A Novel Role of the Sympatho-Adrenergic System in Regulating Valve Calcification

Lana Osman, BSc; Adrian H. Chester, PhD; Padmini Sarathchandra, PhD; Najma Latif, PhD; Wenfang Meng, MD, PhD; Patricia M. Taylor, PhD; Magdi H. Yacoub, FRS

Background—Aortic valve calcification is a progressive process resembling ossification. Recent evidence indicates that the sympathetic nervous system plays an important role in regulating bone deposition and resorption through the β2-adrenergic receptors (β2-ARs). The aim of this study is to determine the level and pattern of expression of β2-ARs in human valve interstitial cells (ICs) and assess their influence on differentiation of the cells into an osteoblast-like phenotype.

Methods and Results—Immunohistochemical analysis demonstrated a high expression of β2-ARs, β1-ARs, β3-ARs and receptor activator of nuclear factor-κB (RANK) in calcified aortic valves. The expression of β2-ARs and β1-ARs mRNA was assessed by real-time TaqMan PCR in cultures of human aortic valve ICs. Human valve ICs treated with the selective β2-AR agonist, salmeterol, in the presence of osteogenic medium showed a significant 5-fold decrease in the alkaline phosphatase (ALP) activity in comparison to cells treated with osteogenic medium only (P<0.05). Immunocytochemical staining of the valve ICs showed a concomitant reduction in osteocalcin expression. In addition, other β2-AR agonists caused a reduction in the protein expression of bone markers including ALP, Cbfa-1, and periostin. Human valve ICs treated with norepinephrine, in the presence of osteogenic medium, did not show a significant reduction in the ALP activity.

Conclusions—These findings suggest an important role of the β2-ARs in regulating valve calcification and may identify potential therapeutic targets. (Circulation. 2007;116[suppl I]:I-282–I-287.)

Key Words: valves ■ calcification ■ β adrenergic receptors ■ differentiation

The prevalence of aortic valve calcification increases with age and other factors like diabetes, hypertension, and cardiovascular disease.¹ In the past, aortic valve calcification was considered to be a passive process involving the deposition of calcium in degenerative tissue. However, more recent studies suggest that it is caused by an active cellular process involving inflammation and bone formation.²³ We have previously shown the ability of human aortic valve ICs to differentiate toward an osteoblast-like cell phenotype,⁴ suggesting that calcification of the aortic valve resembles ossification.

In bone, there is a balance between resorption and formation to maintain skeletal homeostasis. Recent evidence indicates that the sympathetic nervous system plays a crucial role in regulating bone deposition and resorption through the β2-ARs.⁵ These β2-ARs are only expressed on osteoblasts in bone and their stimulation reduces bone formation and increases osteoclastogenesis.⁶ Activated β2-ARs couple to Gsα proteins to activate adenyl cyclase, leading to an increase in intracellular levels of cAMP (cAMP). This leads to activation of protein kinase A (PKA), which can phosphorylate various proteins: transcription factors, kinases, and cell surface receptors, including β2-ARs.⁷⁸ One important target protein is the ATF4, which is an essential factor for osteoblast development and function. Once activated it stimulates the production of receptor activator of nuclear factor-κB ligand (RANKL), which binds to RANK on osteoclast precursors to induce the formation of mature osteoclasts and subsequently the removal of bone mass.⁹¹⁰ This mechanism is fully functional in osteoblasts, however no previous studies have been carried out to investigate the effect of these sympathetic receptors on the osteogenic differentiation of human valve cells.

We hypothesize that activation of the β2-ARs may regulate calcification of the aortic valve by preventing the differentiation of valve ICs into an osteoblast-like cell phenotype. The aims of this study were to determine the level and pattern of expression of β-ARs and RANK in human aortic valve leaflets and to investigate the effect of the β2-ARs stimulation on the osteogenic differentiation of human valve ICs.

