and was about 40% higher than that of nontransgenic female rabbits.

Analysis of plasma lipoproteins in transgenic rabbits indicated that both genders displayed the hallmarks of type III HLP, namely the presence of β-migrating VLDL and significant increases in VLDL and intermediate density lipoprotein (IDL) concentrations. However, gender differences were again noted within the human $apoE_2$ transgenic group, with females having less VLDL and IDL, but more HDL, than males. Moreover, the HDL particles observed in females were larger than those in males and eluted in the HDL_1 size range. Male transgenic rabbits

had much more $apoE_2$ in the VLDL + IDL fractions than did female transgenic rabbits (49% vs. 21%) and also had a greater ratio of human $apoE_2$ to rabbit apoE in β-VLDL (7:1 vs. 4:1). Kinetic studies using ^{125}I-labeled nontransgenic or $apoE_2$ transgenic VLDL revealed that the presence of $apoE_2$ dramatically slowed the clearance of transgenic, as compared with control, VLDL from the plasma of nontransgenic rabbits, suggesting that the hyperlipidemia observed in transgenics was due, in part, to defective interaction of $apoE_2$ with lipoprotein receptors. VLDL lipolysis was also found to be aberrant in $apoE_2$ transgenic rabbits.

To investigate the potential role of sex hormones in modulating type III HLP, as well as to further explain the gender differences seen in $apoE_2$ transgenic rabbits, these researchers treated three male transgenic rabbits with 100 μg of 17α-ethinyl estradiol per kg/day. After 10 days of treatment, this resulted in 73% and 89% reductions in plasma total cholesterol and triglyceride levels, respectively, with HDL increasing in concentration from 24 ± 4 to 40 ± 6 mg/dl and shifting in size to HDL_1. The potential role of the LDL receptor in the estrogen-mediated changes of plasma lipid levels was determined by measuring clearance of the LDL receptor antibody 9D9 from estrogen-treated male transgenic rabbits. Clearance of the 9D9 antibody was significantly enhanced in those animals that were treated with estrogen, suggesting LDL receptor up-regulation with estrogen treatment. Studies with ovariectomized female $apoE_2$ transgenic rabbits complemented those described in estrogen-treated males and indicated that estrogen status was partly responsible for the gender differences noted in $apoE_2$ transgenic rabbits.

Atherosclerosis susceptibility was assessed in both male and female nontransgenic and human $apoE_2$ transgenic rabbits after 11 months on a normal rabbit chow diet (Table 2). Sudan IV staining of aortas from both transgenic groups revealed obvious lesions, especially in the aortic arch and the upper region of the abdominal aorta, whereas nontransgenic rabbits essentially had no stained lesions. Male $apoE_2$ transgenic rabbits had 24% of the aortic arch surface covered by lipid staining, as compared with 10% in transgenic females. There was also less involvement seen in the abdominal aortas of $apoE_2$ transgenic females, with 5% of the surface stained relative to 10% in $apoE_2$ transgenic males; however, none of these gender differences were statistically significant. Thus, it may be concluded from these studies that expression of human $apoE_2$ in transgenic rabbits promotes spontaneous atherosclerosis in both genders, demonstrating the atherogenic potential of β-VLDL.

Apolipoprotein E_3

Recently, Fan and colleagues described the generation of transgenic rabbits expressing the most common isoform of apoE in the human population, $apoE_3$ (Cys_{112}, Arg_{158}) (31). The construct used to generate these rabbits contained the complete human apoE gene together with 5 kb of its 5′-flanking sequence and 1.7 kb of its 3′-flanking sequence, ligated to a 3.8 kb downstream fragment containing the hepatic control region for this gene. This region directs high levels of expression in the liver with little expression in any other tissue (30). These investigators established three independent transgenic rabbit F1 lines that expressed human $apoE_3$ in the plasma at levels of ≈6, ≈11, and ≈13 mg/dl, with the latter group used for most subsequent studies, as well as for the generation of F2 homozygotes. F2 homozygotes had a mean plasma human $apoE_3$ concentration of 26 mg/dl.

