Role of Human Valve Interstitial Cells in Valve Calcification and Their Response to Atorvastatin

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Background—Calcific aortic valve stenosis is a common disease in the elderly and is characterized by progressive calcification and fibrous thickening of the valve, but the cellular and molecular mechanisms are not fully understood. We hypothesized that human valve interstitial cells (ICs) are able to differentiate into osteoblast-like cells through the influence of defined mediators and that this process can be modulated pharmacologically.

Methods and Results—To test this hypothesis, we treated primary cultures of human aortic valve ICs with osteogenic media, bone morphogenic proteins ([BMPs] BMP-2, BMP-4, and BMP-7), and tissue growth factor-β ([TGF-β]) TGF-β1 and TGF-β3) for 21 days. These mediators induced osteoblast differentiation of valve ICs by significantly increasing the activity and expression of alkaline phosphatase ([ALP] P<0.001). A cytokine protein array revealed that atorvastatin treatment (100 μmol/L) of human valve ICs caused a downregulation in levels of expression of BMP-2, BMP-6, TGF-β1, and TGF-β3 after 24 hours. In addition, human valve ICs treated with atorvastatin in the presence of osteogenic media showed a significant reduction in ALP activity in comparison to cells treated with osteogenic media only (P=0.001). This was further confirmed with immunocytochemical staining of valve ICs, whereby atorvastatin markedly reduced the expression of ALP and osteocalcin induced by osteogenic media in comparison to untreated cells.

Conclusions—These findings suggest that human valve ICs are capable of osteoblastic differentiation, by potential mediators which can be pharmacologically targeted by atorvastatin. (Circulation. 2006;114[suppl 1]:I-547–I-552.)

Key Words: inflammation ■ pharmacology ■ statins ■ valves

Aortic valve disease is the most common form of acquired valvular disease, with a prevalence of 1% to 2% in people over the age of 65 years.1 However, unlike atherosclerosis, relatively little is known about the cellular mechanisms that are involved in the onset and progression of the disease. This is reflected by the fact that aortic valve replacement remains the only viable treatment in patients with this disease.2 It has previously been thought to be a degenerative disease, but now valvular calcification is recognized as an active process that may be considered as an inflammatory condition.3 This observation has raised the possibility that it may be amenable to pharmacological treatment.

The amount of calcification in the valve is thought to influence the rate of progression of aortic stenosis4 and could be an independent risk factor for death.5 Valve calcification is characterized by the expression of bone-like lesions on the aortic surface of the valve. These lesions have been shown to express markers normally found in osteoblasts.6 Previous studies have demonstrated the involvement of osteoblast differentiation in vascular smooth muscle cells and vascular calcification.7 However, the exact molecular mechanisms responsible are not understood. Bone morphogenetic protein (BMP)-2 and BMP-4 are known to be potent osteogenic morphogens and have been shown to be present in ossified valves.8 Transforming growth factor-β (TGF-β1) is another important mediator that may contribute to the calcification process, because it has “bone-inducing” properties. Previous immunohistochemical studies demonstrated the presence of higher levels of TGF-β1 in calcified human aortic valve cusps in comparison to noncalcified cusps.9 The origin and site of action of these mediators in the valve is not known. Human valve interstitial cells (ICs) are a heterogeneous group of cells that have unique characteristics, including patterns of gene expression and the ability to perform vital functions, such as secretion of a variety of biologically active molecules.10 It has been shown in a number of clinical studies that patients treated with the cholesterol-lowering drugs, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), seemed to have a reduction in the progression of aortic valve stenosis, as defined by echocardiography.11,12 However, the cellular mechanism and the site of action of statins in valve cells remain unclear. The aim of this study was to investigate the possible role of human valve ICs in calcification and the...
mechanism of action of atorvastatin on the calcification potential of human valve ICs.

Methods

Human Aortic Valve Cell Isolation
Human valves were excised from recipient hearts at the time of transplantation from patients with no previous history of heart valve disease. Six aortic valves (mean age: 62.7±7.5 years) were used. Valve ICs were immediately isolated from explanted human valve leaflets by enzymatic digestion as described previously. Valve ICs were used between passages 4 and 6, and cells were phenotypically characterized by methods described previously.

