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The Watanabe heritable hyperlipidemic (WHHL) rabbit, its characteristics and history of development: A tribute to the late Dr. Yoshio Watanabe

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Abstract

Professor Yoshio Watanabe, who developed the WHHL rabbit, died on December 13, 2008. He had contributed to studies of lipoprotein metabolism and atherosclerosis, and to the development of hypolipidemic and/or anti-atherosclerotic compounds. WHHL rabbits show hypercholesterolemia due to a deficiency of LDL receptors, and a very similar lipoprotein metabolism to humans. The incidences of coronary atherosclerosis and myocardial infarction in the original WHHL rabbits were very low. After three rounds of selective breeding, the coronary plaques changed to fibroatheromas with thin fibrous caps and myocardial infarction developed spontaneously. In studies with WHHL rabbits, plaque-stabilizing effects of statins were proved. In this review, we admire his achievements and describe the history of studies using WHHL rabbits.

Keywords: Atherosclerosis, hypercholesterolemia, LDL receptor deficiency, WHHL rabbit

1. Introduction

The Watanabe heritable hyperlipidemic (WHHL) rabbit is an animal model for hypercholesterolemia due to a deficiency of low-density lipoprotein (LDL) receptors and has contributed to studies about lipoprotein metabolism, hypercholesterolemia, and atherosclerosis, and to the development of compounds for treating hypercholesterolemia (especially inhibitors of HMG-CoA reductase, statins) and atherosclerosis. Yoshio Watanabe (Fig. 1), who developed the WHHL rabbit strain, died on December 13, 2008. He was 81 years old.

Fig 1. A photograph of Dr. Yoshio Watanabe taken by Dr. Yoshio Tsujita in 1990

The first paper about the WHHL rabbit was published in *Atherosclerosis* [1]. After that, many researchers requested WHHL rabbits and Watanabe provided them. One famous study by Goldstein and Brown clarified lipoprotein metabolism in vivo [2]. They proved their LDL receptor pathway hypothesis by using WHHL rabbits and were awarded the Nobel Prize in 1985. In addition, WHHL rabbits have also contributed to the development of statins. As of the end of 2008, a total of 3,338 WHHL rabbits have been provided by Kobe University and 603 papers using WHHL rabbits have been published in international journals. A list of these papers appears at the WHHL rabbit-website (http://www.med.kobe-u.ac.jp/iea/w-index.html).

The late Dr. Watanabe was assigned to Kobe University in 1966 to manage a newly constructed animal center. At that time he was 39 years old. As a clinical veterinarian for domestic cattle, he had been involved in developing a strain of Japanese beef cattle (Tajima cattle) by selective breeding. This experience was to prove useful in developing the WHHL rabbit strain. Although busy establishing a management system for the animal center, Dr Watanabe maintained strong aspirations for research. Seven years later, he accidentally discovered a mutant rabbit showing hypercholesterolemia, from which he developed the WHHL rabbit strain. A man of great patience and perseverance, and strong convictions, he spent almost his entire salary establishing the colony, and devoted himself in maintaining the strain while endeavoring to provide the animals to researchers worldwide. His contribution to the study of lipoprotein metabolism, atherosclerosis, and related diseases is substantial.

In this review, we, as the successors of the WHHL rabbit colony, would like to pay tribute to his scientific achievements by looking back upon the history of studies using WHHL rabbits.

2. Development of the WHHL rabbit strain

Fig. 2 shows the history of the WHHL rabbit's development. While examining the effects of feeding on serum biochemical parameters in rabbits, Yoshio Watanabe accidentally found a male with hyperlipidemia in 1973. The WHHL rabbit was derived from this mutant. The mutant's serum cholesterol level was 447 mg/dl at 8 months of age despite normal levels of

