Valvular Heart Disease

Calcific Aortic Valve Stenosis in Old Hypercholesterolemic Mice

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Background—Hypercholesterolemia and old age are clinical risk factors for development of aortic valve stenosis, and hypercholesterolemia is a putative therapeutic target. We tested the hypothesis that calcification and aortic valve stenosis would develop in genetically hypercholesterolemic old mice.

Methods and Results—Twenty-four low-density lipoprotein receptor–deficient apolipoprotein B-100–only (LDLr<sup>−/−</sup>ApoB<sup>100/100</sup>) mice were fed normal chow from weaning until age 20.1±0.5 months (mean±SE; range 17 to 22 months). Twenty-one age-matched (20.8±0.9 months, range 17 to 25 months) C57Bl/6 mice served as controls. Echocardiographic imaging was used to assess morphology and function of the aortic valve and left ventricle. A subset of 12 mice underwent invasive hemodynamic assessment of aortic valve function. Functionally significant aortic stenosis, with >75% reduction in valve area, occurred in 8 of 24 LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice and in 0 of 21 controls (P=0.01). In the subset that underwent catheterization, mice with echocardiographic evidence of aortic stenosis had a systolic transvalvular gradient of 57±6 mm Hg. In the group of all LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis, left ventricular mass was increased by 67% (P=0.001) and ejection fraction was decreased by 30% (P=0.004) compared with LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis. Von Kossa staining of the aortic valve demonstrated abundant mineralization in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice but not in control mice. Superoxide (oxyethidium fluorescence) was present in valve tissue from all 3 groups of mice and was more abundant in mice with aortic stenosis.

Conclusions—Hypercholesterolemic LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice are prone to develop calcification and oxidative stress in the aortic valve, with functional valvular heart disease, mimicking the clinical syndrome. This discovery holds promise for elucidation of the pathophysiology of aortic valve disease mechanisms and for the design of effective nonsurgical treatment. (Circulation. 2006;114:2065-2069.)

Key Words: valves ■ free radicals ■ cholesterol

Calcific aortic valve stenosis is a large and growing health problem in Western societies. Studies in explanted valves from patients with aortic stenosis reveal histopathological parallels with atherosclerosis: lipid deposition, inflammation, cellular reaction, and matrix remodeling. The epidemiological profile of aortic stenosis also shares common features with atherosclerosis. Hyperlipidemia, age, hypertension, and smoking are all risk factors for both conditions. Hyperlipidemia, in particular, has drawn the interest of clinicians and is a putative therapeutic target in patients with aortic stenosis. In experimental animals, severe hyperlipidemia causes lipid deposition and structural alterations in aortic valves. To date, these studies have not demonstrated hemodynamic impact to a clinically relevant degree, however, nor have they demonstrated the significant impact on left ventricular structure and function known clinically as “valvular heart disease.”

In vascular tissue, superoxide is associated with hypercholesterolemia and modulates several aspects of vascular injury. This complex process includes superoxide-mediated reprogramming of smooth muscle cells to an osteoblastic phenotype. Evidence for increased levels of superoxide and its local tissue consequences have not been reported in heart valves. We hypothesized that hypercholesterolemia would produce calcification in aortic valve tissue and would lead to the syndrome of aortic valvular heart disease in old mice.
Methods

The Office of Animal Resources of the University of Iowa approved all procedures. Low-density lipoprotein receptor–deficient apolipoprotein B-100–only (LDLr<sup>−/−</sup>ApoB<sup>100/100</sup>) mice (n=24) were generated as described previously.12 Wild-type C57Bl/6 mice (n=21) were obtained from Jackson Laboratories (Bar Harbor, Me). All mice were fed normal chow and housed under 12-hour light/dark cycles. Ages were 20.1±0.5 months (range 17 to 22 months) for LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice and 20.8±0.9 months (range 17 to 25 months) for C57Bl/6 mice.

Echocardiography

Mice were sedated with midazolam (0.15 mg SC), after which they remained awake but docile. The anterior thorax was shaved to optimize the acoustic interface. Warmed gel was applied, and the animal was grasped gently by the nape of the neck and cradled in the left lateral recumbent position in the imager’s left hand. A 15-MHz linear-array probe was applied horizontally to the chest. The imaging probe was coupled to a Sonos 5500 imager (Philips Medical Systems, Bothell, Wash), generating 180 to 200 2-dimensional frames per second in both short- and long-axis left ventricular (LV) planes.

Images of the aortic valve were acquired in M mode, at a nominal sampling rate of 1000/s, with 2-dimensional images used for guidance. Pulse-wave Doppler tracings were obtained with depth gates near the ventricular aspect of the mitral valve to measure heart rate. Doppler interrogation of blood velocity through the aortic valve was not possible because the imaging probe does not have continuous-wave Doppler capability. All images were acquired by an operator blinded to mouse genotype.