Intermediate expression of human $apoE_3$ (13 mg/dl) led to significant increases in plasma total cholesterol concentrations in both male (+74%) and female (+38%) transgenic rabbits relative to nontransgenic controls expressing approximately 3 mg/dl of endogenous rabbit apoE. In contrast, male and female $apoE_3$ transgenics had significantly lower levels of plasma triglycerides (−35% ♂, −19% ♀) than did their age- and gender-matched controls. In both genders, HDL cholesterol levels were also increased in the transgenic versus the nontransgenic group (+29% ♂, +16% ♀); however, these differences were not statistically significant. Analysis of plasma lipoproteins revealed that transgenic rabbits had lower VLDL levels, higher LDL levels, and an accumulation of HDL_1 compared with nontransgenic littermates, but that human and endogenous apoE were not differentially distributed among lipoproteins. ApoE was found predominantly in the HDL fraction, with lesser amounts in VLDL, IDL, and LDL. Conversely, the distribution of apoB was shifted by transgene expression, being most abundant in both the IDL and LDL fractions of $apoE_3$ transgenic rabbits. The most striking differences noted between transgenic and nontransgenic lipoproteins were observed in the VLDL fraction. Large VLDL (>36 nm) were nearly absent from the plasma of transgenic rabbits, whereas they accounted for 20% of this fraction in nontransgenic rabbits. This was perhaps due to an increase in receptor-mediated clearance facilitated by apoE.

To explore the role of $apoE_3$ in receptor-mediated clearance of lipoproteins, chylomicron remnant and LDL catabolism were determined in these studies (**Table 3**). Intravenously injected radiolabeled chylomicrons that had been isolated from the thoracic lymph of a cholesterol-fed dog were cleared more rapidly from the plasma of F1 transgenic rabbits than from nontransgenic controls. There was a corresponding increase in the uptake of radioactivity by the livers of transgenic animals, consistent with the ability of apoE to mediate chylomicron remnant clearance from rabbit plasma via receptor-mediated pathways (32–35). In contrast, ^{125}I-labeled human LDL was catabolized at a slower rate by human $apoE_3$ transgenic rabbits, with estimated residence times of 11 and 6 h for transgenic and control animals, respectively. Further studies demonstrated that the clearance of radioactively labeled monoclonal 9D9 LDL receptor antibody from the plasma was similar for transgenic and control rabbits, indicating that down-regulation of LDL receptors was not responsible for the delayed catabolism of LDL observed in transgenics.

The effects of human $apoE_3$ transgene expression on atherogenesis have been addressed in a preliminary report (36). After 10 weeks on a 0.3% cholesterol diet,

TABLE 3. Effects of human apolipoprotein and lipid enzyme transgenes on lipoprotein metabolism in rabbits

Animal	Reference	VLDL FCR	VLDL PR	LDL FCR	LDL PR	HDL FCR	HDL PR	Comments
New Zealand White								
$hApoE_2$	Huang (29)	↓	ND	ND	ND	ND	ND	^{125}I-labeled control or $hApoE_2$ transgenic VLDL were injected into control ♂ rabbits. At 30 min post-injection, 38% of the initial dose of control VLDL remained in the plasma vs. >60% of $hApoE_2$ VLDL.
$hApoE_3$	Fan (31)	ND	ND	↓	ND	ND	ND	^{125}I-labeled human LDL were cleared faster in control vs. transgenic rabbits, with estimated residence times of 6 and 11 h, respectively. In contrast, chylomicron remnant clearance was slower in controls relative to $hApoE_3$ transgenics.
hHL	Fan (51)	↑	ND	ND	ND	ND	ND	^{125}I-labeled rabbit β-migrating VLDL were cleared more rapidly from the plasma of transgenic vs. control rabbits.
hLCAT	Brousseau (45, 46, 48)	↑	↔	↑	↔	↓	↔	LDL apoB-100 FCR (h^{-1}) was increased in transgenic vs. control rabbits on both chow (1.09 ± 0.17 vs. 0.22 ± 0.01) and high-cholesterol (0.131 ± 0.03 vs. 0.031 ± 0.00) diets. HDL apoA-I FCR (d^{-1}) was decreased in transgenic vs. control rabbits on both chow (0.328 ± 0.03 vs. 0.528 ± 0.04) and high-cholesterol (0.282 ± 0.03 vs. 0.496 ± 0.04) diets.
Watanabe Heritable Hyperlipidemic								
hApoA-I	Brousseau[a]	ND	ND	↔	↔	↓hA-I ↔rA-I	↓rA-I	The FCR of hApoA-I (h^{-1}) in transgenics (0.032 ± 0.00) was half that of controls (0.061 ± 0.02); however, the FCR of rabbit apoA-I was similar for transgenic (0.048 ± 0.00) and control (0.047 ± 0.00) animals. The PR of rabbit apoA-I was decreased 40% in trangenics vs. controls.
hLCAT	Brousseau (46, 49)							
LDLr+/−		↑[a]	↔[a]	↑	↔	↓	↑	LDL apoB-100 FCR (h^{-1}) was increased in hLCAT (0.17 ± 0.02) vs. control (0.05 ± 0.01) LDLr+/− rabbits, but not in hLCAT+/LDLr−/− rabbits. LCAT decreased HDL apoA-I FCR ≈40% and increased apoA-I PR ≈2-fold in both LDLr+/− and LDLr−/− rabbits.
LDLr−/−		↔[a]	↔[a]	↔	↔	↓	↑	