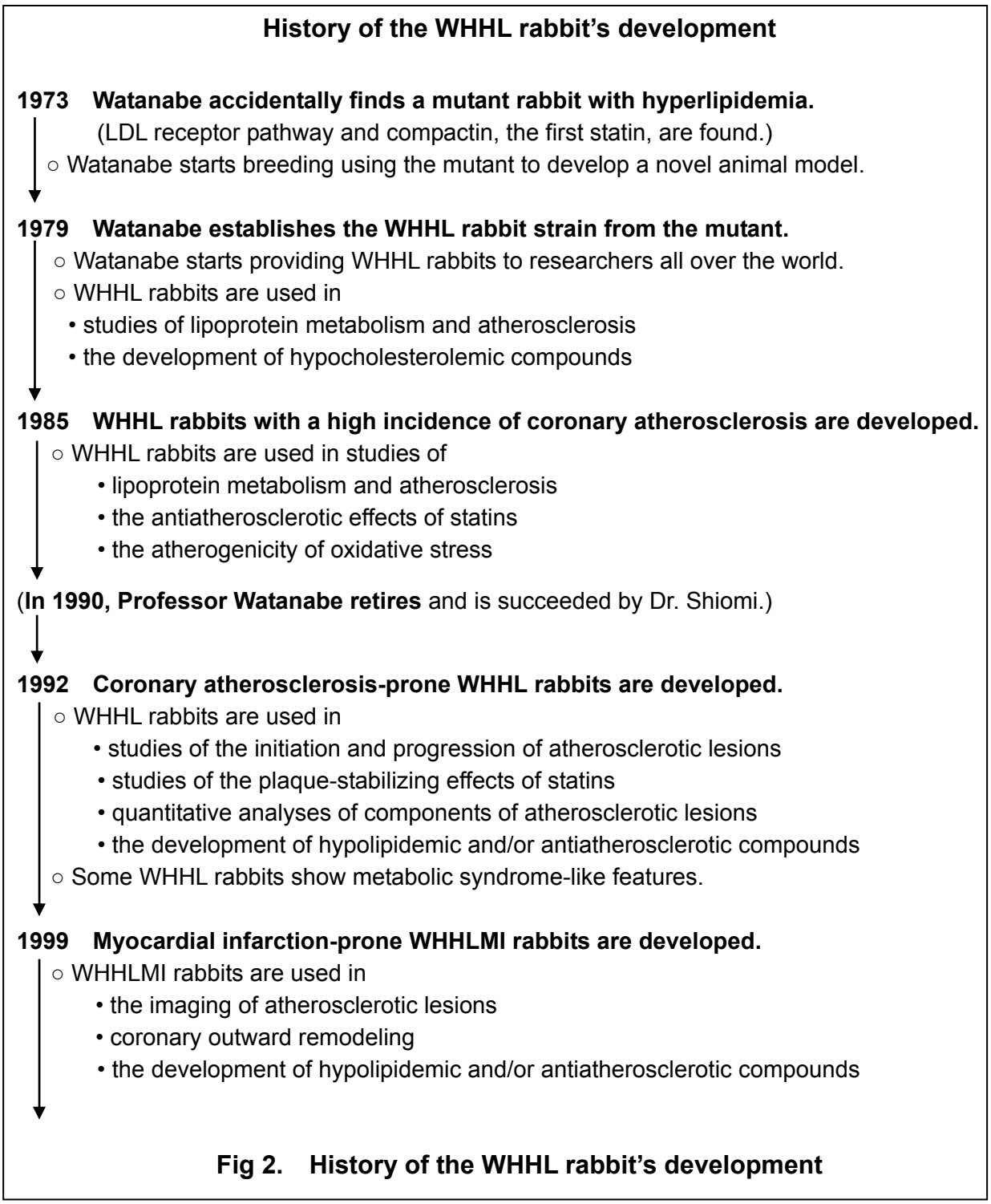

Fig 2. History of the WHHL rabbit's development

other biochemical parameters except lipids [3]. At that time, there was little interest in hyperlipidemia in Japan. However, Watanabe started to develop a new animal model of the disease. First, he examined whether the hyperlipidemia was heritable and followed Mendel's law. After obtaining the fifth generation of rabbits in 1977, he designated the strain the hyperlipidemic rabbit (HLR) and submitted a paper to a Japanese journal [4]. However, they were not interested. Two years later, he submitted a new paper to *Atherosclerosis* which

showed the accumulation of β-lipoprotein, aortic atherosclerosis, and xanthoma at the digital joints. In this paper he renamed the strain as WHHL (Watanabe heritable hyperlipidemic) rabbit following a suggestion by Professor Adams C.W.M., one of the chief editors of *Atherosclerosis* [1]. As 1973 was also the year that the LDL receptor pathway was found by Goldstein and Brown [5] and the first statin, compactin [6], was found by researchers of Sankyo Company (Tokyo, Japan), it was an epoch year in the study of lipoprotein metabolism.

