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Review

Principles and Applications of Rabbit Models for Atherosclerosis Research

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Rabbits are one of the most used experimental animals for biomedical research, particularly as a bioreactor for the production of antibodies. However, many unique features of the rabbit have also made it as an excellent species for examining a number of aspects of human diseases such as atherosclerosis. Rabbits are phylogenetically closer to humans than rodents, in addition to their relatively proper size, tame disposition, and ease of use and maintenance in the laboratory facility. Due to their short life spans, short gestation periods, high numbers of progeny, low cost (compared with other large animals) and availability of genomics and proteomics, rabbits usually serve to bridge the gap between smaller rodents (mice and rats) and larger animals, such as dogs, pigs and monkeys, and play an important role in many translational research activities such as pre-clinical testing of drugs and diagnostic methods for patients. The principle of using rabbits rather than other animals as an experimental model is very simple: rabbits should be used for research, such as translational research, that is difficult to accomplish with other species. Recently, rabbit genome sequencing and transcriptomic profiling of atherosclerosis have been successfully completed, which has paved a new way for researchers to use this model in the future. In this review, we provide an overview of the recent progress using rabbits with specific reference to their usefulness for studying human atherosclerosis.

Key words: Animal models, Hypercholesterolemia, Atherosclerosis, Rabbits, Transgenic

Introduction

Experimental animal models play an important role in studying human atherosclerosis. Until now, many animals have been used in this field, including non-human primates, pigs, rabbits, and mice[1, 2]. Nowadays, genetically modified mice have become the most popular animal model for atherosclerosis research[3]. Which animals researchers use depends on the experimental purposes; however, there are general essential principles that need to be considered when choosing animals: (1) animals 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, (2) the animal should reproduce in a laboratory setting and have a well-defined genetic background, and (3) the animal model should share with humans the most important aspects of lipid metabolism and cardiovascular pathophysiology[4]. Although no animal model meets all of these requirements, rabbits are still valuable models for studying atherosclerosis. Indeed, rabbits were the first used animal model for the study of human atherosclerosis. Pioneer studies to establish rabbit atherosclerosis models were performed by two Russian scientists more than a century ago. In 1908, a Russian physician, Ignatowski, fed rabbits with a diet enriched in animal proteins (milk, meat and eggs) and observed intimal lesions with large clear cell (now referred to as foam cell) accumulation in the aorta[5]. Later, a Russian experimental pathologist, Anitschkow, used a cholesterol diet dissolved in vegetable oil to produce aortic atherosclerosis in rabbits similar with

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Table 1. Comparison of lipid and lipoprotein metabolism features

	Human	Rabbit	Mouse
Major plasma lipoproteins	LDL	LDL	HDL
CETP	Abundant	Abundant	None
Hepatic apoB mRNA editing	No	No	Yes
apoB-48	Chylomicrons	Chylomicrons	VLDLs/LDLs and Chylomicrons
apoB-100	Can be bound to apo(a)	Can be bound to apo(a)	Cannot be bound to apo(a)
HDL	Heterogeneous	Heterogeneous	Homogeneous
apoAⅡ	Dimer	Absent	Monomer
Hepatic LDL receptor activity	Down-regulated	Down-regulated	Usually high
Hepatic lipase	High, liver-bound	Low, liver-bound	High, 70% in circulation
Cholesterol pool	Mainly from hepatic synthesis	Mainly from hepatic synthesis	Mainly from dietary origin
Excretion of bile acid	Low	Low	High
Response to a high cholesterol diet	Sensitive	Sensitive	Resistant

that seen in humans and proposed a causal role of cholesterol in atherosclerosis[6]. Their studies established the basis of atherosclerosis theory that in both humans and experimental animals, dietary cholesterol can induce atherosclerosis. Rabbit models were widely used and disclosed most of the pathophysiological significance between human atherosclerosis and lipid metabolism such as the discovery of LDL receptors and development of statins, the most prescribed lipid-lowering drugs in the world[7]. Compared with mice, rabbits have many features similar with humans, which facilitates the studies of lipid metabolism and atherosclerosis (**Table 1**). For example, like humans but unlike mice, rabbits have abundant plasma cholesteryl ester transfer protein (CETP), which facilitates studying the relationship between CETP and atherosclerosis[8]. For a long time, it has been controversial whether inhibition of CETP is beneficial for the treatment of atherosclerosis. Concurrently, human clinical trials of CETP inhibitors were generally unsuccessful due to off-target side effects (torcetrapib) or lack of efficacy (dalcetrapib and evacetrapib)[9-12]. Merck's anacetrapib was reported to be effective in reducing cardiovascular events in a Phase Ⅲ clinical trial[13]. Recently, we successfully generated CETP KO rabbits using ZFN methods and demonstrated that genetic deletion of the CETP gene in rabbits protects against diet-induced atherosclerosis[14].

