Evidence for Active Regulation of Pro-Osteogenic Signaling in Advanced Aortic Valve Disease

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Objective—To test the hypothesis that valvular calcium deposition, pro-osteogenic signaling, and function can be altered in mice with advanced aortic valve disease.

Methods and Results—“Reversa” mice were given a Western-type diet for 12 months and screened for the presence of aortic valve stenosis. Mice with advanced valve disease were assigned to 1 of 2 groups: (1) those with continued progression for 2 months and (2) those with regression for 2 months, in which lipid lowering was accomplished by a genetic switch. Control mice were normocholesterolemic for 14 months. Mice with advanced valve disease had massive valvular calcification that was associated with increases in bone morphogenetic protein signaling, Wnt/β-catenin signaling, and markers of osteoblastlike cell differentiation. Remarkably, reducing plasma lipids with a genetic switch dramatically reduced markers of pro-osteogenic signaling and significantly reduced valvular calcium deposition. Nevertheless, despite a marked reduction in valvular calcium deposition, valve function remained markedly impaired. Phosphorylated Smad2 levels and myofibroblast activation (indexes of profibrotic signaling) remained elevated.

Conclusion—Molecular processes that contribute to valvular calcification and osteogenesis remain remarkably labile during the end stages of aortic valve stenosis. Although reductions in valvular calcium deposition were not sufficient to improve valvular function in the animals studied, these findings demonstrate that aortic valve calcification is a remarkably dynamic process that can be modified therapeutically, even in the presence of advanced aortic valve disease. (Arterioscler Thromb Vasc Biol. 2010;30:2482-2486.)

Key Words: calcification ■ genetically altered mice ■ heart valves ■ oxidized lipids ■ aortic valve stenosis

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Recently, there have been substantial advances in understanding pathobiological processes in the calcifying aortic valve. Valvular calcification, like vascular calcification, was once thought to be a passive and degenerative process. However, it is now clear that osteoblastlike cells are present in the valves of most patients with aortic valve stenosis,1–3 suggesting that calcium deposition in the valve is an active process.

Nonsurgical treatments aimed at slowing or halting the progression of aortic valve stenosis have proved to be elusive. Studies in hypercholesterolemic animals have demonstrated that lipid-lowering therapy can dramatically reduce osteogenic markers and calcium deposition in the valve4–6; when initiated in the early stages of valve disease, these studies can halt the progression of aortic valve stenosis.4 Three clinical trials5–7 that examined the effects of lipid lowering on the progression of aortic valve stenosis yielded negative results. However, it is difficult to interpret these findings in a biological context. Many cells in the aortic valve and vascular express markers of terminally differentiated osteoblasts,1–3 and it is unclear whether lipid lowering results in dedifferentiation of these cells to a less osteogenic phenotype. Furthermore, although calcifying vascular smooth muscle cells are remarkably labile with regard to their osteogenic activity in vitro,8–10 a recent study11 in cultured valvular interstitial cells demonstrated that stiffening of the extracellular matrix, associated with end-stage aortic valvular stenosis, may reduce the efficacy of anticalcific treatments.

The aim of the current study was to determine whether lipid lowering with a genetic switch12 would reduce pro-osteogenic signaling and calcium deposition in hypercholesterolemic mice with advanced aortic valve disease.

Methods

Animal Model and Protocol

At the age of 6 to 8 weeks, “Reversa” (low-density lipoprotein receptor–deficient/apolipoprotein B100/100/microsomal triglyceride transfer protein (mttp)fl/fl/Mx1-Cre) littermates were assigned to 1 of 3 groups (control, hypercholesterolemic, or regression/reversed), as

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Control mice were given 4 injections of polyinosinic-polycytidylic acid, 225 µg IP, at 2-day intervals, and a chow diet was maintained for 14 months. Mice in the progression group were given a Western diet (Teklad No. TD88137: 42% of calories from fat and 0.25% cholesterol) for 14 months. Mice in the progression/reversed group were given a Western diet for 12 months, then given 4 injections of polyinosinic-polycytidylic acid, 225 µg IP; switched to a chow diet; and observed for an additional 2 months.