3. Lipoprotein metabolism in the WHHL rabbit

Fig. 3 shows the characteristics of the myocardial infarction-prone WHHL (WHHLMI) rabbit, derived from the WHHL rabbit strain. The mechanism of hyperlipidemia in WHHL rabbits

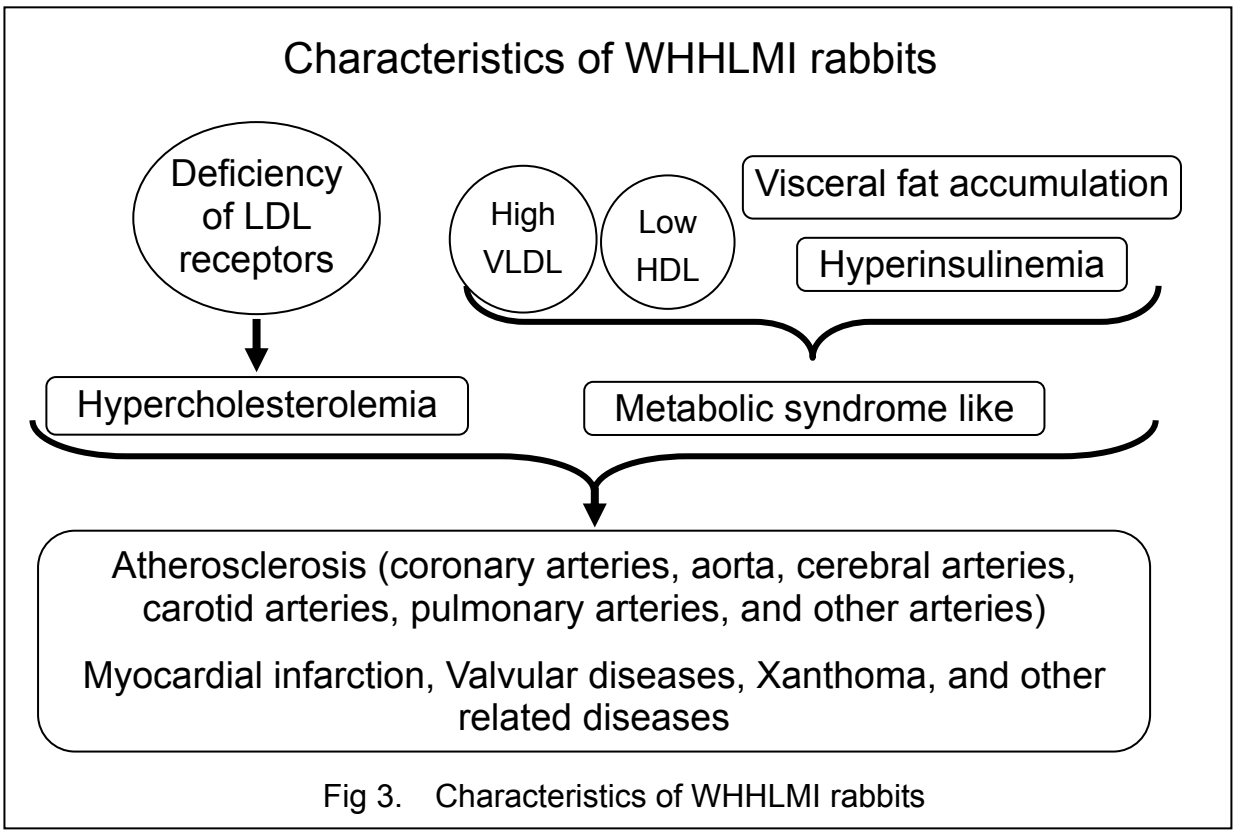

Fig 3. Characteristics of WHHLMI rabbits

was examined from 1980 in collaboration with a research group of Sankyo Company (Japan). Tanzawa et al. [7] found that LDL receptor function was almost deficient in the skin fibroblasts of WHHL rabbits. That study examined the lipoprotein profile of WHHL rabbits and found that almost all of the cholesterol in plasma was accumulated in the LDL fraction. In WHHL rabbits, the disappearance of LDL from plasma was delayed and the LDL-binding activity of skin fibroblasts was almost absent. These results demonstrated that a deficiency of LDL receptor activity resulted in the accumulation of LDL in plasma in this strain. Thereafter, Kita et al. [8] and Attie et al. [9] demonstrated that the LDL receptor activity of WHHL rabbits was deficient in cells of the liver and other major organs. In addition, Goldstein and Brown

[5,10-15] and others [16-19] elucidated lipoprotein metabolism in WHHL rabbits. In 1986, Yamamoto et al. [15] demonstrated that 12 nucleotides were deleted in the LDL-binding domain of LDL receptor cDNA in WHHL rabbits. Their study certified that the hypercholesterolemia of WHHL rabbits is due to the genetic defects in the LDL receptor and the WHHL rabbit is a true animal model of human familial hypercholesterolemia (FH). In addition, Schneider et al. [14] demonstrated that processing of the LDL receptor from the 120-kDa precursor to the 160-kDa mature form was delayed and many of the mature proteins were destroyed in the cytoplasm.

Compared to mouse models (apoE-KO or LDLR-KO) fed standard chow, WHHL or WHHLMI rabbits resemble humans in lipoprotein metabolism (Table 1). Although plasma cholesterol levels are not very high in mouse models without the feeding of a western diet [20], they are extremely high in WHHL and WHHLMI rabbits (700-1200 mg/dl at 