Cholesterol-fed and Transgenic Rabbit Models for the Study of Atherosclerosis

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The rabbit has been extensively utilized as an ideal model of atherosclerosis because of its size, easy manipulation, and extraordinary response to dietary cholesterol. The availability of spontaneously hypercholesterolemic model, Watanabe heritable hyperlipidemic rabbits (WHHL) and St. Thomas rabbits, has also provided insights into understanding human familiar hypercholesterolemia and atherosclerosis. With the advent of genetically engineered rabbits, transgenic rabbits have become a novel means to explore a number of proteins that are associated with cardiovascular diseases including atherosclerosis. To date, transgenes for human apo(a), apoA I, apoB, apoE, hepatic lipase, lecithin:cholesterol acyltransferase (LCAT), lipoprotein lipase, 15-lipoxygenase, as well as for rabbit apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1), have been expressed in rabbits. In addition, human apoA-I, LCAT and apo(a) have been introduced into WHHL rabbits which have deficient LDL receptor function. All of these transgenes have been found to have significant effects on plasma lipoprotein metabolism or/and atherosclerosis. These studies have revealed new insights into the mechanisms responsible for the development of atherosclerosis. In this article, we provide a brief review on the rabbit model for the study of atherosclerosis with emphasis on transgenic rabbit models developed during the past few years. J Atheroscler Thromb, 2000; 7: 26-32.

Key words: Transgenic rabbit, Animal model, Hypercholesterolemia, Atherosclerosis, Lipoproteins

Introduction

Atherosclerosis is a multifactoral disease and its complications are the major causes of mortality in Western society and Japan. Hypercholesterolemia is the leading factor for the development of atherosclerosis in humans and experimental animals (1). There are three kinds of so-called atherogenic lipoproteins in the plasma; low density lipoproteins (LDL), remnant lipoproteins such as \( \beta \)-very low density lipoproteins (\( \beta \)-VLDL) and intermediate density lipoproteins (IDL), and lipoprotein (a) \([Lp(a)]\) whereas high density lipoproteins (HDL) are considered as anti-atherogenic lipoproteins. Elevation of atherogenic lipoproteins and/or reduction of HDLs in plasma are associated with increased incidence of coronary heart disease.

For the study of the pathogenesis of this disease and also for the development of therapeutical drugs, experimental animals, either cholesterol-fed or spontaneously hyperlipidemic, are often used. The ideal animal model of human atherosclerosis should possess several important characteristics (2). It should be easy to acquire and maintain at a reasonable cost, easy to handle, and the proper size to allow for all anticipated experimental manipulations. Ideally, the animal should reproduce in a laboratory setting and have well-defined genetic characteristics. Finally, the animal model should share with man the most important aspects of the disease process.
Lesions should develop naturally while the animal consumes a reasonable diet, and lesions should develop slowly over the animal's lifetime with clinical sequelae in later middle to old age. The natural history of lesion pathogenesis should range from fatty streaks to atheromatous plaques with complications such as calcification, ulceration, hemorrhage, and superimposed thrombosis with luminal stenosis. Vesselinovitch has proposed requirements for the ideal animal model to be used in atherosclerosis research (2). Since there is no single animal model that fulfills all of these requirements to date, several different animal species including mice, rats, rabbits, birds, dogs, pigs and nonhuman primates, have been used. Among these experimental animals, cholesterol-fed rabbits are the first and classical model for the study of lipoproteins and atherosclerosis (3). They have gained renewed popularity with the development of transgenic rabbits with altered expression of specific genes (4-6). In this paper, we summarize rabbit models for the study of atherosclerosis, including cholesterol-fed rabbits, spontaneously hypercholesterolemic rabbits and transgenic rabbits.