Evaluation of Aortic Valve Function
Aortic valve function was evaluated as previously described. Briefly, mice were sedated with midazolam, 0.15 mg SC; each mouse was cradled in the left lateral recumbent position while a 15-MHz linear-array probe was applied horizontally to the chest. The imaging probe was coupled to an imager (Sonos 5500), generating 180 to 200 frames (2D) per second in both short- and long-axis left ventricular planes. Images of the aortic valve were acquired in M mode, at a nominal sampling rate of 1000 frames per second, with 2D images used for guidance. Blood lipid levels, oxidative stress, histological features, and immunohistochemistry results were examined as previously described. Methods for image quantification and immunofluorescent image background correction are described. Methods for image quantification and immunofluorescent image background correction are described.11

Plasma Lipid Levels
Total plasma cholesterol was markedly elevated in hypercholesterolemic versus control mice (571±54 versus 150±10 mg/dL; *P<0.01). Lowering cholesterol levels with a genetic switch at 12 months significantly lowered cholesterol levels in the reversed group (200±9 mg/dL, †P<0.01) versus the hypercholesterolemic group. Values given as mean±SE.

Histological Changes in the Aortic Valve
In control mice, there were small amounts of calcium deposition in the aortic valve at 14 months (Figure 1A). In hypercholesterolemic mice, calcium deposition in the valve cusps was dramatically increased (Figure 1B). Remarkably, calcium deposition in the valve cusps was significantly reduced by lipid lowering for 2 months (Figure 1C).

Procalcific Signaling in the Aortic Valve
Levels of phosphorylated mothers against decapentaplegic homologs 1/5/8 (Smad1/5/8) were relatively low in control mice but were significantly elevated in hypercholesterolemic mice after 14 months of severe hypercholesterolemia (Figure 2A, B). Reducing plasma lipids for 2 months significantly reduced phosphorylated Smad1/5/8 immunofluorescence in reversed mice (Figure 2C).

Immunofluorescence of β-catenin was virtually undetectable in control mice (Figure 2B). β-Catenin immunofluorescence was significantly increased in mice subjected to 14 months of severe hypercholesterolemia and was significantly attenuated by reducing plasma lipids for 2 months (Figure 2F, G).

Immunofluorescence of the Smad1/5/8 target gene core binding factor alpha 1 (CBFA1)/Runx2 was low in control mice but was significantly increased in hypercholesterolemic mice at 14 months (Figure 3A, B). Reducing plasma lipid levels for 2 months significantly attenuated immunofluorescence of the CBFA1/Runx2 gene in the aortic valve cusps (Figure 3C).

Profibrotic Signaling in the Aortic Valve
Levels of phosphorylated Smad2/3 (an indicator of transforming growth factor β signaling) were low in control mice but were
significantly elevated in mice after 14 months of severe hypercholesterolemia (Figure 4A, B). However, reducing plasma lipids for 2 months did not significantly alter phosphorylated Smad2/3 immunofluorescence (Figure 4C). Myofibroblast activation, assessed by immunofluorescence of smooth muscle actin, was low in control mice but was robustly increased in hypercholesterolemic mice at 14 months (Figure 4E, F). Reducing plasma lipids for 2 months significantly reduced myofibroblast activation in reversed mice (Figure 4G).

Levels of phosphorylated Smad2/3 (an indicator of transforming growth factor (TGF)-β signaling) were low in control mice but were significantly elevated in mice after 14 months of severe hypercholesterolemia. However, reducing plasma lipids for 2 months did not significantly alter phosphorylated Smad2/3 immunofluorescence (Figure 4).

**Oxidative Stress in the Aortic Valve**

Superoxide levels were low in control mice at 14 months and were significantly increased in hypercholesterolemic mice (supplemental Figure). However, reducing plasma lipids for 2 months did not significantly reduce superoxide levels in the aortic valve cusps (supplemental Figure).