12 months old) similar to in human FH. The main lipoprotein in plasma is LDL in WHHL/WHHLMI rabbits and human FH, but it was the VLDL fraction with apoB-48 in apoE KO mice [20] and HDL and LDL in LDLR-KO mice [21]. The activity levels of cholesterol-ester transfer protein (CETP) in plasma are high in WHHL rabbits [19], although mice and rats do not have the activity [22]. Therefore, HDL levels in plasma are low in WHHL rabbits but high in mice and rats. ApoB-editing enzyme is not expressed in the liver of rabbits [23], although mice and rats do have apoB-editing activity in the liver [20,24]. Therefore, apoB-48-containing very low-density lipoprotein (VLDL) is secreted from the liver in mice and rats. Li et al.[25] demonstrated that apoB-48-containing VLDL particles disappeared from the circulation rapidly supposedly through remnant receptors of the liver similar to chylomicron remnants. As a

Table 1. Differences in lipoprotein metabolism, atherosclerosis, and myocardial infarction among human familial hypercholesterolemia (FH), WHHLMI rabbits, and apoE-KO and LDLR-KO mice fed standard chow.

	WHHLMI rabbits	Human FH	ApoE-KO mice	LDLR-KO mice
Plasma cholesterol levels	Extremely high	Extremely high	Mildly high	Moderate
Main lipoprotein in plasma	LDL	LDL	VLDL	LDL and HDL
LDL levels	Extremely high	Extremely high	Moderate	Moderate
HDL levels	Low	Low	Low	High
ApoB of VLDL	B-100	B-100	B-48	B-48 and B100
Expression of apoB-editing enzyme in liver	No	No	Yes	Yes
Cholesteryl ester transfer activity in plasma	Yes	Yes	No	No
Development of coronary atherosclerosis	Severe	Severe	Resistant	Resistant
Features of coronary atherosclerosis	Various types*	Various types*	(Not developed)	(Not developed)
Features of aortic atherosclerosis	Complicated lesions	Complicated lesions	Foamy lesions	(Not developed)
Myocardial infarction	Spontaneous	Spontaneous	Resistant	Resistant

* Various types consist of lesions showing a large lipid core covered by a thin fibrous cap, fibroatheroma, lesions with intra-plaque hemorrhage and/or calcification, fibromuscular lesions, and foamy lesions.