Methods

Tissue Collection and Processing

Human aortic valve leaflets were collected from explanted hearts either at time of cardiac transplantation from patients with no previous history of heart valve disease or during aortic valve
replacement surgery. Eight aortic valves were used, 3 from children (noncalcified; mean age 13.7±4.2 years) and 5 from adults (2 noncalcified and 3 calcified; mean age 62.7±7.5 years). Valve ICs were isolated from noncalcified valve leaflets by enzymatic digestion and phenotyped, as previously described. Other calcified and noncalcified leaflets were either snap frozen in liquid nitrogen, fixed in 10% formal saline, or placed in RNasey (Qiagen). The diseased valves were decalcified by neutral EDTA (12.5%) before processing.

Scanning Electron Microscopy and Energy Dispersive X-Ray Microanalysis
Calcified valves from patients with aortic stenosis were excised and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. After 2 buffer washes, specimens were dehydrated in ascending ethanol concentrations ranging from 20% to 100%. Specimens were then treated with Hexamethyl disilazane (HMDS; Sigma) 3 times for 2 minutes, air-dried, and mounted on scanning electron microscopy (SEM) stubs. The stubs were sputter-coated with gold/palladium, viewed in JEOL JSM-5500 LV SEM, and energy dispersive X-ray (EDAX) spectra were collected in the calcified and noncalcified regions of the valve leaflet and calcium/phosphorus content was quantified.

Immunohistochemistry
Sections of tissue and cells were incubated separately for 1 hour with antibodies against β2-ARs (Santa Cruz Biotechnology), β1-ARs, β3-ARs, and RANK (R&D systems), osteocalcin, osteopontin (Abcam), smooth muscle α actin (SMA) (Sigma), calponin, and CD45 (Deko). Negative controls consisted of 3% BSA in PBS. Followed by incubation with biotinylated goat anti-rabbit immunoglobulins for RANK; and biotinylated goat anti-mouse antibodies against 1-AR, and biotinylated rabbit anti-goat immunoglobulins for RANK; and biotinylated goat anti-mouse immunoglobulins for osteocalcin, SMA, calponin, and CD45 (Vector laboratories) for 1 hour. Sections were washed and incubated with Avidin-Biotin Complex ABC (Vector Laboratories). Reactivity was detected using diaminobenzidine tetrahydrochloride (DAB tablets; Sigma; 25 mg/mL) and hydrogen peroxide (0.01% W/V). Sections were counterstained with hematoxylin and viewed on Zieiss Axioskop microscope. Photomicrographs were taken using a Nikon DMX12000 camera.

RT-TaqMan PCR
The RNA was extracted from samples following standard protocols using the RNeasy system (Qiagen). The relative levels of β1-ARs and β2-ARs were quantitated using an ABI/PRISM 7700 system (PE Biosystems). PCR amplifications were detected using gene-specific internal primers that were labeled with a fluorescent group at 1 end and a quenching group at the other (“TaqMan” probe; Applied Biosystems: Catalogue number, Hs00265965-m1 [β1-ARS] and Hs00240532-s1 [β2-ARs]). Different sample sets were compared by ΔΔCt comparative analysis as described by Perkin-Elmer.

Differentiation of Valve ICs
Human valve ICs were cultured in osteogenic medium for 21 days, which contained ascorbate-2-phosphate (50 μg/mL), dexamethasone (10nmol/L), and β-glycerol phosphate (10nmol/L; all purchased from Sigma, UK) in addition to growth medium. Cells were incubated in the absence or presence of a range of β2-AR agonists (1 μmol/L): clenbuterol, fenoterol, salmeterol, iso-proterenol, and salbutamol. An endogenous nonselective β-AR agonist, norepinephrine (1 μmol/L), was also used to treat the cells. The role of β2-AR subtypes was assessed by coinoculation with either IC1118551 (300nmol/L), a selective β2-AR antagonist, or CGP20712A (300nmol/L), a selective β1-AR antagonist; all drugs were purchased from Sigma, UK.

Alkaline Phosphatase Enzyme Assay
Cells were lysed and centrifuged at 9000g for 5 minutes, and enzyme activity was assayed in the supernatant by adding 10nmol/L of Rho-nitrophenyl phosphate as a substrate in 0.1mol/L glycine buffer, pH 10.4, containing 1 mmol/L ZnCl2 and 1 mmol/L MgCl2. The quantity of Rho-nitrophenol formed was read immediately using a spectrophotometer at 405 nm and then monitored every 30 minutes. The alkaline phosphatase (ALP) activity was calculated from a standard curve. Protein content was determined using the BCA protein assay kit (Sigma, UK). The specific activity of ALP was calculated as nmol/min/mg protein.