**Aortic Valve Function**

Cusp separation distance in control mice was 0.84±0.03 mm at 12 months of treatment and did not change significantly from 12 to 14 months (Figure 5). Cusp separation distance was significantly reduced in mice after 12 months of hypercholesterolemia (0.67±0.02 mm) but did not decrease significantly after an additional 2 months of hypercholesterolemia (Figure 5). Cusp separation distance was not significantly altered by reducing blood lipids for 2 months (Figure 5).

**Heart and Lung Weights**

Compared with control mice, both heart weight and heart weight/body weight ratios were significantly increased after prolonged hypercholesterolemia (Table). Reducing plasma lipids for 2 months did not significantly reduce heart or lung wet weight in the reversed group (Table).

**Discussion**

The major findings of this study are as follows: (1) lipid lowering dramatically reduces levels of several pro-
osteogenic proteins in hypercholesterolemic mice with advanced aortic valve disease, (2) lipid lowering significantly reduces calcium deposition in aortic valves of mice with advanced disease, and (3) despite biological and histological improvements in the aortic valve, lipid lowering did not improve aortic valve function or cardiac hypertrophy in hypercholesterolemic mice.

Pro-Osteogenic Signaling and Valvular Calcium Deposition

After 14 months of hypercholesterolemia, we observed large increases in pro-osteogenic proteins in hypercholesterolemic mice. More important, we observed marked activation of both canonical bone morphogenetic protein signaling and Wnt/β-catenin signaling in hypercholesterolemic mice, 2 pathways that are consistently and robustly activated in human aortic valve stenosis.12–14

Increases in procalcific signaling were associated with massive calcification of the aortic valve cusps. Although we did not conduct a detailed analysis of the composition and ultrastructure of these calcium deposits, previous studies13–15 examining tissue from humans with severe aortic valve stenosis identified matrix components that are virtually indistinguishable from bone and often resemble endochondral ossification. Similar observations have been made in calcified arterial plaques of humans and mice,16–21 suggesting that osteoblastlike cells in the cardiovascular system can drive the formation of bonelike tissue.

After activating a genetic switch and normalizing cholesterol levels from 12 to 14 months, pro-osteogenic signaling was markedly reduced in the aortic valve cusps. Remarkably, reductions in pro-osteogenic protein levels were also associated with significant reductions in valvular calcium deposition. These findings contrast with observations from humans with severe calcification of the coronary arteries22 or aortic valve,7,23 in whom lipid-lowering therapy has a negligible impact on calcium burden. However, these findings are consistent with previous observations in animals with early aortic valve disease4 and in primary prevention studies. It is not clear how much the aortic valve and atherosclerotic plaque differ with regard to resorption of calcium and regression of advanced lesions or whether mice have a greater ability to resorb/regress ectopic calcium deposition compared with primates.24

Oxidative Stress

After 14 months of hypercholesterolemia, we observed significant increases in superoxide levels in the aortic valve. This finding is consistent with previous observations that superoxide levels are significantly increased during all stages of aortic valve stenosis2,25 and that there is no “burnout” during terminal stages of the disease.

A surprising finding was that reduction of blood lipids for 2 months did not reduce superoxide levels in the aortic valve. This finding contrasts with a previous study,2 in which it was demonstrated that superoxide levels could be significantly reduced if lipid lowering was initiated during the early stages of aortic valve disease. This finding has 2 important implications. First, increased oxidative stress is not likely to be a primary stimulus for increased procalcific signaling in end-stage stenosis; instead, it may play an important role in amplification of procalcific signaling and matrix remodeling. This concept is supported, in part, by observations in calcifying vascular smooth muscle cells, where the addition of exogenous oxidative stress accelerates calcium deposition and nodule formation only in the presence of calcifying media.9,26 Second, it is possible that sustained increases in valvular oxidative stress after normalization of plasma lipids in advanced aortic valve disease may be secondary to increased mechanical stresses/pressures placed on the valve.27