**Cholesterol-fed rabbit models for the study of atherosclerosis**

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, plasma cholesterol levels for both New Zealand White (NZW) and Japanese White (JW) rabbits are in the range of 30-90 mg/dl at the age of 12-16 weeks while young animals usually have cholesterol levels in the upper portion of this range (4). The plasma cholesterol concentrations are higher in females than males, decrease with age in males and remain unchanged in females. In addition, cholesterol levels show greater seasonal variation in females than males and are lower in pregnant and lactating females than in non-pregnant, non-lactating females (7). Therefore, males are used more often than females for atherosclerosis study. On a standard chow diet, it is generally believed that most strains of rabbits do not develop spontaneous atherosclerosis. However, the rabbits rapidly develop severe hypercholesterolemia leading to premature atherosclerosis in response to dietary manipulation (3). In 1908, Ignatowsky produced intimal lesions resembling those of human atherosclerosis, for the first time, by feeding rabbits diets of milk, meat, and eggs, and concluded that the lesions were due to the high protein content of the diets (8). However, the results of later studies incriminated dietary fat, and it is now known that cholesterol was the atherogenic portion of the diet. The usual experimental diet consists of commercial rabbit chow diet supplemented with 0.3 to 2% cholesterol and 4 to 8% fat by weight (9). On a diet containing cholesterol >1%, rabbits develop severe hypercholesterolemia with plasma cholesterol levels as high as 1,500 to 3,000 mg/dl, which is often criticized as not "physiological" since such extremely high cholesterol levels are almost never seen in humans. In addition, rabbits with such severe hypercholesterolemia accumulate fat and cholesterol in many organs. Therefore, it is generally recommended that feeding rabbits 0.3-0.5% cholesterol diet results in a reasonable elevation of plasma cholesterol (<1,000 mg/dl) without interfering with the animal's health. There is an important relationship between dietary cholesterol and fat in the production of atherosclerosis in rabbits. Addition of cholesterol to the diet without additional fat results usually in the development of more severe atherosclerosis than the addition of both cholesterol and fat; presumably failure to add supplementary dietary fat leads to mobilization of endogenous fat stores which are more saturated than common dietary fats. Currently, our laboratory uses a diet supplemented with 0.3% cholesterol and 3% soybean oil reported by others (10) and feeds JW rabbits for 16 weeks ad libitum. The average cholesterol levels at the termination of 16 weeks are ~700 mg/dl (11). Induction of hypercholesterolemia in the rabbit with dietary cholesterol results in elevated levels of β-VLDLs, remnant lipoproteins; which are enriched in cholesteryl esters and migrate as β-lipoprotein on agarose gel electrophoresis (12). Accumulation of this atherogenic lipoprotein (β-VLDL) in plasma is the hallmark of human type III hyperlipoproteinemia (13). Hypercholesterolemia with predominant LDL-rich particles in rabbits can be induced by feeding diets that are rich in casein (14). In response to this casein-rich diet, rabbits have a greater reapportionment 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 (15). 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 and results in the development of atherosclerotic lesions (16, 17). The lesion area and volume in casein-fed rabbits are; however, usually smaller than those of cholesterol-matched cholesterol-fed rabbits even though there is no difference in topographic distribution (16). It should be pointed out that the distribution of atherosclerotic lesions in the rabbit fed an atherogenic diet differs from that in humans. Rabbits tend to develop atherosclerotic lesions in the aortic arch and thoracic aorta rather than in the abdominal aorta in man. Most studies on cholesterol-fed rabbits were focused on the aortic trunk and investigated the surface involvement and intimal thickness. Our laboratory has also tried to study other vascular beds such as coronary arteries, carotid arteries, and iliac arteries, which are almost impossible to investigate in mice (11). Cere-
bral atherosclerosis can also be induced in cholesterol-fed rabbits in the presence of hypertension (18), suggesting that an additional factor(s) is needed for the lesion development in these arteries.

**Genetically hypercholesterolemic rabbits**

Important genetic variants of the rabbit have been identified that confirm the link between hypercholesterolemia and atherosclerosis. In 1980, Dr. Watanabe et al. in Kobe University established a line of rabbits with hypercholesterolemia, named as Watanabe heritable hyperlipidemic rabbits (WHHL) (19). These rabbits have defective LDL receptor function due to a spontaneously arising deletion in exon 4 of the LDL receptor gene that encodes a 4-amino acid deletion in the cysteine-rich ligand-binding domain of the protein (20). Homozygous WHHL rabbits are markedly hypercholesterolemic from birth and suffer from tendon xanthoma and atherosclerosis, both of which exhibit remarkable pathological resemblance to those observed in human familiar hypercholesterolemia (21). The WHHL rabbits develop complicated lesions in the aorta, coronary artery and cerebral artery. Another rabbit model, the St. Thomas Hospital strain of hyperlipidemic rabbits, has increased levels of VLDL, and IDL, and LDL due to an apparent overproduction of these lipoproteins, making this variant a potential model for familiar combined hyperlipidemia (22). The St. Thomas rabbits develop atherosclerotic lesions on a chow diet and the strongest predictor of aortic atherosclerosis is an elevated level of IDL (23).

