A Novel Role of Extracellular Nucleotides in Valve Calcification
A Potential Target for Atorvastatin

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Background—Calcific aortic valve disease is a common condition and is associated with inflammatory changes and expression of osteoblast-like cell phenotypes, but the cellular mechanisms are unclear. Recent studies identified extracellular ATP and P2Y receptor cascade as important regulators of bone remodeling, whereas its breakdown product, adenosine, is known to have anti-inflammatory properties. We hypothesize that extracellular ATP and adenosine have important roles in regulating osteoblast differentiation in human valve interstitial cells, and that this can be a potential target for therapy.

Method and Results—Primary cultures of human valve interstitial cells (ICs) treated for 21 days with osteogenic media, ATP, and ATP-γ-S (a stable agonist of the P2Y receptor) revealed a significant increase in alkaline phosphatase (ALP) (an osteoblast marker) activity and expression as measured using spectrophotometric assay and immunocytochemistry staining. Valve ICs treated with adenosine alone did not cause an increase in ALP activity; however, adenosine treatment decreased the ALP activity and expression induced by osteogenic media after 21 days of incubation. In addition, atorvastatin inhibited the activity of ALP induced by ATP in human valve ICs, and enzyme studies revealed that atorvastatin upregulated the breakdown of extracellular ATP into adenosine in human valve ICs after 24-hour treatment.

Conclusion—These findings identify a novel role for extracellular nucleotides in inducing osteoblast differentiation in human valve ICs in vitro and provide a potential therapeutic target for preventing the disease progression. (Circulation. 2006;114[suppl I]:I-566–I-572.)

Key Words: adenosine ■ enzymes ■ statins ■ valves

Aortic valve disease is a common problem among the elderly and is characterized by calcification of the leaflets.1 It has been shown that valve calcification is associated with inflammatory changes and expression of osteoblast-like cell phenotype.2 However, the cellular mechanism of the disease process remains unclear. Extracellular ATP (adenosine 5'-triphosphate) is known to be involved in osteoblast formation. It is one of the most important extracellular regulatory molecules in the skeleton for bone remodeling.3 Several studies have shown that bone cells release nucleotides into the extracellular environment in response to mechanical or physiological stimuli to provide highly localized and transient signals that regulate bone formation and bone resorption.4

Extracellular ATP is known to bind as a signaling molecule to the purinergic G-protein-coupled P2Y receptors triggering pathways that can mediate inflammation.5 However, adenosine, which is the end product of ATP breakdown, binds to P1 purinergic receptor, triggering anti-inflammatory mechanisms.6 The opposite effects of extracellular ATP and adenosine raise the possibility that they might play a differential role in the development of calcified valves by regulating the transformation of normal valve cells to osteoblast-like cells.

The precise site of action of these mediators in heart valve is not known. Human valve interstitial cells (ICs) spell 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,7 and may have the capacity to participate in inflammatory reactions. Previous clinical studies have demonstrated the beneficial effects of 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (Statins) in delaying the disease progression of aortic stenosis, possibly through their lipid-lowering and anti-inflammatory actions.8 However, the exact mechanism of action of statins in valve calcification is unknown. We previously studied the functional effect of atorvastatin on...
the degradation process of extracellular nucleotides in human endothelial cells (human umbilical vein endothelial cells and human microvascular endothelial cells) and showed that atorvastatin enhanced the breakdown of extracellular ATP and the formation of adenosine. However, no studies have been performed to investigate the effect of atorvastatin on extracellular nucleotides in human valve ICs.

The purpose of this study was to examine whether extracellular ATP is able to induce the differentiation of human valve ICs to osteoblast-like cells and assess whether adenosine has any inhibitory effects on this process. We also aimed to investigate whether the mechanism of action of atorvastatin in valve disease may involve in its effect on the breakdown of extracellular nucleotides.

**Methods**

**Human Aortic Valve Cell Isolation and Culture**

Cells were cultured from cusp tissue excised from recipient hearts at the time of transplantation and placed immediately into DMEM. All patients had no previous history of heart valve disease and were undergoing transplantation for either ischemic heart disease or cardiomyopathy. 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 and cultured as previously described.9 Before the experiments, the phenotype of the cells were assessed using a panel of mouse monoclonal antibodies. Cultures that stained positively with antibodies against smooth muscle α-actin (Sigma, Poole, UK), fibroblast surface antigen and prolyl 4-hydroxylase (Dako, Cambridge, UK) and negatively for CD31 and von Willebrand factor (Dako) were judged to be valve interstitial cells as previously reported.9 These cells have previously been shown to retain their phenotype and functional characteristics after response in culture.