Expression of Bone Markers by Western Blotting
Total protein homogenates (15 μg) were denatured, separated on 10% Bis-Tris gels (Invitrogen, UK), and transferred to nitrocellulose Hybond C (Amersham, UK). Nitrocellulose membranes were blocked (3% wt/vol nonfat dried milk in PBS containing 0.05% Tween-20) and then probed using primary antibodies against bone alkaline phosphatase (AbCam, UK), Cbfa-1, and periostin (R&D Systems). Visualization of the protein bands was accomplished by using enhanced chemiluminescence (ECL; Amersham, UK) and captured on Hyperfilm (Amersham, UK).

Statistical Analysis
Results are presented as mean±SEM. Statistical analyses were performed using 1-way ANOVA. All statistical analyses were performed using SigmaStat software (version 2.03). A probability value of <0.05 was considered as statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Scanning Electron Microscopy of Calcified Human Aortic Valve
Scanning electron microscopy revealed areas of calcification spanning throughout the spongiosa of the aortic valve leaflet (Figure 1A). In the noncalcified regions, EDAX spectra revealed an absence of calcium and phosphorous (Figure 1B). However, in the calcified region of the valve leaflet, elevated levels for calcium (Ca) and phosphorous (P) were observed (Figure 1C), confirming the calcification process and determining its chemical constitution.

Localization and Expression of β-ARs and RANK in Human Aortic Valves
Normal aortic valve leaflets demonstrated a weak expression of β2-ARs, β1-ARs, β3-ARs, and RANK. Less than 2% of the cells in the noncalcified regions expressed these receptors (Figure 2A.I). However, 60% of the cells adjacent to the calcified regions expressed β2-ARs, 50% of cells in the calcified region of the valve leaflet expressed β1-ARs, and 30% of cells in the calcified region expressed β3-ARs (Figure 2A.II). Finally, 20% to 30% of cells near the calcified regions expressed RANK (Figure 2A.II). Bone (osteocalcin, osteopontin) and smooth muscle markers (smooth muscle α actin, calponin) were similarly expressed in the pericalcification region, where positive β-ARs and RANK staining were observed. The expression of CD45 was negative in these regions. No staining was observed when the primary antibody was omitted, as shown in the negative control (Figure 2C).

Expression of mRNA for β-ARs in Cultured Cells
Using RT-TaqMan PCR, mRNA for the β2-ARs and β1-ARs were detected in primary cultures of human aortic valve ICs. However, when cells were treated with osteogenic medium for 21 days, the level of expression of β2-ARs mRNA was...
upregulated 3.17-fold in comparison to control (1.05±0.38 versus 3.33±0.61; *P*<0.05), but the level of mRNA expression of the β2-ARs remained unchanged (1.14±0.59 versus 1.24±0.26), as shown in Figure 2C.

**Effect of β2-ARs Agonists on Differentiation of Human Valve ICs**

ALP activity was significantly increased 13.6-fold from 0.8±0.4 nmol/min/mg protein in the control ICs to 11.7±1.2 nmol/min/mg protein in the osteogenic treated ICs. Salmeterol alone had no effect on the ALP activity and was similar to control values. When valve ICs were coincubated with salmeterol, the selective β2-ARs agonist, and osteogenic medium, the ALP activity was significantly reduced to 5.2±1.1 nmol/min/mg protein in comparison to cells treated with osteogenic medium only (*P*<0.05). The inhibitory effect of salmeterol on osteogenic medium was reversed by coinubcation of valve ICs with ICI118551. In contrast, CGP20712A enhanced further the inhibitory effect of salmeterol (Figure 3A). Immunocytochemical staining for osteocalcin expression of human valve ICs demonstrated a high expression of osteocalcin in the osteogenic treated cells in comparison to normal valve ICs (Figure 3C and 3B, respectively). This was markedly reduced when cells were coincubated with osteogenic medium and salmeterol (Figure 3D). Salmeterol alone did not increase the expression of osteocalcin in the valve ICs (Figure 3E). Similar effects on ALP activity were also seen with different β2-AR agonists (clenbuterol, feneterol, and salbutamol; Figure 4). However, the nonselective β2-AR agonist isoproterenol did not induce an