Osteoblast Differentiation
Primary cultures of human valve ICs were grown in normal media as a negative control and treated with osteogenic media for 21 days. This osteogenic media contained DMEM supplemented with 4 mmol/L L-glutamine, 100 U/mL penicillin, 100 μmol/L streptomycin, 10% FCS, 10 mmol/L dexamethasone, and 10 mmol/L β-glycerol phosphate (all purchased from Sigma).

Atorvastatin Treatment
Atorvastatin, 7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1 H-pyrrol-1-yl]-3,5-dihydroxy-heptanoic acid (calcium salt; Pfizer), was dissolved in 100% DMSO and was further diluted with fresh appropriate media to prepare different concentrations (1 to 100 μmol/L). The volume of DMSO never exceeded 0.1% (v/v) in all of the prepared concentrations. Confluent cultures of human valve ICs were treated with atorvastatin at the appropriate concentration prepared and were incubated at 37°C. Control cells were grown in normal media but in the absence of atorvastatin.

Alkaline Phosphatase Enzyme Assay
Primary cultures of human aortic valve ICs were seeded into a 24-well plate and were grown in osteogenic media for 21 days with and without atorvastatin (100 μmol/L) to induce osteogenesis. Valve ICs were also plated onto 24-well plates and treated for 21 days with recombinant BMP-2, BMP-4, and BMP-7 proteins (100 ng/mL; R&D Systems); recombinant TGF-β1 and TGF-β3 (10 ng/mL; R&D Systems); osteogenic media; and control media. Samples were centrifuged at 10,000 rpm for 5 minutes, and enzyme activity was assayed in the supernatant by adding 10 mmol/L of p-nitrophenyl phosphate as a substrate in 0.1 mol/L glycine buffer (pH 10.4), containing 1 mmol/L ZnCl2 and 1 mmol/L MgCl2. The quantity of p-nitrophenol formed, which is a yellow color, 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. In the supernatant, protein content was also measured using the BCA protein assay kit (Sigma) using bovine albumin as a standard sample. Total protein homogenates (15 μg) were denatured, separated on 10% Bis-Tris gels (Invitrogen), and transferred to nitrocellulose Hybond C (Amersham). Nitrocellulose membranes were blocked (3% wt/vol nonfat dried milk in PBS containing 0.05% Tween 20) and then probed using primary antibodies against human ALP (Sigma). Membranes were washed and then incubated with horseradish peroxidase–conjugated secondary antibody (Dako) for 1 hour. Visualization of the protein bands was captured on Hyperfilm (Amersham).

Expression of ALP by Western Blotting
Cultured human valve ICs were solubilized and homogenized in 1% SDS containing protease inhibitors (Roche). Protein content was determined using the BCA protein assay kit (Sigma) using bovine albumin as a standard sample. Total protein homogenates (15 μg) were denatured, separated on 10% Bis-Tris gels (Invitrogen), and transferred to nitrocellulose Hybond C (Amersham). Nitrocellulose membranes were blocked (3% wt/vol nonfat dried milk in PBS containing 0.05% Tween 20) and then probed using primary antibodies against human ALP (Sigma). Membranes were washed and then incubated with horseradish peroxidase–conjugated secondary antibody (Dako) for 1 hour. Visualization of the protein bands was captured on Hyperfilm (Amersham).

Capacity of Human Valve ICs to Secrete Growth Factors
Cultured human aortic valve ICs were grown in a 24-well plate with and without atorvastatin (100 μmol/L). After 24 hours of atorvastatin treatment, media was collected and immediately frozen. To determine the expression of cytokines in the media, RayBio Human Cytokine Array C series 1000 membranes VI and VII were used (RayBio). Signal intensities were quantitated by densitometry using Quantity One (Biorad).

Statistical Analysis
Results are presented as mean ± SEM. Statistical analyses were performed using 1-way ANOVA. All of the statistical analyses were performed using GraphPad Prism TM software (version 3.0). A P value of <0.05 was considered statistically significant.

We had full access to the data and take full responsibility for its integrity. We have read and agree to the article as written.