**Osteoblast Differentiation**

Primary cultures of human valve ICs were grown in normal media as a negative control and were treated for 21 days with osteogenic media containing DMEM supplemented with 4 mmol/L L-glutamine, 100 U/mL penicillin, 100 μmol/L streptomycin, and 10% fetal calf serum; and ascorbate-2-phosphate (50 μmol/L), 2-Methylthio-ADP (2-Methylthio-(3-thiotriphosphate), -S (Adenosine 5'-nucleotidase (Sigma, Poole, UK), was dissolved in 100% DMSO and was further diluted with fresh appropriate media to prepare different concentrations (1 to 100 μmol/L). 2-Methylthio-ADP (2-Methylthio-ADP Trisodium salt) (10 μmol/L), which are 2 stable agonists to the P2Y receptor (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, UK) was dissolved in 100% DMSO and was further diluted with fresh appropriate media to prepare different concentrations (1 to 100 μmol/L).

**Alkaline Phosphatase Enzyme Activity**

Cells were washed and lysed with lysis buffer (0.2% NP-40, 1 mmol/L MgCl₂). Lysates were centrifuged and enzyme activity was assayed in the supernatant by adding it to 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, and incubated at 30°C. The quantity of p-nitrophenol product formed was recorded by monitoring absorbance at 405 nm at regular time intervals. Protein content was determined using the bicinchoninic acid protein assay kit (Sigma, UK). The specific activity of alkaline phosphatase (ALP) was expressed as nmol/min per milligram of protein.

**Expression and Localization of ALP by Immunocytochemistry**

Before immunostaining, cells were washed, then fixed and permeabilized with ice-cold acetone for 5 minutes. Cells were blocked in 2% bovine serum albumin (BSA) and incubated with anti-human alkaline phosphatase antibody (Sigma, UK) for 1 hour at room temperature. Cells were then washed with phosphate-buffered saline (PBS) and incubated for another 1 hour with the secondary goat anti-mouse antibody horse-radish peroxidase–conjugate (Sigma, UK) and DAPI (nuclear stain) (Sigma, UK). Cells were washed in PBS and mounted on glass slides in Permafluor aqueous mounting fluid (Coulter).

**Capacity of Valve ICs to Degrade Extracellular ATP**

Immediately after 24-hour treatment of human valve ICs with atorvastatin (1 to 100 μmol/L), cultured media was removed from the 24-well plate and cells were washed. Substrate (10 μmol/L of ATP containing 10 μmol/L of AA) was added to the cell media and concentrations of ATP, ADP, AMP, and adenosine were analyzed and measured in the media (nmol/min/mg protein) using the high performance liquid chromatography method.10

**Assays of Extracellular Nucleotide Degradation Enzymes**

Enzyme activities of ATPase, ADPase, and ecto-5'-nucleotidase (eN) were performed for 5 minutes and terminated by the addition of 20 μmol/L of 1.3 mol/L HClO₄. Samples were then neutralized with 3 mol/L KPO₄, centrifuged, and were finally separated by HPLC. Lactate dehydrogenase activity was measured in the cell homogenates and media using standard spectrophotometric procedure. Total protein content of cell homogenates was assayed using the bicinchoninic acid protein assay kit (Sigma, UK).

**Statistical Analysis**

Results are presented as mean ± SEM. Statistical analyses were performed using 1-way ANOVA or 2-way ANOVA for repeated measures when appropriate. P < 0.05 was considered as statistically significant.

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

**Results**

**Effect of Extracellular ATP on ALP Activity in Human Valve ICs**

Treatment of human valve ICs with osteogenic media, ATP (100 μmol/L), and ATP-γ-S (10 μmol/L) caused a significant increase in ALP activity compared with control (untreated cells) after 21 days (P < 0.001, n = 3) as demonstrated in Figure 1. However, treatment of valve ICs with 2-Methylthio-ADP (10 μmol/L) did not cause a significant increase in ALP activity after 21 days. Valves ICs treated with osteogenic media significantly increased ALP activity from 3.1 ± 1.0 nmol/min per milligram of protein in control cells to 14.4 ± 1.4 nmol/min per milligram of protein. In addition, both ATP and ATP-γ-S increased the ALP activity to 12.1 ± 1.9 nmol/min per milligram and 11.1 ± 1.5 nmol/min per milligram of protein, respectively. This increase in ALP only reached 5.9 ± 1.2 nmol/min per milligram of protein when the valve ICs were treated with 2-Methylthio-ADP.
Effect of Extracellular Adenosine on ALP Activity in Human Valve ICs

Treatment of human valve ICs with adenosine (30 μmol/L) in the presence of inhibitors of its metabolism (deoxycoformycin and AA) resulted in a significant reduction in the ALP activity induced by osteogenic media. Maximum ALP activity was observed in cells treated with osteogenic media at 10.4±0.8 nmol/min per milligram of protein in comparison to 2.3±0.7 nmol/min per milligram of protein in the untreated cells (control) after 21 days of treatment. The effect of osteogenic media was reduced to 3.4±0.6 nmol/min per milligram of protein in the presence of adenosine. Valve ICs treated with adenosine alone did not show any increase compared with control (2.5±0.9 nmol/min per milligram of protein) (Figure 2).