**Figure 1.** Scanning electron micrograph (SEM) and X-ray microanalysis spectra of calcified and noncalcified areas of calcified aortic valve (A). An analysis of calcium and phosphorous content using EDX spectra in noncalcified region (B) and in calcified region of the aortic valve leaflet (C).

**Figure 2.** Immunohistochemical staining of noncalcified (I) and calcified human aortic valves (II) for β1-ARs, β2-ARs, β3-ARs, and RANK (A); smooth muscle α actin (SMA), calponin, CD45, osteocalcin (Osc), osteopontin (Osp), and negative control (Neg) (B). Positive staining is indicated by arrows, and * demonstrates calcified regions (magnification ×40). Gene expression (AU) for β1-ARs and β3-ARs in cultured human valve ICs (control) and in human valve ICs treated with osteogenic medium (OST) for 21 days, by using RT-TaqMan PCR (C) (*P*<0.05 versus Control; n=3).
Discussion
In this study we have demonstrated that human aortic valve leaflets express β-ARs, and that valves affected with calcific disease demonstrate enhanced β-ARs and RANK expression in the pericalcification regions. The predominant receptor subtypes expressed in calcified valves were β2-ARs and β1-ARs with a lower expression of β3-ARs. In addition, the target protein RANK was expressed at higher levels in the calcified aortic valves as compared with the noncalcified valves. Immunohistochemical analysis showing a preponderance of smooth muscle cells in the same pericalcification regions, suggests that smooth muscle cells or myofibroblasts express β-ARs and RANK. Furthermore, the presence of bone markers in similar regions suggests that the same cells transdifferentiate into an osteoblast-like cell phenotype, as demonstrated in our in vitro experiments. Primary cultures of human aortic valve ICs expressed mRNA for the β2-ARs and β1-ARs, however when the valve ICs were treated with osteogenic medium the level of expression of the β2-ARs mRNA was tripled, whereas the β2-ARs mRNA was unchanged. Stimulation of the β2-ARs reduced the osteogenic differentiation of the valve ICs by decreasing the expression of bone markers including ALP, osteocalcin, and the osteoblast-specific transcription factors Cbfa-1 and periostin. These findings suggest an important novel protective role of the β2-ARs in regulating heart valve calcification.

The enhanced expression of β2-ARs in the degenerative calcified regions of the valve leaflets suggests that they might be involved in regulating the process of valve calcification. Indeed, β2-ARs play a key role in the regulation of bone formation via the induction of osteoclast formation. These events are known to occur continuously in bone in a balanced manner through the interaction of hormones, sympathetic nervous system, and transcription regulators. It has been shown that the antiresorptive function of leptin, a hormone secreted mainly from adipocytes, is mediated by the sympathetic nervous system (SNS) acting via the β2-ARs and causing bone resorption. Our observations showed that the expression of the β2-ARs and RANK, a marker of osteoclasts, was upregulated in the pericalcified regions of the aortic valve, suggesting that they may play an important role in regulating progression of calcification.

It has previously been reported that human cardiac valves have a distinct pattern of innervation by immunohistochemical characterization of nerves in human valve leaflets. These comprised both primary sensory and autonomic components, and the presence of a range of neuropeptides such as vasoactive intestinal peptide and substance P was demonstrated, in addition to the sympathetic and parasympathetic nerve markers. The presence of these distinct nerve terminals in the valve leaflet and the close association of varicose nerve fibers with endothelial, smooth muscle, and fibroblast cells correlate with our findings, where the β2-ARs on the aortic valve leaflets may be stimulated via neurotransmitters released by the SNS. It has been shown that valve ICs are responsive to stimulation by a number of neurotransmitters
and paracrine mediators such as 5HT, angiotensin II and dopamine agonists.\textsuperscript{18–20}