Aortic Valve Function

Despite its dramatic effects on procalcific signaling and calcium deposition in the valve, reducing cholesterol levels failed to improve aortic valve function in the present study. This observation is relevant to the principal findings of 3 recent clinical trials,7–9 which demonstrated no benefit of statin therapy in patients with advanced aortic valve disease. There are several potential explanations for this observation. First, reductions in valvular calcium may not have passed a “threshold” for improving valve function because calcium deposition in the regression group mice remained higher than what was previously observed.4 Second, it is possible that valvular fibrosis plays an important role in determining valve function in advanced stages of disease. Our data demonstrating that transforming growth factor β signaling and myofibroblast activation do not decrease after lipid lowering are consistent with the persistence of a profibrotic environment in the aortic valve.28 Although we did not assess changes in valvular fibrosis in the present study, per previous data,4 valvular fibrosis does not improve after 6 months of lipid lowering; it is unlikely that fibrosis improves with this shorter period. Finally, it is possible that stenotic aortic valves may reach a “point of no return,” at least in relation to lipid lowering, at which impairment in valvular function is sustained indefinitely, even after reversal of its initiating stimulus and amelioration of pro-osteogenic signaling.

Limitations

Although hypercholesterolemia is a risk factor for the development of aortic valve stenosis, there are other risk factors (including age) that have stronger associations with the development of aortic valve stenosis. However, the experimental model is unique because it captures 2 key aspects of human valve disease: (1) hemodynamically significant impairments in aortic valve function and (2) activation of pro-osteogenic signaling cascades, which is similar to what is observed in human disease. Although there is likely to be
context-dependent signaling for several pro-osteogenic signaling cascades (ie, modulation by the presence/absence of hypercholesterolemia), this nevertheless serves as a powerful model to facilitate studies of development and treatment of aortic valve stenosis.

There are methodological limitations associated with the present investigation, many of which are inherent to working with mice. Because only a small amount of tissue is available from mouse aortic valves, we are unable to perform Western blotting to assess protein levels of pro-osteogenic signaling molecules. Also, the number of sections available for staining is small because the number of animals per group is limited by the extended duration needed to generate animals. Consequently, we were not able to determine whether osteoclasts were present in aortic valves during progression or regression of calcification. Nevertheless, the data are consistent with the hypothesis that calcium deposition in the aortic valve is an active and labile process.

In conclusion, the major findings of this study are that profound reductions in pro-osteogenic signaling and valvular calcium deposition can occur in mice with advanced aortic valve disease. The data suggest that aortic valve calcification in end-stage disease remains an active process that can be modified therapeutically. Thus, although lipid lowering per se does not improve aortic valve function, the finding that pro-osteogenic signaling and calcium deposition are labile even in advanced aortic valve stenosis implies that it may be feasible to improve valve function despite advanced aortic valve disease.

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Disclosures

None.

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In the article, “Evidence for Active Regulation of Pro-Osteogenic Signaling in Advanced Aortic Valve Disease ” by Miller et al, which appeared in the December 2010 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;30:2482–2486; DOI: 10.1161/ATVBAHA.110.211029), an error occurred in the final, printed version of the article. On page 2484, the 1st full paragraph (“Levels of phosphorylated Smad2/3 . . .”) should have been omitted.

The online version has been corrected.

The publisher sincerely regrets the error.

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Online Supplementary Methods

Animals. At 6–8 weeks of age, “Reversa” (ldlr-/-/apoB<sup>100/100</sup>/mttp<sup>fl/fl</sup>/Mx1-Cre) littermates were assigned to either “control”, “progression,” or “regression” groups, as described previously<sup>1</sup>. Control mice were given 4 injections of polyinosinic-polycytidylic acid (pI-pC, 225 μg, i.p.) at two-day intervals and maintained on a chow diet for 14 months. Progression mice were placed on a Western diet (Harlan Teklad #TD88137, 42% of calories from fat, 0.25% cholesterol). After 12 months of hypercholesterolemia/progression, a subset of mice were selected for this study based on the presence of moderate to severe stenosis (cusp separation distance less than 0.7mm), and randomized to two groups: 1) a group which underwent sustained progression/hypercholesterolemia for 2 months, and 2) a group which underwent normalization of lipids by a genetic switch for 2 months (i.e., “regression”/”reversal” group). As described previously, the regression/reversed mice were given 4 injections of pl-pC (225 μg, i.p.) over the course of 8 days to drive Cre recombinase expression and conditionally inactivate the microsomal triglyceride transfer protein and were switched to a chow diet. All data are the average of 5-8 animals per group.