Although the rabbit model has brought about many breakthroughs in the history of atherosclerosis research[7], apolipoprotein E (apo E) and LDL receptor gene knock-out (KO) mice became the major models to study atherosclerosis from 2000[7]. In spite of this, rabbits are the second most-used animal models for the study of atherosclerosis based on research papers published in high-profile journals. The importance of using rabbits has been extensively reviewed in previous articles[4, 7, 15-17], and in this short review, we will focus on how to use rabbits for cardiovascular research.

Methods to Induce Atherosclerosis in Rabbits

In general, aortic atherosclerotic lesions in rabbits can be easily induced by feeding a high cholesterol diet alone or in combination with arterial balloon injury. Compared with other animals, especially rodents, laboratory rabbits are sensitive to a high cholesterol diet and rapidly develop hypercholesterolemia[7]. Therefore, it is much easier to induce hypercholesterolemia in rabbits than in other animals by feeding a high cholesterol diet. Dietary cholesterol can range from 0.3%~1% depending on the research purposes. We recommend the use of 0.3~0.5% cholesterol in the diet for most experiments because it produces less health problems such as liver toxicity. If cholesterol content is too high (>1%), rabbits develop massive hypercholesterolemia (plasma cholesterol levels >1200 mg/dl), which is never observed in humans. Plasma cholesterol levels that are too high often lead to systemic lipid accumulation and liver dysfunction in rabbits, which is often criticized as "not physiological" by many researchers. Therefore, it is not recommended to feed rabbits with a diet containing cholesterol higher than 1% for more than 4 weeks. In addition to cholesterol, oil, such as soybean or corn oil, is essential in the diet. This is because oil in the diet helps in absorption of intestinal cholesterol. There are many commercially available dietary oils that may be used for rabbit experiments. However, coconut oil, as an exception, is rich in saturated fatty acids, unlike other vegetable dietary oils that are rich in polyunsaturated fatty acids. Coconut oil can be used for insulin resistance studies[18, 19]. It should be noted that cholesterol diets without the addition of oil lead to mobilization of internal fat tissue for absorption of

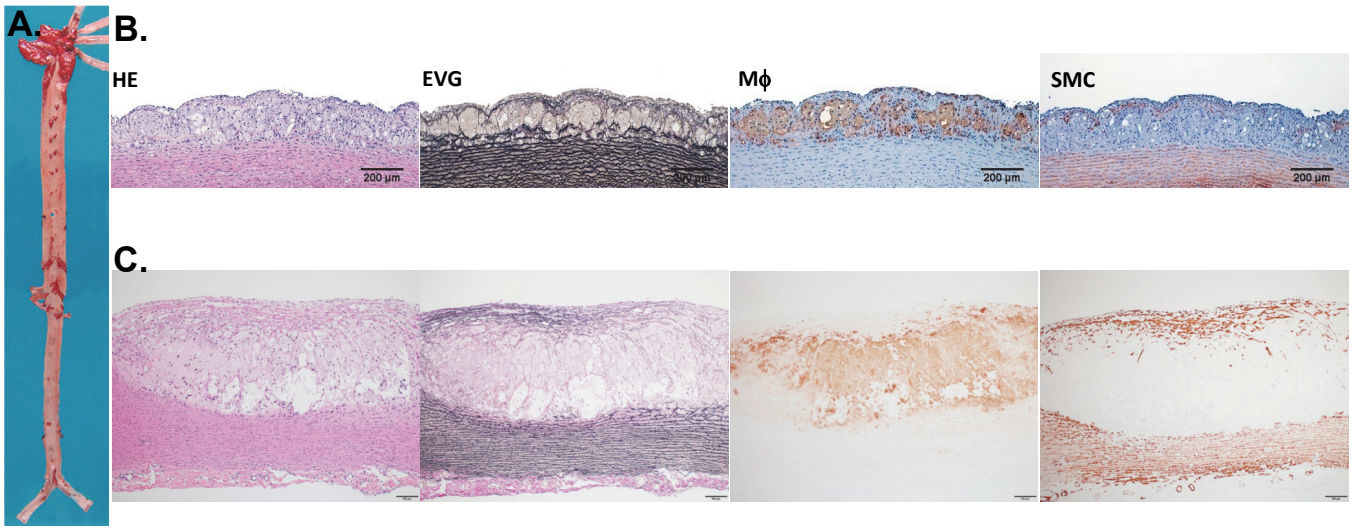

Fig. 1. Representative aortic lesions in cholesterol-fed rabbits. A. Gross lesions of the aorta stained by Sudan Ⅳ (visualized as red area). B. Representative micrographs of early stage lesions (fatty streaks). C. A typical fibrous plaque with a lipid core covered by a fibrous cap. Serial paraffin sections of the aortic arch were stained with hematoxylin and eosin (HE) and elastica van Gieson (EVG), or immunohistochemically stained with monoclonal antibodies (mAbs) against either macrophages (Mϕ) or α-smooth muscle actin for smooth muscle cells (SMC).