Effect of Extracellular Nucleotides on the Expression of ALP in Human Valve ICs

Immunocytochemical staining of human valve ICs (Figure 3) treated with osteogenic media (Figure 3B) revealed much higher ALP expression than in untreated cells (Figure 3A). A similar observation was found in valve ICs treated with ATP (Figure 3C) and ATP-γ-S (Figure 3D), whereby a high expression of ALP protein was present in comparison to control. However, less expression of ALP was observed in valve ICs treated with 2-Methylthio-ADP (10 μmol/L) (Figure 3E) and adenosine (30 μmol/L) combined with osteogenic media (Figure 3F) for 21 days. There was no expression of ALP in untreated cells (control).

Effect of Atorvastatin on ALP Activity Induced by ATP in Human Valve ICs

Treatment of human valve ICs with ATP (100 μmol/L) caused a significant increase in ALP activity from 2.1±0.6 nmol/min per milligram of protein in the untreated cells to 11.4±1.4 nmol/min per milligram of protein (Figure 4). This increase was significantly reduced in these treated cells in response to atorvastatin (100 μmol/L) to 4.5±1.9 nmol/min per milligram of protein (P<0.001, n=3).

Effect of Atorvastatin on the Extracellular ATP Breakdown in Human Valve ICs

Treatment of cultures of human valve ICs with atorvastatin (1 to 100 μmol/L) for 24 hour caused a significant increase in the rate of ATP conversion to its metabolites ADP, AMP and adenosine (Figure 5). Atorvastatin induced changes that resulted in accelerated breakdown of extracellular ATP and in the formation of the anti-inflammatory adenosine in a dose-dependent manner. The rate of extracellular ATP breakdown in valve ICs pretreated with the highest atorvastatin concentration (100 μmol/L) was significantly increased from 4.2±1.1 nmol/mg protein in the nontreated cells to 13.5±2.8 nmol/mg protein. This was followed by a marked increase in adenosine formation when cells were pre-incubated with 100 μmol/L atorvastatin in comparison to control, from 21.6±1.5 nmol/mg protein to 34.2±3.4 nmol/mg protein.

Effect of Atorvastatin on Extracellular Nucleotide Enzymes in Human Valve ICs

Treatment of human valve ICs with atorvastatin (1 to 100 μmol/L) for 24 hours significantly increased the activities of ATPase, ADPase, and eN extracellular enzymes in a dose-dependent manner, as shown in Figure 6. These enzyme activities were determined in the cell homogenates after treatments. Valve ICs treated with 100 μmol/L of atorvastatin had the highest activity of ATPase at 17.8±0.6 nmol/min per milligram of protein, in comparison with untreated cells (10.2±0.7 nmol/min per milligram of protein, n=8). This increase in ATPase activity was also observed with the 1 μmol/L and 10 μmol/L atorvastatin treated valve ICs (Figure 6A). ADPase activity in cultured valve IC homogenates was also increased by 2-fold in the 100 μmol/L atorvastatin treated cells compared with control (2.7±0.2 nmol/min/mg protein) (Figure 6B). Treatment of valve ICs with atorva-
statin also induced a significant increase in the eN activity after 24 hours, whereby 100 μmol/L of atorvastatin increased eN activity from 81.3±13.09 nmol/min per milligram of protein in the untreated cells to 152.3±11.98 nmol/min per milligram of protein after 24 hours (P=0.001 versus control, n = 8), as demonstrated in Figure 6C. No change in lactate dehydrogenase activity was observed in any conditions, as shown in Figure 6D.

**Discussion**

In this study we have demonstrated the effect of extracellular ATP and adenosine on the expression of the osteoblast cell marker, ALP, in human valve ICs. Treatment of valve ICs with extracellular ATP and agonists of the P2Y receptors enhanced the activity and expression of ALP, whereas adenosine treatment reduced the activity and expression of ALP induced by osteogenic media. In addition, we demonstrated that atorvastatin reduces ALP activity induced by ATP. Atorvastatin also upregulated the breakdown of extracellular ATP into adenosine. These findings demonstrate a novel role for extracellular nucleotides in regulating the expression of osteoblast cell markers, which is a potential therapeutic target for valve calcification disease.