Echocardiographic Image Analysis

Images were archived offline and analyzed in blinded fashion with custom-designed software (Freeland Medical Systems, Louisville, Colo). Endocardial and epicardial borders were traced in the short-axis plane at end diastole and end systole. The lengths from left ventricular outflow tract to endocardial apex and epicardial apex, respectively, were measured at end diastole and end systole. Left ventricular mass was calculated by the biplane area-length method, which was previously validated in our laboratory.13 This method was also used to calculate end-diastolic and end-systolic left ventricular volumes and ejection fraction. M-mode images of the aortic valve systolic orifice were analyzed with electronic calipers.

Hemodynamic Assessment of Aortic Valve Function

After echocardiographic imaging, mice were anesthetized with ketamine/acepromazine (90/1.8 mg/kg intraperitoneally) The right common carotid artery was exposed, and a 1.4F microtransducer-tipped catheter (Millar, Houston, Tex) was inserted. Pressure was recorded continuously as the catheter was advanced into the left ventricle. After left ventricular pressure was recorded, the catheter was withdrawn into the ascending aorta, and pressure was again recorded. Peak aortic valve gradient was defined as the difference between left ventricular peak pressure and aortic peak pressure, averaged over at least 3 beats from each location.

Tissue Analysis

Mice were euthanized with pentobarbital sodium (150 mg/kg intraperitoneally). The fresh, unfixed aortic valve tissue was frozen in OCT compound for detection of superoxide and for histological staining.

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to localize superoxide (O<sub>2</sub><sup>−</sup>) in the aortic valves as described previously.14 Briefly, transverse sections (30-μm thickness) were cut in a cryostat and placed on glass slides. Samples were then incubated at room temperature for 30 minutes with DHE (0.002 mmol/L) and protected from light. DHE reacts with O<sub>2</sub><sup>−</sup> to form oxythidium, a fluorescent product that can be detected by confocal microscopy.

Images were obtained with a Bio-Rad MRC-1024 laser (krypton/argon) scanning confocal microscope (excitation and emission spectra of 488 and 610 nm, respectively; Bio-Rad Laboratories, Hercules, Calif). Fluorescence was detected with a 585-nm long-pass filter. Relative increases in oxythidium fluorescence were determined with ImageJ software (version 1.32j; National Institutes of Health, Bethesda, Md) as described previously.14 Adjacent sections of the frozen tissue (10-μm thickness) were used for analysis of tissue mineralization (Von Kossa stain).

Statistical Analysis

Group data are reported as mean±SE, and comparisons between groups were made with ANOVA. The proportion of LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis was compared with the proportion of C57 mice with aortic stenosis using the comparison of 2 proportions test.15 The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> Mice Develop Moderate Hypercholesterolemia

Serum cholesterol was 271±12 mg/dL for LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice and 110±17 for C57 mice (P<0.001).

LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> Mice Develop Aortic Stenosis and Valvular Heart Disease

Systolic aortic valve orifice diameter was reduced by >50%, predicting a 75% reduction in valve area, in 8 of 24 LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice versus 0 of 21 age-matched wild-type mice (P=0.01; Figure 1). Of the 8 mice with echocardiographic evidence of aortic stenosis, 5 were included in the subset of 12 mice that underwent invasive hemodynamic assessment. In that subset, echocardiographic evidence of aortic stenosis correlated with a transvalvular systolic gradient of 57±6 mm Hg during invasive hemodynamic assessment (Figure 2). LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis developed left ventricular hypertrophy, because LV mass was 67% greater in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis (P=0.001). In LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis, LV ejection fraction was 30% lower than in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis (P=0.004; Figure 3).
Calcification and Oxidant Stress

LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice demonstrate abundant calcification in valve leaflet tissue and at the attachment points to the aorta (Figure 4). A few foci of calcification also were seen in wild-type mice, but the density and anatomic extent were minimal. Superoxide (oxyethidium fluorescence) was present in aortic valve tissue from all 3 groups of mice (Figure 5) and was 42±9% greater in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis (n=4) than in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis (n=4; P=0.007).

**Discussion**

The most important finding of the present study is that old mice with a moderate, clinically relevant degree of hypercholesterolemia are prone to develop physiologically important aortic valvular stenosis and the syndrome of valvular heart disease. Furthermore, LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis have oxidant stress in valve tissue, a feature of aortic valve disease not reported previously.

The correlation between the ≈75% decrease in aortic valve area and a hemodynamically significant transvalvular gradient in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice reproduces the relationship seen in clinical disease. The findings of left ventricular hypertrophy and impairment of systolic function in LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis verify the pathophysiological responses to pressure overload. These findings are not epiphenomena of the genotype, because LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis displayed neither feature.

**Figure 2.** Hemodynamic evaluation of aortic valve function. Left and center panels depict representative pressure tracings during left ventricle-to-aorta catheter “pullback.” Right panel depicts the correlation between peak systolic valve gradient (catheter) and echocardiographic assessment of systolic valve orifice dimension (Echo-Cath; n=12).