**Transgenic rabbits for the study of atherosclerosis**

In addition to cholesterol-fed rabbits, several features of the rabbit make it an excellent model for assessment of the effects of human transgenes on lipoprotein metabolism and atherosclerosis susceptibility (5). Compared to the most widely used transgenic model, the mouse, rabbit has different lipoprotein metabolism features as summarized in Table 1. For example, 1) rabbit lipoprotein profiles are similar to humans; 2) rabbit liver does not edit apoB mRNA and thus, produces apoB-100 only as does the human; 3) the rabbit has abundant cholesteryl ester transfer protein (CETP) in plasma as does the human; and 4) as mentioned above, rabbits are susceptible to cholesterol-rich diet-induced atherosclerosis. Additionally, the rabbit has relatively lower hepatic lipase activity compared with murine, which is thought to be responsible for its susceptibility of diet-induced atherosclerosis (24). During the past years, as shown in Table 2 and 3, transgenes for human apo(a), apoA-I, apoB, apoE2, apoE3, hepatic lipase, lecithin : cholesterol acyltransferase (LCAT), lipoprotein lipase, 15-lipoxygenase, as well as for rabbit apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1) have been expressed in rabbits [see reviews (5-6)]. In addition, human apoA-I, LCAT and apo(a) have been introduced into the WHHL rabbits which have deficient LDL receptor function.

**Apolipoprotein A-I transgenic rabbits**

Duverger and colleagues reported the generation of five lines of transgenic NZW rabbits expressing human apoA-

### Table 1. Comparison of rabbits and mice.

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Mice</th>
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<tr>
<td>Lipoprotein profile</td>
<td>LDL-rich</td>
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<tr>
<td>CETP</td>
<td>HDL-rich</td>
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<tr>
<td>Hepatic apoB editing activity</td>
<td>no</td>
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<tr>
<td>Hepatic lipase</td>
<td>liver-bound</td>
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<tr>
<td>Cholesterol diet</td>
<td>sensitive</td>
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### Table 2. Transgenic rabbits expressing human apolipoproteins.

<table>
<thead>
<tr>
<th>Transgene expressed</th>
<th>Major findings</th>
<th>References</th>
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<tbody>
<tr>
<td>Human apoAl</td>
<td>Anti-atherosclerosis</td>
<td>Duverger et al. 1996</td>
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<tr>
<td>Human apoB 100</td>
<td>Increase in LDL and decrease in HDL</td>
<td>Fan et al. 1995</td>
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<tr>
<td>Human apoE2</td>
<td>Type III hyperlipoproteinemia and spontaneous atherosclerosis</td>
<td>Huang et al. 1997</td>
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<td>Human apoE3</td>
<td>Reduction of VLDL and accumulation of LDL in plasma</td>
<td>Fan et al. 1998</td>
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<tr>
<td>Human apo(a)</td>
<td>Formation of Lp(a) particles</td>
<td>Fan et al. 1998, Rouy et al. 1998</td>
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### Table 3. Transgenic rabbits expressing enzymes involved in the lipid metabolism and atherosclerosis.

<table>
<thead>
<tr>
<th>Transgene expressed</th>
<th>Major findings</th>
<th>References</th>
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<tbody>
<tr>
<td>Human hepatic lipase</td>
<td>IDL and HDL metabolism, and inhibition of diet-induced atherosclerosis</td>
<td>Fan et al. 1994</td>
</tr>
<tr>
<td>Rabbit apoB mRNA editing protein</td>
<td>Liver dysplasia and tumor formation</td>
<td>Yamanaka et al. 1995</td>
</tr>
<tr>
<td>15-lipoxygenase</td>
<td>Anti-atherosclerosis</td>
<td>Shen et al. 1995</td>
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<tr>
<td>Human LCAT</td>
<td>Hyperalphalipoproteinemia and anti-atherosclerosis</td>
<td>Hoeg et al. 1996</td>
</tr>
<tr>
<td>Human Lipoprotein lipase</td>
<td>Reduction of VLDL</td>
<td>Fan et al. 2000</td>
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I in the liver. The plasma levels of human apoA-I in transgenic rabbits ranged from 8-100 mg/dl. When these transgenic rabbits were fed a cholesterol-diet (0.48 g cholesterol per 120 g of diet) for 14 weeks, the atherosclerotic lesions in the thoracic aorta were reduced by 50% compared to those in control rabbits (15±12% vs 30±8%) (25). Their study showed that the protective effects of human apoA-I on diet-induced atherosclerosis in rabbits were associated with HDL levels via the mechanism of reverse cholesterol transport.