result, LDL lipid levels in mice and rats are low. In apoE-KO mice, which are hypercholesterolemic, the main lipoprotein fraction was not eluted at the position of the LDL fraction in HPLC and included apoB-48 [26]. Since apoE is a ligand of remnant receptors, apoB-48-containing VLDL particles are not bound to remnant receptors in apoE-KO mice. As a result, apoB-48-containing VLDL accumulates in the plasma of apoE-KO mice. Another hypercholesterolemic model is the LDL receptor-KO mouse. Ishibashi et al. [21] demonstrated that LDL levels were high in LDL receptor-KO mice compared to wild-type mice, and serum cholesterol levels were 225 ± 27 mg/dl in the mice fed standard chow. Such serum cholesterol levels are markedly low compared to levels in human familial hypercholesterolemia homozygotes and WHHLMI rabbits. After the administration of a cholesterol-containing diet, serum lipid levels of LDL receptor-KO mice increased to 1583 ±120mg/dl. However, the main lipoprotein fraction was not LDL in a HPLC analysis. Therefore, in lipoprotein metabolism and the pathophysiological features of hypercholesterolemia, mice are markedly different from humans, but WHHL and WHHLMI rabbits resemble humans.

4. Atherosclerosis

Atherosclerotic lesions develop spontaneously in WHHL and WHHLMI rabbits due to hypercholesterolemia even in animals fed normal chow. In the original WHHL rabbits, prior to 1985, atherosclerotic lesions mainly developed in the aorta and the incidence of coronary atherosclerosis was very low. Therefore, many studies of atherosclerosis were carried out using the aorta of WHHL rabbits. The first detailed analysis was carried out by Buja et al.[27], who showed the accumulation of foam cells derived from macrophages and fibrous caps in the intimal plaques of WHHL rabbits. The mechanism of atherogenesis has been examined histopathologically by using specimens from WHHL rabbits. At the initiation of atherogenesis, arterial endothelial cells express adhesion molecules and circulating monocytes adhere to the arterial endothelial cells [28]. These monocytes infiltrate the sub-endothelial region [27-32] and transform into macrophages. Macrophages express scavenger receptors, remnant receptors and VLDL receptors [33,34] and take in degenerated lipoproteins, such as oxidized lipoproteins, and then transform into foam cells [27-32]. Several research groups [35,36] demonstrated peroxidized lipoproteins in atheromatous lesions of WHHL aortas and that an anti-oxidant, probucol, suppresses the development of atherosclerotic lesions of WHHL aortas. Using ultra-rapid freezing techniques, Frank and Fogelman [37] demonstrated that the diameter of 80% of lipid particles in intimal lesions of WHHL aortas was between 70 and 160 nm. These particles are equivalent to VLDL particles. Recent studies have indicated that remnant lipoproteins including VLDL are atherogenic, similar to oxidized LDL [38]. Foam cells collapse and the accumulated lipids are scattered into the extracellular matrix [39] and then a necrotic lipid core appears [27]. With aging, atherosclerotic lesions grow and the cell components decrease (Fig. 4) [40]. Consequently, atherosclerotic lesions change into rupture-prone plaques having a large lipid core covered with a thin fibrous cap on exposure to risk factors for long periods (Fig. 4-F and G), whereas atherosclerotic lesions change into

stable fibromuscular lesions on hypolipidemic treatment [41-46]. WHHL rabbits contributed to studies about the initiation and development of atherosclerotic lesions.