**Figure 3.** Echocardiography-derived estimation of left ventricular mass and ejection fraction. Data from wild-type mice (C57, n=21), LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis (No AS, n=16), and LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis (AS, n=8). *P=0.001 for LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis compared with LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis. **P=0.004 for LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice with aortic stenosis compared with LDLr<sup>−/−</sup>ApoB<sup>100/100</sup> mice without aortic stenosis.
To begin to examine potential mechanisms of development of aortic valvular stenosis, we sought evidence of oxidant stress in valve tissue. Oxidant stress plays a key role in the pathophysiology of atherosclerosis, possibly including initiation of the sequence of events that lead to arterial calcification. We observed high levels of superoxide in aortic valve tissue of LDLr\(^{-/-}\)/ApoB\(^{100/100}\) mice with aortic stenosis, which provides the first evidence for an association between oxidant stress and valve disease. Although the data do not demonstrate a causative role for oxidative stress in the disease process, the findings suggest that calcific aortic stenosis, even with “end-stage” mechanical dysfunction, continues to be an active disease process.

Explanted valves from patients with aortic stenosis contain oxidized LDL, a putative initiator of tissue calcification. The high levels of superoxide in valve tissue of mice with aortic stenosis may suggest that at least some oxidized LDL may be formed in situ, thus implicating local cellular processes as initiators of valve calcification.

Many other investigators have examined vascular consequences of hyperlipidemia in genetically engineered mice, and pathological changes in valves have been observed incidentally. Recent studies have also focused on the aortic valve in hypercholesterolemic animals. A statistically significant reduction in aortic valve area has been observed, which corresponds to an average valve gradient of \(\approx 7\) mm Hg (range \(\approx 4\) to \(\approx 17\) mm Hg) in cholesterol-fed rabbits that also received very high doses of vitamin D. Morphological changes and Doppler indices indicating a valve gradient of \(\approx 5\) mm Hg also have been observed in mice with diet-induced metabolic syndrome. Changes in valve morphology have been described in ApoE\(^{-/-}\) mice, but high blood velocity across the aortic valve (\(\approx 425\) cm/s) was observed in only 1 of 45 mice studied. In that report, ApoE\(^{-/-}\) mice as a group apparently did not demonstrate a statistically significant increase in blood velocity across the aortic valve compared with wild-type mice. Thus, in previous studies of experimental hypercholesterolemia, only very mild aortic stenosis has been observed, hemodynamic confirmation has not been reported, and the impact of valve disease on left ventricular mass and systolic function was not investigated.

It is perhaps surprising that the present study is the first to report a predisposition toward spontaneous development of the full syndrome of aortic valvular heart disease in a laboratory animal. The reasons for the novelty of the observation are probably manifold. First, we studied mice at an older age than is typically reported. This may have influenced disease prevalence and severity, as suggested by studies of patients. Second, the anatomic distribution of atherosclerotic changes may vary among hyperlipidemia-related genotypes. We observed extensive atherosclerosis in the aortic root in LDLr\(^{-/-}\)/ApoB\(^{100/100}\) mice, adjacent to the aortic valve. We have not systematically compared the magnitude of aortic root plaque formation nor tested for hemodynamically significant aortic valve dysfunction in old hyperlipidemic mice with other genotypes. Third, echocardiographic and hemodynamic characterization of these mice is technically challenging and thus may not have been pursued in previous studies of atherosclerotic vascular disease.

The present study also has an important methodological implication. Noninvasive characterization of valve function with echocardiography correlates with invasive hemodynamic assessment in reliable, although semiquantitative, evaluation of valve function. This newly validated capability should facilitate longitudinal studies of progression/regression of aortic valve disease in mice.

Hyperlipidemia can be “switched off” when LDLr\(^{-/-}\)/ApoB\(^{100/100}\) mice are bred with mice homozygous for both a conditional allele of the gene encoding for microsomal triglyceride transfer protein and the inducible Mx1-Cre transgene. After Cre expression is induced by treatment with polyinosinic-polycytidylic RNA, plasma lipid profiles rapidly normalize. This capability should facilitate future studies to determine whether treatment of hypercholesterolemia does or does not slow progression of aortic valve stenosis. The finding of high superoxide levels in

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**Figure 4.** Von Kossa staining of aortic valve tissue. Mineralization of tissue appears as discreet dark foci. L indicates leaflet; LA, leaflet attachment. Bar left=0.3 mm.

**Figure 5.** Superoxide in aortic valve tissue. Images depict oxyethidium fluorescence, a reporter for tissue superoxide, as discrete bright white pixels. L indicates leaflet; LA, leaflet attachment. In the image on the far right, there appears to be fusion of the attachment surfaces of adjacent valve leaflets (larger white arrow). Bar=0.3 mm.
aortic valve tissue may prompt future studies designed to examine molecular mechanisms of valvular calcification and to test the efficacy of antioxidant strategies.

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Disclosures

None.

References

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