Information is provided only for those studies in which lipoprotein kinetics were assessed. Changes are expressed relative to nontransgenic controls, unless otherwise indicated. All data are from studies in which rabbits were consuming a standard rabbit chow diet.

[a]Unpublished observations.

plasma cholesterol concentrations were approximately one-third greater in $apoE_3$ transgenics relative to controls, due largely to an accumulation of cholesterol in the IDL and LDL fractions. An initial analysis of aortic atherosclerosis showed that, while lesion areas were about 40% greater in human $apoE_3$ transgenic versus control rabbits, lesion thickness was substantially reduced in the former group. Further studies will be required to better understand the role of $apoE_3$ in atherosclerosis susceptibility.

HUMAN APOLIPOPROTEIN TRANSGENES EXPRESSED IN THE WHHL RABBIT

Apolipoprotein A-I

In 1993, Hoeg and colleagues (37) described the generation of the lipoprotein field's first transgenic rabbit, an LDL receptor-deficient model containing the transgene for human apoA-I. Three founder rabbits, expressing relatively low levels of human apoA-I, were identified and used for the production of F1 progeny. As shown in Table 1, transgenic F1 progeny had plasma human apoA-I concentrations ranging from 2 to 59 mg/dl, and HDL cholesterol levels that were significantly increased relative to those of nontransgenic controls (24 ± 2 vs. 6 ± 1 mg/dl, respectively). Detailed kinetic studies using radiolabeled human and rabbit apoA-I revealed that the clearance of rabbit apoA-I was similar in transgenic and control animals, resulting in a residence time of 21 hours for both groups (**Fig. 1A**). In contrast, the catabolism of human apoA-I was significantly delayed in the transgenic group relative to the control group, with plasma residence times of 32 and 18 h, respectively (Fig. 1B). It is important to note that radiolabeled rabbit and human apoA-I were catabolized at similar rates when injected into nontransgenic rabbits, lessening the likelihood that the divergent clearance of human apoA-I observed between the transgenic and control groups was due to differential handling of the rabbit and human proteins. Atherosclerosis susceptibility was not determined in these studies.

The production of human apoA-I transgenic WHHL rabbits was subsequently reported in abstract form by another group of investigators (38). In these studies, human