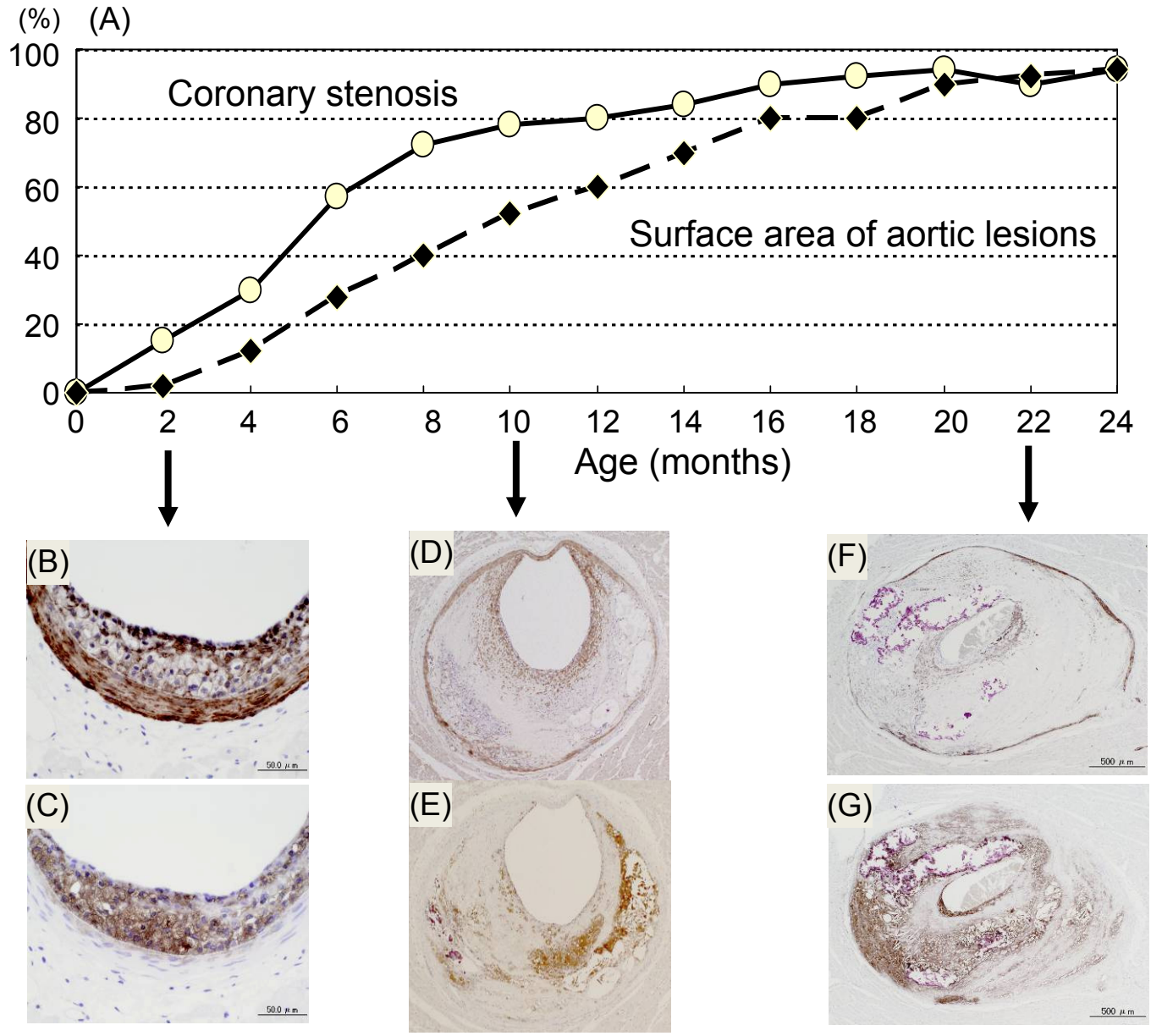

Fig 4. Progression of atherosclerosis in WHHLMI rabbits. (A) Degree of cornary (cross-sectional narrowing,——) and aortic (percentage of surface damaged in the lumen, - - -) atherosclerosis. (B) 1A4 and (C) RAM-11 immunohistochemical staining of an early coronary lesion. (D) 1A4 and (E) RAM-11 immunohistochemical staining of an established coronary lesion (10 months old). (F) 1A4 and (G) RAM-11 immunohistochemical staining of an advanced coronary lesion (22 months old).