**Apolipoprotein B transgenic rabbits**

The development of transgenic rabbits expressing human apoB-100 was described by Fan et al. in 1995 by using an 80 kb human apoB genomic DNA (26). Four lines of transgenic rabbits were generated and their plasma levels of human apoB-100 ranged from 12-94 mg/dl. Expression of human apoB-100 in transgenic rabbits resulted in a 2-3-fold increase of total cholesterol and triglycerides compared to age- and sex-matched control rabbits. Nearly all of the cholesterol and human apoB-100 was in the LDL fraction, with striking enrichment of triglyceride content. Transgenic rabbit LDL was further found to contain large amounts of apoC-III and apoE. Atherosclerosis susceptibility was not determined in these animals. Recently, these transgenic rabbits were crossbred with hepatic lipase transgenic rabbits (27).

**Apolipoprotein (a) transgenic rabbits**

Elevated plasma levels of lipoprotein (a) [Lp(a)] constitute an independent risk factor for coronary heart disease, stroke, and restenosis (28). However, apo(a), a unique component of Lp(a), is naturally present exclusively in Old World monkeys, humans, and the hedgehog. Therefore, there are no convenient experimental animal models of Lp(a). Studies on transgenic mice expressing human apo(a) revealed that murine apoB cannot bind to human apo(a) to form Lp(a) particles (29). To investigate the Lp(a) assembly and its possible role in atherosclerosis, we and others have reported the generation of transgenic rabbits expressing human apo(a), using either artificial chromosome clone (YAC) containing the human apo(a) gene (30) or the human apo(a) cDNA under the control of the mouse transferrin promoter (31). The human apo(a) levels of transgenic rabbits from these studies were 2.5 mg/dl in transgenic rabbits with YAC vector and 1.8 to 4.5 mg/dl in transgenic rabbits with apo(a) cDNA. These studies showed that transgenic rabbits expressing human apo(a) exhibited efficient assembly of human Lp(a)-like particles, suggesting that these models can be used as a possible model for the study of Lp(a) (31). To examine the effect of Lp(a) on the development of atherosclerosis, we studied transgenic rabbits expressing human apo(a) (mean concentration of apo(a) is 11 nM in the plasma). We did not find any atherosclerotic lesions in transgenic rabbits on a regular chow diet, suggesting that lower plasma apo(a) is not atherogenic. On a 0.3% cholesterol diet for 16 weeks, human apo(a) transgenic rabbits had more extensive atherosclerotic lesions than nontransgenic rabbits although the cholesterol levels in the plasma of both groups of rabbit were similarly elevated. Compared to the lesions in nontransgenic control rabbits, the areas of atherosclerotic lesions in human apo(a) transgenic rabbits were increased 1.65-fold in the aorta, 3.4-fold in the iliac artery, and 2-fold in the carotid artery. Furthermore, we found that human apo(a) transgenic rabbits on a cholesterol-rich diet had a greater degree of coronary atherosclerosis than control rabbits (11). The study is being extended currently to reveal the mechanism(s) responsible for atherogenicity of apo(a) in transgenic rabbits.

**Apolipoprotein E2**

Transgenic rabbits expressing high levels of human apoE2 (Cys122, Cys372), an apoE variant associated with the human genetic disorder type III hyperlipoproteinemia were generated in 1997 by Huang and coworkers (13). Huang’s study demonstrated that overexpression of human apoE2 (30-70 mg/dl) resulted in a marked accumulation of β-VLDL (intestinal and hepatic remnant lipoproteins), a hallmark for type III hyperlipoproteinemia. These rabbits fed a normal diet showed developed atherosclerosis in the aortic arch and proximal abdominal aorta. A more interesting finding from their study was that male transgenic rabbits showed more extensive atherosclerosis, suggesting that sex hormones play an important role in modulating type III hyperlipoproteinemia.

**Apolipoprotein E3**

Fan and coworkers generated transgenic rabbits expressing human apoE3 (Cys122, Arg158) using human apoE3 genomic DNA together with the hepatic control region (32). Three lines of transgenic rabbits were established and human apoE3 levels were 6, 11, and 13 mg/dl, respectively. Analysis of these transgenic rabbits revealed that increased expression of human apoE3 results in reduced VLDL and accumulation of LDL, which is apparently different from transgenic mice expressing the same transgene (33). The mechanism(s) responsible for this phenomenon were investigated and the results showed that apoE-rich particles confer a greater affinity for cell surface receptors, thereby increasing remnant clearance from plasma. In addition, these particles appear to compete more effectively than LDL for receptor-mediated binding and clearance, resulting in delayed clearance and the accumulation of LDL in plasma. The effects of human apoE3 expression on atherosclerosis have been described in a preliminary report (34). Further studies will be required to better understanding the role of apoE3 in atherosclerosis susceptibility.