exogenous cholesterol[4]; therefore, rabbits become extremely thin and sometimes develop cachexia. On a high cholesterol diet, rabbits develop aortic atherosclerosis by 12~16 weeks. For more advanced lesions, the length of cholesterol diet feeding can be extended for more than 28 weeks[20]. As rabbits are out-bred animals, it is not unusual for some rabbits to exhibit inert responses to a high cholesterol diet, and are referred to as hypo-responders[21]. Watanabe heritable hyperlipidemic (WHHL) rabbits are genetically deficient in LDL receptor functions, and develop hypercholesterolemia and atherosclerosis spontaneously on a chow diet[22]. Although it is difficult to transport WHHL rabbits to many institutions, it is now possible to produce LDL receptor knock-out rabbits by CRISPR-Cas9 methods. Recently, we successfully generated LDL receptor KO rabbits that show a similar phenotype with WHHL rabbits (Liang *et al.* unpublished data).

Methods to Analyze Atherosclerosis

The rabbit aorta is mainly used for the analysis of atherosclerosis because it is easy to isolate and both gross and microscopic evaluation of aortic lesion size and quality can be made. The lesions usually start to form from the aortic arch followed by the intercostal orifice area and thoracic aorta. Abdominal aortic lesions are less frequently seen, which is different from human atherosclerosis. As the aortic lesions are large, it is also possible to collect a part of the lesions to analyze protein and mRNA expression using Western blotting and RT-PCR methods. If these analyses are required, these samples should be immediately frozen in nitrogen liquid and stocked at −80℃.

Gross analysis usually requires Sudan Ⅳ staining to visualize atherosclerotic lesions followed by image analysis to calculate the lesion percentage of each aortic segment[23] (**Fig. 1**). Based on the gross observations, one can decide which parts to select to make paraffin-embedded sections for microscopic observations. Usually, the whole aortic arch and thoracic aorta will be cut into 10-12 segments (1-mm thick) for paraffin embedding[23]. Serial sections can be routinely used for hematoxylin-eosin (HE) staining and elastic van Gieson (EVG). HE staining is essential to evaluate the quality of the lesions such as cellular and extracellular matrix component features and plaque vulnerability. To quantitate the intimal lesion area on the sections, EVG staining is useful because it can visualize the internal elastic lamina (IEL), making it easy to measure intimal lesions and medial lesions separately. Paraffin sections can be used for direct immunohistochemical staining in most cases using most commercial antibodies. Routinely, it is essential to evaluate macrophages and smooth muscle cells using RAM-1 and smooth muscle α-actin antibodies (**Fig. 1**).

If analysis of protein and mRNA expression is required, a piece of fresh tissue proceeded in liquid nitrogen at the time of aorta collection should be collected. In hypercholesterolemic rabbits, iliac arteries, carotid arteries and subclavian arteries seldom exhibit atherosclerosis; however, coronary lesions of rabbits can

Table 2. Transgenic rabbits for the study of human cardiovascular diseases

Genes	Expressing cells	Major phenotypes	References
Apolipoproteins:			
Apo(a)	Liver	Atherogenic	33-37
ApoA-I	Liver	Athero-protective	38, 39
ApoA-II	Liver	Athero-protective	40, 41
ApoB-100	Liver	LDL ↑, HDL ↓	42
ApoCIII	Liver	VLDL ↑	43
ApoE2	Liver	Atherogenic	44
ApoE3	Liver	Atherogenic	45, 46
Enzymes:			
Hepatic lipase	Liver	Athero-protective	47
apoB mRNA editing protein	Liver	LDL ↓	48
LCAT	Liver	Athero-protective	49, 50
Lipoprotein lipase	Universal	Athero-protective	51
PLTP	Universal	Atherogenic	52
Endothelial lipase	Liver	Athero-protective	53
Vascular factors:			
Lipoprotein lipase	Macrophage	Atherogenic	54
MMP-12	Macrophage	Atherogenic	20, 55
C-reactive protein	Liver	Thrombogenic	24, 56
15-lypooxygenase	Macrophage	Athero-protective	57
VEGF	Liver	hemangiomas and impaired glomerular functions	58, 59

ND: not done. LCAT, lecithin: cholesterol acyltransferase; PLTP, phospholipid transfer protein; MMP, matrix metalloproteinase; VEGF, vascular endothelial cell growth factor.

be observed at the large branches such as the left and right coronary arteries. The method for rabbit coronary lesion analysis has been described in detail in the previous review[7]. Balloon injury of aortas or wire injury of iliac arteries in rabbits is sometimes used to produce intimal lesion models. However, these lesions induced by injuries are mainly composed of proliferating smooth muscle cells forming so-called "neo-intima", which is different from fatty streaks induced by cholesterol diet feeding. However, balloon injury may also be performed in cholesterol-fed rabbits, which will accelerate the formation of the lesions[24].