5. Coronary atherosclerosis-prone WHHL rabbits

Ultimately an animal model for hypercholesterolemia needs to include myocardial infarction. However, the original WHHL rabbit developed in 1979 did not develop myocardial infarction and had a very low incidence of coronary atherosclerosis [47]. To improve WHHL rabbits as a model for myocardial infarction, Watanabe et al. [47] carried out selective breeding. After five years, the incidence of coronary atherosclerosis was markedly increased. However, the degree of coronary stenosis was mild. Professor Watanabe retired from Kobe University in 1990. His successor attempted to achieve this final goal, the development of myocardial infarction. After a second round of selective breeding, WHHL rabbits with severe coronary atherosclerosis were obtained [48]. However, the incidence of ischemic myocardial lesions was still very low. In a quantitative analysis of the components of atherosclerotic lesions using imaging [40], the coronary plaques of those WHHL rabbits were found to be fibrous and different from the aortic plaques, fibroatheroma. In addition, the cerebral arterial lesions were more fibrous than the coronary lesions in WHHL rabbits [49].

6. Development of myocardial infarction-prone WHHL rabbits

To develop myocardial infarction-prone WHHL rabbits, we selected the descendants of rabbits showing severe coronary lesions mainly consisting of macrophages and foam cells in addition to high plasma cholesterol levels [50] because Van der Wall et al. [51] showed that macrophages and T-lymphocytes accumulated in ruptured plaques in humans. After seven years of selective breeding, we obtained a colony of myocardial infarction-prone WHHL rabbits (designated the WHHLMI rabbit) [50]. The cumulative incidence of myocardial infarction at the age of 30 months was increased from 23 to 97% [50]. Fig. 4 illustrates the progression of atherosclerosis in WHHLMI rabbits [52]. Coronary atherosclerosis is detected from the age of 2 months [52,53]. The lesions are mainly composed of macrophage-derived foam cells. The coronary stenosis (cross-sectional narrowing) was >70% at 10 months and >90% at the age of 20 months [53]. The coronary plaques of WHHLMI rabbits were changed to fibroatheromas from the fibrous lesions of the original WHHL rabbits by selective breeding [50, 53]. WHHLMI rabbits showed various coronary plaques [52], including plaques with intra-plaque hemorrhage, calcified nodules, and the denudation of endothelial cells, and fibromuscular lesions. Furthermore, in the coronary plaques, oxidized lipoproteins were accumulated [45, 54] and macrophages expressed high levels of matrix metalloproteinases, and interleukin-1 [45,54]. These findings suggest that coronary plaques of WHHLMI rabbits mimic typical human vulnerable plaques. However, no ruptured coronary plaques were detected in WHHLMI rabbits [52]. These observations suggest that not only structural properties of vulnerable plaques but also additional factors or triggers for evoking rupture are required. Myocardial infarction in WHHLMI rabbits is characterized by both new and old infarcts [50].

7. Contribution of WHHL rabbits to the development of compounds for treating hypercholesterolemia and atherosclerosis

It is important that animal models can be used in translational research for human diseases and the development of new drugs, devices, or techniques for therapeutics. WHHL or WHHLMI rabbits have been used in studies of several compounds with hypocholesterolemic and/or anti-atherosclerotic effects (Table 2), including statins, the general term for inhibitors of HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis. More than 20 million patients worldwide take statins, one of the most potent drugs for preventing acute coronary syndromes [55]. Studies using WHHL rabbits elucidated the mechanism whereby a reduction in serum cholesterol levels stabilized atherosclerotic lesions [41-44]. The first statin was compactin [6], found in 1973. Although it was not effective in mice and rats, Watanabe et al. [56] demonstrated dose-dependent hypolipidemic effects of compactin in 1981. After the development of compactin was discontinued, a study using WHHL rabbits [57] showed the hypolipidemic effects of pravastatin, a metabolite of compactin. In 1988, Watanabe et al. [58] demonstrated that lowering serum cholesterol levels with pravastatin suppressed the development of coronary atherosclerosis in WHHL rabbits. Their in vivo study was the first direct evidence of anti-atherosclerotic effects of statins. Furthermore, studies using WHHL rabbits showed that the reduction in serum lipid levels caused by statins altered the composition of coronary plaques from macrophage-rich unstable plaques to fibrous stable plaques [41-44]. In addition, synergistic anti-atherosclerotic effects of treatments combining statins with resin, thiazolidinedione, or ACAT inhibitor, or an angiotensin II receptor inhibitor were also suggested in studies using WHHL rabbits (Table 2). Similar results were obtained in a study of squalene synthase inhibitor, another inhibitor of cholesterol biosynthesis [45].