Extracellular ATP is known to exert a broad range of regulatory effects, mediated by its cell surface, G-protein-coupled P2Y receptors. Activation of these receptors triggers...
phospholipase C pathway and results in elevation of intracellular calcium [Ca$^{2+}$]. This, in turn, leads to transcription factor activation and upregulation of RANK/RANKL and Cbfa1 expression.$^{11}$ RANK/RANKL and Cbfa-1 have both been shown to be expressed in animal models of aortic stenosis.$^{12}$

In this study we were able to demonstrate a significant increase in the activity of ALP in response to ATP, which was inhibited by statins. We have measured ALP activity as a marker for osteoblast differentiation. Osteoblast cells have also been shown to express a number of other markers, including osteopontin, bone sialoprotein, osteocalcin, osteonectin, as well as osteoblast-specific transcription factor Cbfa1.$^{13}$ Whereas ALP is expressed in other cell types, this enzyme plays a key role in the calcification process in valve cells. It has been shown that inhibition of ALP activity prevents the formation of calcified nodules and osteonectin expression in human valve ICs grown in an osteogenic media.$^{14}$

In contrast, adenosine exhibited a protective action on valve ICs by inhibiting the effects of osteogenic media. Physiological actions of extracellular adenosine are often opposite to the effects of extracellular ATP or ADP. It is known that increased activity of 5'-nucleotidases involved in formation of adenosine has protective effects on ischemia-induced endothelial injury and endothelial barrier function. It supports vasodilation and suppress leukocyte adhesion to the vascular endothelium.$^{15}$ These anti-inflammatory effects are mediated by Gs-protein coupled adenosine (P1) receptors. The concentration of ATP in the valve interstitial fluid has never been measured, but it probably reflects that in plasma, which is sufficient to trigger P2Y receptors. Factors such as inflammation or platelet deposition which are common in valve calcification could lead to elevation of ATP concentration in valve interstitium.

We were able to demonstrate that atorvastatin treatment leads to increased activity of the enzymes of the extracellular nucleotide degradation in human valve ICs. Ecto-ATPase/CD39 is responsible for the breakdown of ATP to ADP and then into AMP while eN converts AMP produced by ecto-ATPase into adenosine.$^{16}$ This is consistent with previous studies reporting a 6-fold increase in CD39 mRNA after exposure of HUVECs to atorvastatin.$^{17}$ A consequence of increasing adenosine production by atorvastatin is a reciprocal reduction in levels of ATP. Thus, atorvastatin treatment will shift metabolism of extracellular nucleotides toward formation of adenosine and will result in a reduction of extracellular ATP available to trigger P2Y receptors. This

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**Figure 5.** Extracellular ATP breakdown into its metabolites by atorvastatin treatment in human valve ICs. Valve ICs treated with 10 μmol/L of ATP in the absence (black circle) or in the presence of 1 μmol/L atorvastatin (white circle), 10 μmol/L atorvastatin (filled triangle), or 100 μmol/L atorvastatin (white triangle) for 24 hours. Samples were analyzed for ATP breakdown (A) and formation of ADP (B), AMP (C), and adenosine (D). Values are expressed as the mean concentration in nmol/mg of protein±SEM and experiment was performed in quadruplicate. *P<0.001; ‡P<0.01 vs control (black circle).
The regulatory effect of atorvastatin may help to attenuate the differentiation of normal valve ICs into an osteoblast-like cell phenotype. The concentrations of atorvastatin used in the current study were higher than plasma concentration during clinical application of the drug. However, it is difficult to directly equate in vivo and in vitro concentrations, because plasma concentration could be much lower than concentration of the drug in the tissues or inside the cells, particularly for lipophilic drugs such as Atorvastatin.

This study supports recent clinical observations where rosuvastatin has been shown to reduce the progression of aortic valve disease and improve inflammatory markers in patients with asymptomatic aortic stenosis. However, the SALITRE trial recently reported a lack of effect of atorvastatin treatment on the rate of progression of aortic stenosis in patients with the disease. The discrepancy between these 2 clinical trials may be caused by the fact that the SALITRE trial was performed on patients with pre-existing calcific aortic stenosis. It has been suggested that advanced calcific aortic stenosis is an irreversible process. The lack of effect of statins in the SALITRE trial suggests that early initiation of treatment is required to reveal a clinical benefit.

This in vitro study highlights a potential novel role for extracellular ATP in enhancing the expression of osteoblast cell markers such as ALP in human valve ICs and demonstrates the inhibitory effects of adenosine on this process. Using atorvastatin, we were able to target these effects, possibly by upregulating the breakdown of extracellular ATP and increasing the formation of adenosine in human valve ICs. The precise role of extracellular nucleotides within the valve, and mechanisms that may alter their rate of degradation or the expression of purinergic receptors requires further investigation. These studies will enhance our understanding of the mechanisms of valve calcification and identify new therapeutic target for heart valve disease.

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Disclosures

None.

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