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Progression of calcific aortic valve sclerosis in WHHLMI rabbits

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ABSTRACT

Background and aims: Aortic valve stenosis (AS) is the most common valvular heart disease and can be life-threatening. The pathogenesis of aortic valve calcification remains largely unknown, primarily due to the lack of an adequate animal model. The high-cholesterol diet-induced AS model in rabbits is one of the established models, but it has the significant limitation of liver dysfunction leading to low survival rates. We hypothesized that a myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbit, an animal model of familial hypercholesterolemia and atherosclerosis, is a useful animal model of AS.

Methods: WHHLMI rabbits, aged 20 months and 30 months (n = 19), and control Japanese White rabbits (n = 4), aged 30 months, were used and evaluated by echocardiography under anesthesia. Pathological evaluation and quantitative analyses by polymerase chain reaction (PCR) were also performed.

Results: The lipid profile was similar between 20 months and 30 months. Two rabbits died due to spontaneous myocardial infarction during the study. Thirty-month-old WHHLMI rabbits exhibited significantly smaller aortic valve area ($0.22 \pm 0.006\ cm^2$ *vs.* $0.12 \pm 0.01\ cm^2$, $p < 0.05$) and higher maximal transvalvular pressure gradient (7.0 ± 0.32 *vs.* 9.9 ± 0.95 mmHg, $p < 0.05$) than 20 month-old rabbits. Macroscopic examination of excised aortic valves demonstrated thickened and degenerated valve leaflets at 30 months. Histological evaluation confirmed thickened leaflets with calcified nodules at 30 months. Real-time PCR of resected aortic valve also showed increased expression level of calcification-related molecules including osteopontin, Sox9, Bmp2, RANKL, osteoprotegerin, and Runx2 ($p < 0.05$ each) in 30-month-old rabbits.

Conclusions: WHHLMI rabbits may be useful models of early-stage AS *in vivo*.

1. Introduction

Severe and symptomatic aortic valve stenosis (AS) is life-threatening and has a poor prognosis. Symptoms include chest pain, syncope, and heart failure; without valve replacement, the average survival time after appearance of these symptoms is 5, 3, and 2 years, respectively [1]. Traditionally, open-heart aortic valve replacement has been the gold standard for AS, and the only option for definitive treatment. Due to the potential complications of open-heart surgery, 40–60% of patients with severe AS do not receive valve replacement [2,3]. Although the recent introduction of transcatheter aortic valve implantation as a new therapeutic option has enabled aortic valve replacement in patients who are poor candidates for open-heart surgery, there is no established pharmaceutical treatment for AS because the pathogenesis of calcific valve degeneration is poorly understood. Therefore, understanding the molecular mechanism of calcific aortic valve disease and establishing a new, low-cost therapeutic strategy is highly desirable. An optimal animal model of aortic valve calcification is vital to identife the factors that contribute to the development of AS.

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Although AS has been considered as a degenerative disease of the valve, accumulating evidence suggests a link between atherosclerosis and calcific aortic valve disease. There are several common risk factors between atherosclerosis and AS, including age, hypertension, smoking, dyslipidemia, and diabetes. Previous reports established that a rabbit fed a high-cholesterol diet is an acceptable animal model for AS [4–8], but this model is significantly limited by liver dysfunction and high mortality due to cholesterol overload [9,10].

In 1973, we identified a strain of rabbit with an inherited hyperlipidemic state, and successful serial breeding established the Watanabe heritable hyperlipidemic (WHHL) rabbits. The WHHL rabbits show abnormally increased serum levels of cholesterol and triglyceride throughout their lives, and they are prone to spontaneous development of atherosclerotic plaques [11]. In WHHL rabbits, the low-density lipoprotein (LDL) receptor is dysfunctional and the clearance of LDL is delayed [12,13]. We subsequently developed a new strain of WHHL rabbits with severe coronary atherosclerosis that are prone to spontaneous myocardial infarction, called myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits [14]. The WHHLMI rabbits have been widely accepted, and have been used as a large animal model of familial hypercholesterolemia and atherosclerosis worldwide [15–18]. Since patients with familial hypercholesterolemia have a high prevalence of subclinical aortic valve calcification, often with extensive involvement [19], we hypothesized that a WHHLMI rabbit, an established animal model of familial hypercholesterolemia and atherosclerosis [11,20,21], could also be a useful animal model of AS.

2. Materials and methods

2.1. Animals

We used 19 WHHLMI rabbits [14], aged 20 or 30 months. The WHHLMI rabbits were bred and kept at the Kobe University Graduate School of Medicine under previously reported conditions [22,23]. Four Japanese White (JW) rabbits, aged 30 months, were used as controls. This study was approved by the Kobe University Animal Care and Use Committee (approval number: P150705-R1), and animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe University [22,23]. Rabbits were euthanized by intravenous injection of sodium pentobarbital (150 mg/kg) after the echocardiographic study.