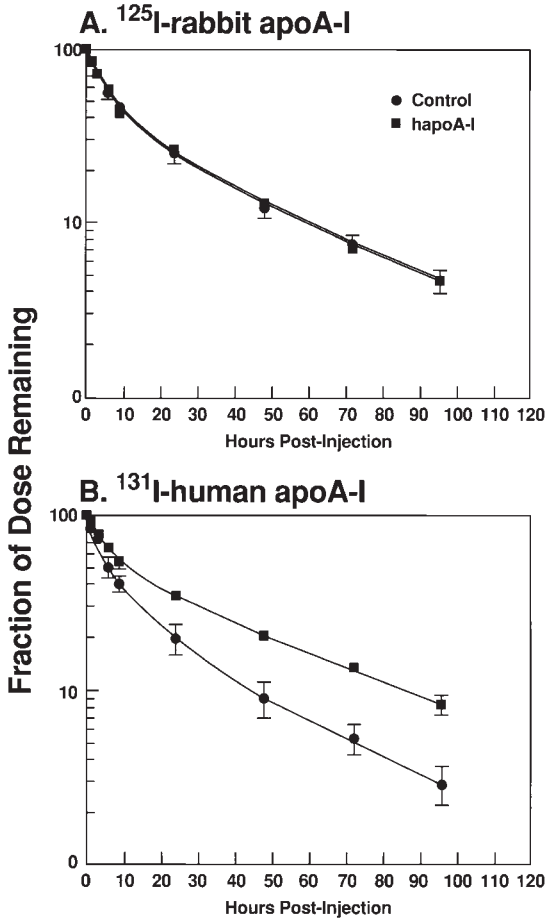

Fig. 1. In vivo metabolism of rabbit apoA-I (A) and human apoA-I (B) in control and human apoA-I transgenic WHHL rabbits. Purified rabbit and human apoA-I were radiolabeled with ^{125}I and ^{131}I, respectively, and were reassociated with autologous rabbit plasma. The radioactivity decay curves for control (●) and human apoA-I transgenic (■) WHHL rabbits are illustrated using a two-log scale as the ordinate. The clearance of rabbit apoA-I from the plasma was nearly identical in control and human apoA-I transgenic WHHL rabbits. In contrast, the catabolism of human apoA-I was significantly delayed in human apoA-I transgenic rabbits relative to controls, with rabbit and human apoA-I catabolized at a similar rate in control WHHL rabbits.

apoA-I transgenic NZW rabbits, described previously in this review (15), were crossed with WHHL rabbits. Human apoA-I transgenic WHHL rabbits had a mean human apoA-I concentration of 101 ± 6 mg/dl and a mean rabbit apoA-I concentration of 60 ± 10 mg/dl. The latter level was not significantly different from that of the nontransgenic group. Plasma HDL cholesterol levels (mg/dl) were significantly greater in transgenics (46 ± 4) relative to controls (25 ± 4), with no differences noted in plasma VLDL + LDL cholesterol concentrations (1176 ± 222 vs. 962 ± 170 mg/dl, respectively). Analysis of fatty streak lesions of thoracic aortas showed that transgenic rabbits had a 36% reduction in the total surface area of the aorta covered by lesions as compared with nontransgenic controls (47 ± 4% vs. 73 ± 12%, respectively), indicating that human apoA-I expression protected against atherogenesis in this model.

LIPID ENZYME TRANSGENES EXPRESSED IN THE NZW RABBIT

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1

Transgenic mice and rabbits expressing rabbit apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1, APOBEC-1, were generated by Yamanaka and colleagues (39) to determine whether hepatic expression would reduce plasma LDL cholesterol concentrations. A full-length cDNA fragment of rabbit APOBEC-1 was microinjected into fertilized oocytes of NZW rabbits (40), and transgene copy number was estimated by Southern blot analysis using the endogenous rabbit APOBEC-1 gene as a control. Two transgenic rabbit founders were identified, one having one copy of the APOBEC-1 transgene and the other 17 copies. The latter transgenic rabbit appeared normal at birth but grew at a slower rate than its nontransgenic littermate, and, after 8 weeks, became weak and immobile and was killed. In contrast, the founder with a single copy of the APOBEC-1 transgene was healthy and grew normally.

In the transgenic rabbit founders, APOBEC-1 was expressed mainly in the liver. Primer-extension analysis showed that 78% of the apoB mRNA was edited in transgenic rabbit liver, whereas no significant editing of apoB mRNA occurred in control rabbit liver. The total plasma cholesterol concentration of the transgenic rabbit having one copy of the APOBEC-1 transgene was 127 mg/dl, as compared with 163 mg/dl for a nontransgenic littermate control. The plasma lipoprotein profile of this animal was characterized by reductions in VLDL-, IDL-, and LDL-cholesterol concentrations, with an increase in the level of HDL cholesterol. Despite the favorable lipoprotein profile, it must be concluded that the therapeutic potential of APOBEC-1 is compromised by the fact that high copy number was associated with liver dysplasia.

Hepatic lipase

Transgenic rabbits overexpressing human hepatic lipase were described by Fan and coworkers in 1994 (41). The construct used for generation of these animals contained liver-specific expression sequences from the human apoE gene, the hepatic control region of the apoE/C-I locus, and a full-length human HL cDNA (30). Nine founder rabbits were identified by Southern blot analysis, with 6 of them having significant expression of human HL in post-heparin plasma. Total RNA isolated from 10 different tissues confirmed that transgene expression was only found in the liver. Post-heparin HL activity was 7- to 81-fold greater in transgenic plasma than in control plasma, with a level of HL activity which was similar to that reported for humans (42). Consistent with the relative deficiency of HL activity in the rabbit model, endogenous rabbit HL activity was barely detectable in control animals. Transgenic F1 progeny from founders having human HL specific activities (μeq FA per mg HL per h) ranging from 14,000 to 27,000 were used for all further studies (**Table 4**).