Studies of the anti-atherosclerotic effects of probucol in WHHL rabbits were dramatic [59,60], demonstrating that oxidative stress plays an important role in atherogenesis and anti-oxidants prevent the development of atherosclerosis. Furthermore, several compounds were examined for hypocholesterolemic or hypolipidemic effects and/or anti-atherosclerotic effects (Table 2). WHHL and/or WHHLMI rabbits have contributed to the development of hypocholesterolemic and/or anti-atherosclerotic drugs. There are two types of studies using WHHL or WHHLMI rabbits to examine the anti-atherosclerotic effects of compounds. One is the plaque prevention study [45, 58-60]. The other is the study of plaque-stabilizing effects [41-44]. With the former protocol, treatments are started at 2 months of age when atheromatous plaques are absent or in the early stages, and atherosclerotic lesions are examined at about 10 months when the plaques are established. With the latter protocol, treatments are started from about 10 to 20 months of age when the plaques are unstable and complicated.

Recently, WHHLMI rabbits have been used in studies of the imaging of atherosclerotic lesions by MRI [61], PET [62], and intra-vascular ultrasound (IVUS) [63]. These techniques are promising for the identification of patients with coronary atherosclerosis and would be

useful to prevent acute coronary syndromes.

Table 2. Studies using WHHL or WHHLMI rabbits to develop compounds with hypocholesterolemic and anti-atherosclerotic effects.

	Hypocholesterolemic effects	Anti-atherosclerotic effects	
		Aortic lesion	Coronary lesion
Statin	○	○ X	○
Anion resin	○	○	n.d.
Statin + resin	○ synergistic	○ synergistic	○ synergistic
Squaren synthase inhibitor	○	○	○
MTP inhibiotr	○	n.d.	n.d.
ACAT inhibitor	○ X	○ X	○ X
Probucol	○	○	n.d.
M-CSF and GM-CSF	○	○	n.d.
ApoE	○	○	n.d.
Fibrate	X	n.d.	n.d.
Fish oil or ω3 fatty acids	○ X	○ X	n.d.
Thiazolidinedione	X	X	X
Thiazolidinedione+statin	○	○ synergistic	○ synergistic
Ca^{2+} antagonist	X	X	X
β-blocker	X	X	X
ACE inhibitor	X	○	n.d.
A-II receptor antagonist	X	○	n.d.
Gene therapy	○	n.d.	n.d.

○: effective; X: no effect; ○X: inconsistent; n.d.: not determined
References are from the WHHL rabbit-website (http://www.med.kobe-u.ac.jp/iea/w-index.html).

8. Transgenic WHHL rabbits

Transgenic or knockout mice are useful for studying the functions or roles of genes. Transgenic WHHL rabbits have been developed since 1996 [64]. Genes introduced to date include those for 15-lipoxygenase [64], LCAT [65], lipoprotein(a) [66], lipoprotein lipase [67], and CRP [68]. Interestingly, Fan and Watanabe [69] pointed out that opposite phenotypes were observed for transgenic rabbits and transgenic mice even when the same genes were introduced. Therefore, care is needed when interpreting results from studies with transgenic animals. Transgenic WHHL/WHHLMI rabbits will be useful for studying gene functions relating to atherosclerosis.

9. Conclusion

In humans, clarification of the mechanism of acute coronary syndromes and the development of therapeutics are critical. To accomplish the late Professor Watanabe's goal, we attempted to induce the rupturing of coronary plaques and subsequent formation of thrombi in WHHLMI rabbits. In addition, the development of transgenic WHHLMI rabbits expressing various MMPs or cytokines may help to clarify the mechanisms behind the destabilization of atheromatous plaques, rupturing of plaques, formation of thrombi, and acute coronary syndromes. The WHHL or WHHLMI rabbit will continue contributing to studies of hypercholesterolemia, atherosclerosis, myocardial infarction, and related diseases. Dr. Watanabe's contribution to progress in studies of lipoprotein metabolism and atherosclerosis was substantial and he will be greatly missed.

10. Acknowledgements

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