In this study we have shown that stimulation of the $\beta_2$-ARs in cultured human valve ICs by various $\beta_2$-AR agonists (salmeterol, clenbuterol, fenoterol, and salbutamol) caused a decrease in the expression of bone markers, leading to a reduction in osteogenic differentiation of the cells. This effect was reversed by blocking the $\beta_2$-ARs. Our findings are supported by a previous study which showed that stimulation of the $\beta$-receptors caused osteoclastogenesis by mediating osteoclast differentiation in mouse bone marrow cells.\textsuperscript{21} It has also been reported that blocking the $\beta_2$-ARs with propranolol enhances bone formation in osteoblast-like rat osteosarcoma cell line.\textsuperscript{22} However, when valve ICs were treated with isoproterenol or norepinephrine there was no significant decrease in the ALP activity, which maybe explained by its lack of selectivity to the $\beta_2$-ARs. In our study, when $\beta_2$-ARs were blocked using CGP20712A, isoproterenol achieved a significant inhibition of the induction of ALP activity by osteogenic medium. The effect seen with the $\beta_1$-AR agonists appear to be indicative of a proosteoblastic response. However, additional work is required to fully examine the nature of this response and the signaling mechanisms involved for both $\beta$-AR subtypes.

In conclusion, we have identified a novel role for the $\beta_2$-ARs in reducing the differentiation of valve ICs into osteoblast-like cells. However, the exact mechanism of action of these $\beta_2$-ARs in reducing osteoblast differentiation in the valve ICs needs further investigation to define the signaling pathways by which $\beta_2$-ARs cause this effect. It is known that $\beta_2$-ARs can signal through the Gi or the Gs proteins. Because it appears the $\beta_1$-ARs, that signal via Gs, may be procalcific, the role of mediators downstream to Gi activation warrants further investigation. It is hoped that our study will help in identifying new therapeutic strategies for preventing and reducing the differentiation of valve ICs into osteoblast-like cells.

\textbf{Figure 4.} ALP activity in: (A) normal valve ICs (c) treated with osteogenic medium (OST) or with $\beta_2$-AR agonists alone; (B) normal valve ICs treated with $\beta_2$-AR agonists (1\,\mu mol/L) in the presence of osteogenic medium; (C) normal valve ICs treated with $\beta_2$-AR agonists in the presence of osteogenic and with $\beta_2$-AR antagonist (ICI 118551; 300nmol/L); (D) normal valve ICs treated with $\beta_2$-AR agonists in the presence of osteogenic and $\beta_1$-AR antagonist (CGP 20712 A: 300nmol/L). Clen indicates Clenbuterol; Fen, Fenoterol; Iso, Isoprotenerol; Sal.B, Salbutamol. Values are presented as nmol per minute per mg of protein±SEM ($^*P<0.05$ vs control, #P<0.05 vs osteogenic treatment; n=3).

\textbf{Figure 5.} ALP activity in normal valve ICs (c), normal valve ICs treated with osteogenic medium (OST), normal valve treated with norepinephrine alone (NE), and valve ICs treated with norepinephrine in the presence of osteogenic medium (NE+OST) for 21 days. Values are presented as nmol per minute per mg of protein±SEM ($^*P<0.05$ vs control; n=3).

\textbf{Figure 6.} Protein expression of periostin, alkaline phosphatase (ALP), and Cbfa-1 in normal valve ICs (C), valve ICs treated with $\beta_2$-AR agonists in the presence of osteogenic medium: Salbutamol (SB), Fenterol (Fen), Salmeterol (SM), Clenbuterol (Clen); and in valve ICs treated with osteogenic medium only (OST). All samples were treated for 21 days. Expression of GAPDH in all samples was also measured (n=3).
healing aortic valve calcification, and possibly in tissue engineering heart valves resistant to calcification.

**Sources of Funding**

This study was supported by the Magdi Yacoub Institute.

**Disclosures**

None.

**References**


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doi: 10.1161/CIRCULATIONAHA.106.681072

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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