2.2. Echocardiography

Transthoracic echocardiography was performed using a Philips Envisor C echocardiograph (Philips Inc., Eindhoven, the Netherlands) under mild sedation by intramuscular injection of ketamine hydrochloride (15 mg/kg, Daiichi-Sankyo Co. Ltd., Tokyo, Japan) plus midazolam (1 mg/kg, Dormicum, Astellas Pharma Inc., Tokyo, Japan) [4,24]. The aortic valve area (AVA) was measured by the standard continuity equation. The indexed aortic valve area ($AVAi_{index}$) was calculated by dividing AVA by body surface area (BSA). BSA was calculated using Meeh's formula as follows; BSA (m^2) = Kx (weight x 1000)$^{2/3}$/10000 (k = 12.0 for rabbits) [25]. A parasternal long-axis view was used to measure the diameter of the left ventricular outflow tract. An epigastric approach with pulsed-wave Doppler was used to obtain stroke volume proximal to the aortic valve. Continuous-wave Doppler was used to measure the maximal transvalvular pressure gradient at the aortic valve.

2.3. Histology

Aortic valves were excised after euthanasia. The three leaflets of the valve were separated and processed to obtain (1) paraffin-embedded sections, (2) frozen sections, and (3) extracted RNA. For paraffin-embedding, the leaflet was immersion-fixed with a 10% neutral buffered formalin solution and embedded in paraffin. Sections were serially sliced at 5-μm thickness. Sections were stained with Elastic van Gieson to assess elastic fibers, Azan for collagen staining, Victoria blue hematoxylin and eosin (HE) for gross histology, and Alizarin Red S for calcification. Serial sections were incubated with RAM-11 (Dako A/S, Glostrup, Denmark) antibody to stain rabbit macrophages and with smooth muscle actin (SMA) antibody (clone 1A4, Dako A/S) to stain myofibroblast cells.

For frozen sections, the leaflet was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Japan). Serial 6 μm-thick cryostat sections were obtained for histological analysis. Frozen sections were incubated with *anti*-osteocalcin (Abcam) antibody and covered with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratory, Burlingame, USA). Stained sections were observed under an all-in-one type of fluorescence microscope (BZ-X710; Keyence, Japan), using the associated software (BZ Analyzer Software; Keyence, Japan). Thickness of the aortic valve leaflet was measured at the largest point in the section. The section at the center of the leaflet was used for the quantitative analysis.

2.4. Assay of serum lipid levels

Blood samples were taken after 15 h of fasting. Serum total cholesterol and triglyceride levels were assayed enzymatically with kits. Serum LDL-cholesterol and HDL-cholesterol levels were measured by high performance liquid chromatography (LipoSEARCH®; Skylight Biotech, Inc., Akita, Japan).

2.5. RNA isolation and RT-PCR

Total RNA was extracted from leaflets by the guanidine thiocyanate method using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription polymerase chain reaction (PCR) was performed as previously reported [26,27]. The values obtained were normalized to 20-month values and expressed as percentage of the 20-month value. The following primers were used; *Sox9*, forward, 5′-GGTGCTCAAGGGCTACGACT-3′ and reverse, 5′-GGG TGGTCTTTCTTGTGCTG-3'; *osteopontin (OPN)*, forward, 5′GCTCAG CACCTGAATGTACC-3′ and reverse, 5′-CTTCGGCTCGATGGCTAGC-3'; *Runx2*, forward, 5′-CCTTCCACTCTCAGTAAGAAGA-3′ and reverse, 5′TAAGTAAAGGTGGCTGGATAGT-3'; *RANKL*, forward, 5′-CAACAC CTGAGAGCCGAGAA3′ and reverse, 5′-ACGGGAACCTGATGGGATGT-3'; *osteoprotegerin (OPG)*, forward, 5′ACAACCGTGTGTGTGAATGC-3′ and reverse, 5′-GTATTCCGCTCTGGGGTTCC-3'; *Bmp2*, forward, 5′-CGCCTCAAATCCAGCTGTAAG-3′ and reverse, 5′-GGGCCACAATCC AGTC GTT-3'; *GAPDH*, forward, 5′-TCGGAGTGAACGGATTTGGC-3′ and reverse, 5′TGCCGTGGGTGGAATCATAC-3'.

2.6. Statistics

All experiments were conducted at least three times, and the results are expressed as means ± standard error of the mean (SEM). The assumption of normality was tested using Kolmogorov-Smirnov (KS) normality test. Statistical comparisons between two groups were evaluated by the unpaired *t*-test for parametric values (echocardiographic data) and the Mann-Whitney *U* test for nonparametric values (RT-PCR experiments). For comparison between two groups within the same animal (serial echocardiographic study), the paired *t*-test was used after confirming parametric distribution. A p value < 0.05 was considered statistically significant (GraphPad Prism; GraphPad Software Inc., La Jolla, CA, USA).