Measurement of whole blood glucose, plasma cholesterol, and plasma insulin levels. Animals were anesthetized with an overdose of inhaled sevoflurane, and the chest cavity was rapidly opened. The inferior vena cava was severed, and blood was taken for analysis. Plasma cholesterol was measured using a colorimetric kit (Wako Diagnostics).

Measurement of histological changes in the valve. Serial sections (10 μm thickness) were taken from tissue frozen in OCT. Lipid deposition was measured using Oil Red O (Sigma, France). Tissue calcification was measured using Alizarin Red staining. Images were obtained using light microscopy at 4x and 10x magnification (Olympus BX51 Digital Light Microscope, Olympus, Japan). For analysis, Adobe Photoshop CS2 (version 7, Adobe Systems Inc. San Jose, CA) was used to select only pixels expressing red histological staining. Valve cusps were traced to obtain a measurement of valve area. Data are expressed as the percentage of valve area that displays positive staining.

Measurement of valvular oxidative stress. Superoxide in the valve was evaluated using ex vivo staining for dihydroethidium fluorescence (DHE, Molecular Probes, Inc.). Tissue samples were frozen in OCT compound and 10 μm transverse sections were cut through the aortic valve using a cryostat. Sections were incubated in 0.002 mmol/L DHE and protected from light for 30 minutes at room temperature. Images were obtained using a Bio Rad MRC-1024 laser scanning confocal microscope at 4x magnification to detect fluorescence (Ex/Em: 488/585 nm). To examine specificity of the stains for superoxide, adjacent sections were incubated with polyethylene glycol superoxide dismutase (PEG-SOD). To evaluate the fluorescent intensity of positively stained areas, only positively stained cells/nuclei were thresholded using Image J software (version 1.42; National Institutes of Health, Bethesda, MD) and expressed data as the difference between the inhibited and non-inhibited fluorescent images (peg-SOD-inhibitable DHE; mean relative light units (RLU’S)/pixel).
Immunohistochemistry. Immunohistochemistry was used to detect the pro-osteogenic markers phospho-Smad1/5/8, CBFA1, and β-catenin. Immunohistochemical detection of proteins was carried out as follows: P-Smad1/5/8 (Cell Signaling Technologies, #9511, 1:300 + TSA kit), CBFA1 (Santa Cruz, #C-19, 1:50), and β-catenin (BD Transduction # 610153, 1:200). Fluorescent images were acquired using a confocal microscope. Immunofluorescence was quantified using ImageJ software as described previously\(^1, 2\). Background immunofluorescence/autofluorescence was calculated from sections taken from a subset of animals in each group which were not treated with primary antibodies. Immunofluorescent data are expressed as background-corrected fluorescent intensity.

Echocardiographic evaluation of aortic valve function. Aortic valve function was evaluated as described previously\(^1\). Briefly, mice were sedated with midazolam (0.15 mg subcutaneously); each mouse was cradled in the left lateral recumbent position while 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 180–200 two-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 frames/second, with two-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. All images were acquired by an operator blinded to the treatment groups.

Statistical analyses. All data are reported as mean ± SE. Significant differences between groups were detected using an analysis of variance, and Bonferroni-corrected T-tests were used for post hoc testing.
Figure I. Effects of reducing plasma lipids on superoxide levels in the aortic valve. Note that valvular superoxide is not altered by reduction of blood lipids. CTRL = control, HCHOL = high fat/hypercholesterolemic group, REV = “reversed” group.
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