HL expression had significant effects on plasma lipid

TABLE 4. Effects of lipid enzyme transgenes on plasma lipid concentrations in transgenic rabbits

Animal	Reference	Range of Plasma Enzyme Activity[a]	Chow Diet				High Cholesterol Diet			
			TC	LDL-C	HDL-C	TG	TC	LDL-C	HDL-C	TG
New Zealand White										
rAPOBEC-1[b]	Yamanaka (39)	ND	↑	↓	↑	ND	ND	ND	ND	ND
hHL	Fan (41) Taylor (36)	14,000–27,000[c]	↓	↔	↓	↔♂,↓♀	↓	↓	↓	ND
hLCAT	Hoeg (43, 47)	541–1,593[d]	↑	↓	↑	↔	↓	↓	↑	↓
Watanabe Heritable Hyperlipidemic										
hLCAT	Brousseau (49)									
LDLr+/−		185–369[d]	↔	↓	↑	↓	ND	ND	ND	ND
LDLr−/−		215–233[d]	↔	↔	↑	↓	ND	ND	ND	ND

Changes are expressed relative to nontransgenic control values; ND, not determined.

[a] Enzyme activity range of F1 generation.

[b] Data are from one transgenic animal that had one copy of the rabbit APOBEC-1 gene. Another founder animal with 17 copies of the gene was killed 8 weeks after birth due to illness.

[c] Human HL specific activity (μeq FA per mg of HL per hour). hHL specific activity data are from 3 founder lines that were used to generate transgenic F1 progeny for all further studies. hHL activity data were not provided for the latter animals.

[d] α-LCAT activity (nmol/ml/h), as determined by a proteoliposome assay. The mean value for nontransgenic NZW rabbits was 101 ± 9, whereas those for nontransgenic LDLr+/− and LDLr−/− rabbits were 55 ± 11 and 62 ± 4, respectively.

levels in rabbits maintained on a standard rabbit chow diet, with a mean decrease of 42% for total cholesterol and of 58% for triglycerides in female transgenic rabbits relative to controls. HDL cholesterol concentrations were also dramatically reduced in HL transgenic (4 ± 2 mg/dl) versus control (25 ± 10 mg/dl) rabbits, whereas little effect was noted on nonHDL cholesterol levels. A detailed analysis of plasma lipoproteins confirmed that human HL transgenic rabbits had strikingly reduced HDL concentrations, with all major classes of HDL decreased in quantity. VLDL and IDL fractions were also reduced in female transgenic rabbits relative to controls, but male transgenics did not have a decrease in VLDL content. Notable reductions were observed in the amounts of apoA-I, apoC-III, and apoE in the HDL fractions of transgenic rabbits, while apoB was reduced in the IDL fraction but was prominent in β-migrating lipoproteins.

In order to determine the susceptibility of the HL transgenic rabbit to atherosclerosis, 3-month-old transgenic and control rabbits were fed a diet containing 0.3% cholesterol and 3% soybean oil (36). The total cholesterol concentration in nontransgenic animals increased from approximately 50 mg/dl at baseline to an average of 1,200–1,500 mg/dl at week 5, after which point it stabilized. However, in the HL transgenic rabbits, the response to dietary cholesterol was attenuated, with a mean plasma total cholesterol concentration that was about one-third that of the control group. Both IDL and HDL concentrations were reduced in the HL transgenic group as compared with the nontransgenic control group. The extent of aortic atherosclerosis was assessed after 10 weeks on the high-cholesterol diet. Approximately 15% of the aortic surface was covered with thick, raised lesions in the control group. Additional studies were performed in which the level of cholesterol in the diet of nontransgenic rabbits was reduced to yield a mean plasma total cholesterol concentration that was similar to that of the HL transgenic group. In both groups, about 8% of the aortic surface had atherosclerotic lesions. Further studies will be required to clarify the role of HL in atherosclerosis susceptibility in this model.