Results

Effect of Osteogenic Media, BMPs, and TGF-β on ALP Activity in Human Valve ICs
Treatment of valve ICs with BMP-2, BMP-4, or BMP-7 (100 μmol/L); TGF-β1 or TGF-β3 (100 μmol/L); or osteogenic media for 21 days all caused a significant increase in ALP activity in comparison with untreated cells (P<0.001), as shown in (Figure 1). Valve ICs treated with osteogenic...
media significantly increased ALP activity from 1.4 ± 1.0 nmol/min per milligram of protein in control cells to 12.4 ± 1.4 nmol/min per milligram of protein. In addition, BMP-4 treatment increased the ALP activity to 7.8 ± 0.6 nmol/min per milligram of protein, and a similar increase in ALP activity was observed in valve ICs treated with BMP-2 and BMP-7. The ALP activity was also increased in valve ICs treated with TGF-β1 to 8.7 ± 2.1 nmol/min per milligram of protein and with TGF-β3 to 6.6 ± 0.3 nmol/min per milligram of protein in comparison with control.

Effect of Osteogenic Media, BMPs, and TGF-β on ALP Expression in Human Valve ICs
Immunocytochemical staining of human valve ICs (Figure 2) treated with BMP-2, BMP-4, BMP-7, TGF-β1, TGF-β3, and osteogenic media showed a high localization of ALP expression in cells treated with osteogenic media in comparison with control cells. A similar observation was found in valve ICs treated with BMP-2, BMP-4, BMP-7, TGF-β1, or TGF-β3, whereby a high expression of ALP protein was present in those treated cells compared with control (similar finding to that in osteogenic treated cells). All of the mediators increased ALP staining in valve ICs after 21 days of treatment.

Capacity of Human Valve ICs to Secret Cytokines and Influence of Atorvastatin
Human valve ICs demonstrated to be the source of cytokine secretion and a target for cytokine action. Atorvastatin downregulated both the secretion and the effect of these cytokines, whereby at 100 µmol/L concentration, it decreased the signal intensities in numerous cytokines and growth factors after 24 hours of treatment (Figure 3C) compared with normal media (Figure 3B). The expression of cytokines was also determined in media only (negative control; Figure 3A). Atorvastatin significantly downregulated the expression of platelet-derived growth factor-BB, membrane cofactor protein (MCP)-4, TGF-β1, TGF-β3, tumor necrosis factor (TNF)-α, BMP-4, and insulin-like growth factor binding protein-4 (P < 0.001), as demonstrated in Figure 3D, after 24 hours of treatment. In addition, levels of expression of other cytokines in the array relevant to the inflammatory response, such as interleukin (IL)-10, IL-13, MCP-1, IL-16, MCP-3, IL-1α, IL-1β, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-2, TNF-β, BMP-6, IL-3, IL-6, and IL-7, showed no significant difference with atorvastatin treatment.

Effect of Atorvastatin on ALP Activity and Protein Expression in Human Valve ICs
Atorvastatin was able to significantly inhibit the induction of ALP activity at a 100-µmol/L concentration by 14-fold, in comparison with valve ICs grown in osteogenic media in the absence of atorvastatin (Figure 4A). ALP activity was reduced from (15.3 ± 1.5 nmol/min per milligram of protein) in the absence of atorvastatin to (1.2 ± 0.4 nmol/min per milligram of protein) in the presence of atorvastatin. Minimal ALP activity of (0.2 ± 0.15 nmol/min per milligram of protein) was observed in the control cells. This was further confirmed by carrying out Western blotting on the same treated valve ICs (Figure 4B). Levels of ALP (130 kDa) were highly expressed in the osteogenic-treated cells in comparison with the control cells, which had very low expression of ALP protein. This high production of ALP levels was markedly reduced in the valve ICs treated with atorvastatin (100 µmol/L) after 21 days as shown in Figure 1B.

Effect of Atorvastatin on Expression of ALP and Osteocalcin in Human Valve ICs
Immunocytochemical staining demonstrated that atorvastatin (100 µmol/L) caused a decrease in the expression of both ALP and osteocalcin induced by osteogenic media in the human valve ICs (Figure 5E and 5F) compared with cells incubated in osteogenic media only (Figure 5C and 5D). Both ALP and osteocalcin were expressed in 90% to 95% of the valve ICs that were incubated with osteogenic media only. The expression was reduced to 10% by incubating the cells with atorvastatin. Control valve ICs showed no expression of ALP or osteocalcin (Figure 5A and 5B).
Discussion

In this study, we have demonstrated the capacity of human aortic valve ICs to express osteoblast cell markers after treatment with osteogenic media, BMP-2, BMP-4, BMP-7, TGF-β1, and TGF-β3. These mediators enhanced the activity and expression of ALP after 21 days. We also demonstrated that atorvastatin treatment in human valve ICs downregulated the expression of a number of proinflammatory cytokines, including platelet-derived growth factor-BB, MCP-4, TGF-β1, TGF-β3, TNF-α, BMP-4, and insulin-like growth factor binding protein–4. In addition, human aortic valve ICs treated with osteogenic media in the presence of atorvastatin showed a marked reduction in the ALP activity and in the expression of ALP and osteocalcin. This effect of atorvastatin was observed at the 100-μmol/L concentration. These findings suggest that human valve ICs play a major role in the process of osteogenesis in response to potential mediators, which are prevented by atorvastatin treatment.