**Hepatic lipase transgenic rabbits**

Transgenic rabbits overexpressing human hepatic
lipase (HL) were reported by Fan et al. in 1994 (35). The rationale for using rabbits specifically is that rabbits have lower levels of HL activity which have been considered to be responsible for their susceptibility to diet-induced atherosclerosis. The construct used for transgenic rabbits was composed of human HL cDNA and human apoE/CI hepatic control region. HL expression in transgenic rabbits had a significant effect on plasma lipid and lipoprotein levels. Total cholesterol and triglyceride levels were reduced by 42% and 58% in transgenic rabbits compared to nontransgenic controls. Lipoprotein analysis revealed that overexpression of HL led to a remarkable reduction of HDL, VLDL and IDL. When HL transgenic rabbits were fed a diet containing 0.3% cholesterol and 3% soybean oil, they showed a attenuated hypercholesterolemia compared to control rabbits. Preliminary study showed that reduced hypercholesterolemia in HL transgenic rabbits was associated with a diminished extent of aortic atherosclerosis (34).

**LCAT transgenic rabbits**

In 1996, Hoeg and colleagues reported the production of human LCAT transgenic rabbits using the human LCAT genomic DNA construct (36). Several papers regarding the effects of human LCAT on the lipoprotein metabolism have been published using this model (see their review (6)). Human LCAT overexpression in transgenic rabbits resulted in a substantial change in plasma lipid and lipoprotein profiles; plasma total, free, and esterified cholesterol, as well as phospholipid, concentrations were significantly increased in both low and high expressor F1 progeny compared to control rabbits. The elevation of plasma total cholesterol content was due to a marked increase in HDL cholesterol concentration. On a 0.3% cholesterol diet for 17 weeks, LCAT transgenic rabbits had significantly reduced atherosclerosis compared to littermate control (37).

**Lipoprotein lipase transgenic rabbits**

Transgenic rabbits expressing human lipoprotein lipase (LPL) were generated in our laboratory recently, using human LPL cDNA construct with a chicken β-actin promoter which was used for transgenic mice (38). Preliminary studies revealed that these rabbits had lower VLDL levels associated with reduced total cholesterol and triglycerides in the plasma (Fan, unpublished data). Characterization of these transgenic rabbits is currently under investigation.

**15-lipoxygenase (15-LO) transgenic rabbits**

Shen et al. reported the generation of transgenic rabbits expressing human 15-LO gene driven by a lysozyme macrophage-specific promoter (39). Fed a diet containing 10% corn oil and 0.25% cholesterol for 13.5 weeks, transgenic rabbits had significantly smaller lesion areas than their littermates even when their levels of hypercholesterolemia were similar (40). This result was unexpected and surprising since it is contrary to the general notion that oxidative modification of LDL increases LDL atherogenicity (41). These authors speculated that 15-LO may exert protective effects relating to the expression of redox sensitive genes and/or that the effects of 15-LO at different stages of lesion development may differ considerably.

**WHHL transgenic rabbits**

Three human transgenes have been introduced into WHHL rabbits to study the relationship between LDL receptor activity and these genes. This included apoA-I, LCAT (42) and apo(a) in WHHL rabbits (43). The advantages of using WHHL rabbits are two-fold; to study these protein functions in the setting of LDL receptor defects; and their relationship with hypercholesterolemia and atherosclerosis without feeding cholesterol diet. In this respect, we recently found that in WHHL transgenic rabbits expressing human apo(a), there was an increased level of plasma Lp(a) compared to control transgenic rabbits with normal LDL receptor functions, suggesting that LDL receptor is involved in the catabolism of Lp(a) in rabbits (43).

**Conclusions**

Cholesterol-fed rabbits are still a useful model for the study of lipoprotein metabolism and atherosclerosis. The development of transgenic rabbits with modified gene expression has provided a novel tool to evaluate individual genes in the lipoprotein metabolism and pathogenesis of atherosclerosis. One can predict that in the next few years, most genes which are involved in lipoprotein metabolism and atherosclerosis will be introduced into this model. We need to generate transgenic rabbit models expressing specific products such as VEGF and matrix metalloproteinases in the arterial wall, directing towards our understanding the mechanism(s) for coronary syndrome, plaque stability and rupture. With these models, we can develop new therapeutic drugs to treat and prevent this disease.

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