Lecithin:cholesterol acyltransferase

In 1996, Hoeg and colleagues (43) reported the production of human LCAT transgenic NZW rabbits. These studies were undertaken to evaluate the impact of the overexpression of this enzyme on plasma lipid and lipoprotein concentrations in an animal model expressing CETP, unlike the murine model. A 6.2 kb human genomic DNA construct consisting of the entire LCAT gene, including 0.85 and 1.134 kb of the 5′- and 3′-flanking regions, respectively, was used in the generation of these animals (44). Five transgenic founder rabbits were produced, 3 of which expressed the human LCAT gene. Northern blot analysis revealed that the liver was the principal site of human LCAT expression, with detectable quantities of LCAT mRNA noted in the brain, heart, and muscle as well.

Human LCAT overexpression in NZW rabbits resulted in substantial changes in plasma lipid and lipoprotein concentrations (Table 4). As with the original founder, plasma total, free, and esterified cholesterol, as well as phospholipid, concentrations were significantly increased in both low and high expressor F1 progeny relative to littermate controls. The elevation in plasma total cholesterol content was due to a marked increase in HDL cholesterol concentration (mg/dl) in low (71 ± 6) and high (79 ± 6) human LCAT expressors as compared with controls (32 ± 1). The lipoprotein profile of transgenic rabbits was characterized by the presence of a large cholesteryl ester- and phospholipid-enriched HDL, analogous to HDL_1, with a virtual absence of apoB-containing lipoproteins. In contrast, plasma cholesterol was distributed in particles corresponding to VLDL + IDL, LDL, and HDL in nontransgenic rabbits, and their HDL was much smaller in size than that of the transgenic group. Plasma apoA-I and apoE concentrations were significantly increased in human LCAT transgenics, whereas those of apoB were reduced.

In order to elucidate the mechanisms responsible for the hyperalphalipoproteinemia observed in transgenic rabbits, HDL apoA-I kinetics were assessed in age- and gender-matched groups of rabbits with high, low, or no human LCAT expression (45). These studies demonstrated that in vivo apoA-I catabolism was delayed in a gene dose-dependent manner in human LCAT transgenic rabbits, such that the fractional catabolic rate (FCR) of apoA-I was slowest in high expressors, followed by low expressors, and, lastly, by controls (Table 3). HDL particle size also correlated with the level of LCAT expression, suggesting that LCAT-induced changes in HDL composition and size ultimately reduced apoA-I catabolism by altering apoA-I conformation and/or particle regeneration. No differences in apoA-I production rates were observed in these studies. Similarly, the reductions seen in plasma LDL apoB concentrations in LCAT transgenic rabbits were found to be the result of alterations in LDL apoB-100 catabolism, with no differences in production rates (46). On a standard rabbit chow diet, the FCR of LDL apoB-100 in LCAT transgenic rabbits was nearly 5-times greater than that of nontransgenic rabbits (Table 3). These findings demonstrated for the first time that LCAT not only plays an important role in HDL metabolism but in that of apoB-containing lipoproteins as well.

The role of LCAT overexpression in susceptibility to diet-induced atherosclerosis was next examined in these rabbits (47). On a 0.3% cholesterol diet, plasma HDL cholesterol concentrations (mg/dl) increased from baseline in both control (24 ± 1 to 39 ± 3) and human LCAT transgenic rabbits (161 ± 5 to 200 ± 21). NonHDL cholesterol concentrations also rose in both groups of rabbits but to a far lesser extent in the transgenic (196 ± 14) versus control (509 ± 57) group. Metabolic studies using radiolabeled HDL and LDL revealed that the differences observed in plasma lipoprotein concentrations were attributable to distinctive rates of catabolism, with no alterations in the production rates of either apoA-I or apoB (48) (Table 3). Compared with nontransgenic controls, LCAT transgenic rabbits were protected from diet-induced atherosclerosis, with significant reductions determined by both quantitative planimetry (−86%) and quantitative immunohistochemistry (−93%) (47).