Transgenic Rabbits

In addition to cholesterol-fed rabbits, transgenic (Tg) rabbits have also been used for the study of human cardiovascular disease and lipoprotein metabolism during the last two decades. However, in contrast to Tg mice, Tg rabbits were only used by a few institutions to study atherosclerosis. Elucidation of gene functions in terms of lipoprotein metabolism and atherosclerosis using Tg rabbits was essentially based on the premise that rabbits bear features more similar with those of humans such as metabolism and susceptibility to atherosclerosis (**Table 1**). Transgenes are usually expressed in the liver or macrophages to study their functions (**Table 2**). To date, more than 20 genes have been introduced into Tg rabbits, and have provided considerable insight into the molecular mechanisms associated with their functions in lipoprotein metabolism and atherosclerosis[25].

Knock-Out Rabbits

For a long time, generation of knock-out rabbits has been a dream for researchers because rabbit embryonic stem (ES) cells are not available and genomic information for rabbits is lacking. Rabbit genome sequencing was initially completed by a European group[26], and two years later, we along with Chinese and US groups, successfully sequenced three rabbit genomes (New Zealand White, Japanese White and Watanabe heritable hyperlipidemic rabbits) and completed transcriptomic profiling of atherosclerotic lesions and the liver for these rabbits[27]. These genomic and RNA seq data will provide many insights into understanding the pathogenesis of atherosclerosis in rabbits. Another advancement in this field is the emergence of novel gene-editing technologies (ZFN, TALENs, CRISPR-Cas9) in succession, which has made it possible to make KO rabbits just as KO mice. The first KO rabbits were

Table 3. KO rabbits recently reported

Genes	Methods	Research models	References
ApoCⅢ	ZFN	Atherosclerosis	29
ApoE	ZFN/CRISPR/Cas9	Atherosclerosis	32, 60
CETP	ZFN	Atherosclerosis	14
αA-Crystallin/GJA8	CRISPR/Cas9	Congenital Cataracts	61, 62
Sex-determining region Y	CRISPR/Cas9	Male-to-female sex reversal syndrome	63
Fumarylacetoacetate hydroxylase	TALENs	Tyrosinemia	30
PHEX	CRISPR/Cas9	Inheritable rickets	64
Myostatin	CRISPR/Cas9	Muscle hypertrophy	65

generated by the ZFN method to target the IgM gene[28], and later, we along with others, successfully generated apoCⅢ[29] and CETP KO rabbits[14] using the same method. Although TALENs were still used to create KO rabbits[30], CRISPR-Cas9 has now been become the main method for generation of KO rabbits in this field, such as apoE and LDL receptor KO rabbits[31, 32] (**Table 3**). Therefore, one can predict that in the next few years, through these novel technologies, KO rabbits will be generated and used for translational studies of cardiovascular diseases.

Advantages and Disadvantages

The rationale of using rabbits rather than mice is dependent upon the research purpose, as described above. As shown in **Table 1**, like humans but unlike mice, rabbits have a unique lipid metabolism system which facilitates the study of the human lipid metabolism (**Table 1**). Second, rabbit atherosclerosis lesions resemble those of humans, from early to advanced lesions. Third, adult rabbit body weights (2000~3500 g) are ~100-fold larger than mice (20~30 g), thus the large size of rabbits (including their arteries) enables preclinical studies for surgical experiments such as studies on stents and catheters. The large amount of tissue also makes it possible to perform many analyses using a single animal. Finally, it is important to use rabbits to perform translational research such as development of new lipid-lowering therapeutics. Rabbits should not be considered as a simple substitute for rodents because compared with mice, rabbits may have disadvantages. The individual price of adult rabbits (150~200 US$ in Japan) is 10-fold higher than for mice (15~20 US$). Second, the breeding expense of rabbits (0.6~1$ /rabbit/day) is 10-fold more expensive than for mice (0.06$ per day). Rabbits are kept in individual cages, whereas mice are kept in a cage with a group of 8~16 mice; therefore, a large space is required for rabbits compared with mice. Being aware of these disadvantages is very important for one to make a decision regarding whether to use rabbits. The most critical point is whether experiments are designed to solve clinical problems such as for translational research.

In conclusion, rabbits are still a powerful model for the study of human atherosclerosis. With the advances in nuclease-editing technology, KO rabbits will provide a new means for translational research in this field. These new models will help develop novel therapeutics and diagnostics for cardiovascular disease in the future.

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Conflicts of Interest

None.

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