ALP expression and activity were used as markers to measure osteoblast differentiation; other markers of osteo-
blast formation include osteopontin, bone sialoprotein, osteocalcin, osteonectin, and the osteoblast-specific transcription factor Cbfa1.6 Although these additional markers were not examined in this study, ALP plays a key role in the calcification process in valve cells, because it has been shown that inhibition of its activity by Levamisol prevents the formation of calcified nodules and osteonectin expression in human valve ICs grown in an osteogenic media.14 In this study, we were able to detect a significant rise in the activity of ALP in response to osteogenic media that was inhibited by statins. Our study also demonstrated that expression of members of the TGF-β and BMP families was downregulated by atorvastatin and that BMP-2, BMP-4, BMP-7, TGF-β1, and TGF-β3 were able to induce an increase in the expression of ALP in human valve ICs. The effect of BMPs and TGF-β in the phenotypic transition of normal valve ICs to become osteoblast-like cells provides further evidence that they may play a role in the progression of aortic stenosis by enhancement of the calcification process. Previous studies have demonstrated the presence of the cytokine TGF-β1 in diseased cardiac valves.9 It is believed that the accumulation of TGF-β1 in the extracellular matrix of the valve tissue is because of the ongoing endothelial injury associated with platelet and inflammatory cell infiltration. TGF-β1 may contribute to the calcification process by initiating apoptosis associated with mineralization of aortic valve ICs.9

We have shown that human valve ICs were capable of secreting proinflammatory cytokines, which could be important in the calcification process. However, atorvastatin downregulated the expression of these proinflammatory cytokines and reduced the expression of osteoblast-like cell phenotypes in human valve ICs. These pleiotropic effects of statins are believed to be mediated by their ability to block the production of isoprenoid intermediates, such as geranyl pyrophosphate and farnesyl pyrophosphate, which are important in the activation of small GTP-binding proteins, including Rho, Ras, and Rac, through their isoprenylation and may play a role in the regulation of the cell cycle.15 Evidence suggests that inhibition of Rho isoprenylation may mediate many of the cholesterol-independent effects of statins, not only in vascular wall cells but also in leukocytes, bone, and perhaps in valves cells as well.16

In contrast to our findings, previous studies suggested that statins can induce osteoblastic differentiation in bone cells, and can also reduce the risk of fractures.17,18 These findings, together with our results, highlight a paradoxical effect of
statins between valve and bone cells, suggesting that statins may achieve discordant effect in different tissue, through differential actions on specific cell phenotypes.

Our in vitro studies need to be validated in different tissues in in vivo models. The in vitro observations relating to the action of statins on bone formation failed to translate into clinical benefits in patients with osteoporosis, highlighting the potential shortcoming of these studies. However, in vitro studies do have the advantage of being able to elucidate cell-specific mechanisms that could be useful. Our in vitro study provides possible mechanisms of action of statins in reducing the progression of aortic valve calcification. This further supports recent clinical studies where rosuvastatin has been shown to reduce the progression of aortic valve disease and improve inflammatory markers in patients with asymptomatic aortic stenosis. In contrast, a recent clinical trial revealed no significant difference in the rate of progression of aortic stenosis between control patients and those treated with statins. However, it is important to note that the statin-treated group already had a pre-existing calcific aortic stenosis, which could render the disease progress irreversible. The lack of effect of statins in this trial suggests that early initiation of treatment might be required to delay the onset and the progression of aortic valve calcification.

The present study strongly suggest that human valve ICs may play an important role in valve calcification by secretory proinflammatory cytokines and changing their phenotype to an osteoblast-like cell in response to specific mediators. Both of these processes are counteracted by statins. It is hoped that these findings will help in further understanding the mechanisms of the disease, as well as developing novel therapies.

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**Disclosures**

None.

**References**

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