HUMAN LIPID ENZYME TRANSGENES EXPRESSED IN THE WHHL RABBIT

Lecithin:cholesterol acyltransferase

To further assess the role of LCAT in the prevention of atherosclerosis, the human LCAT transgene was introduced into the WHHL rabbit, an animal model at high risk for the development of spontaneous atherosclerotic lesions (49). As FH is associated with significantly reduced concentrations of both HDL and apoA-I concentrations, we hypothesized that expression of LCAT in WHHL rabbits would improve the observed hypoalphalipoproteinemia and, possibly, atherosclerosis susceptibility. The human LCAT gene was introduced into WHHL rabbits by selective breeding, and experimental groups were defined by the presence or absence of the human LCAT gene, as well as by LDL receptor (LDLr) status. Heterozygosity (LDLr+/−) or homozygosity (LDLr−/−) for the LDL receptor defect was established by PCR, amplifying a 306 bp portion of exon 4 of the rabbit LDLr gene which includes a 12 bp mutant region containing a *Bgl* I restriction site. In LDLr+/− rabbits, the mean HDL cholesterol level was significantly greater in the LCAT transgenic group (62 ± 8 mg/dl) relative to the nontransgenic group (21 ± 1 mg/dl). This was similarly the case for LDLr−/− rabbits, with mean HDL cholesterol concentrations of 27 ± 2 and 5 ± 1 mg/dl for transgenic and nontransgenic animals, respectively. As was the case for human LCAT transgenic NZW rabbits, HDL particle size was increased in both LDLr+/− and LDLr−/− LCAT transgenic rabbits, with the former group having comparatively larger HDL than the latter group. Kinetic experiments using ^{131}I-labeled rabbit apoA-I demonstrated that the FCR of apoA-I (d^{-1}) was substantially delayed in LCAT transgenic (0.376 ± 0.025) versus nontransgenic (0.588) LDLr+/− rabbits, as well as in transgenic (0.666 ± 0.033) versus nontransgenic (1.194 ± 0.138) LDLr−/− rabbits. In contrast to NZW rabbits, human LCAT expression increased apoA-I production rate (PR, $mg \cdot kg \cdot d^{-1}$) relative to nontransgenic controls in both LDLr+/− (10 ± 2 vs. 6) and LDLr−/− (9 ± 1 vs. 4 ± 1) rabbits. Thus, LCAT overexpression in WHHL rabbits corrected the dual metabolic defects responsible for the hypoalphalipoproteinemia observed in LDL receptor deficiency, identifying LCAT as a potential gene therapy candidate for FH patients.

In order to explore the mechanism(s) responsible for the unexpected LCAT-induced reductions of apoB-containing lipoprotein levels, we next assessed LDL apoB-100 kinetics in LDLr+/+, LDLr+/−, and LDLr−/− human LCAT transgenic and nontransgenic rabbits (46). Because we had previously observed that LCAT enhanced LDL catabolism without influencing production rate, we hypothesized that the reduced LDL concentrations observed in both our chow- and cholesterol-fed LCAT transgenic rabbits might be due to up-regulation of the LDL receptor pathway. If correct, one would predict that no differences would be noted in LDL cholesterol levels, or metabolism, between human LCAT transgenic and nontransgenic LDLr−/− rabbits, who lack normal LDL receptor function. This, in fact, proved to be the case. Human LCAT transgenic LDLr+/+ and LDLr+/− rabbits had approximately 70% reductions in LDL apoB pool size relative to nontransgenic controls of the same LDLr status due to significant increases in LDL apoB-100 FCR (Table 3). Conversely, LDL apoB pool size and LDL apoB-100 FCR were nearly identical when LCAT transgenic and nontransgenic LDLr−/− rabbits were compared. Consistent with the preceding findings, neither aortic lipid concentrations nor the extent of aortic atherosclerosis were significantly different between the LDLr−/− groups, with 84 ± 3% of the aortic surface covered by plaque (50). From these data it can be concluded that LCAT modulates LDL metabolism via the LDL receptor pathway, ultimately, in-

fluencing atherosclerosis susceptibility. Moreover, LCAT's antiatherogenic effect requires only a single functional LDL receptor allele, making it an attractive gene therapy candidate for the majority of dyslipoproteinemic patients.

CONCLUSIONS

The development of transgenic rabbit technology has provided a unique tool with which to assess the effects of individual genes on lipoprotein metabolism and atherosclerosis susceptibility. Thus far, only the human apoA-I and LCAT transgenes have conclusively been shown to decrease atherogenesis in the rabbit. Future studies in transgenic rabbit models will undoubtedly enhance our understanding of the human atherosclerotic process, improving treatment strategies for individuals at risk for premature cardiovascular disease.

DEDICATION

This work is dedicated to the memory of my beloved mentor and friend Jeff Hoeg. Jeff was not only an excellent scientist and mentor but a wonderful human being as well. I am truly blessed to have worked with him and to have had his friendship and guidance. I will miss him deeply.

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