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PRE-CLINICAL RESEARCH

Pro-Osteogenic Phenotype of Human Aortic Valve Interstitial Cells Is Associated With Higher Levels of Toll-Like Receptors 2 and 4 and Enhanced Expression of Bone Morphogenetic Protein 2

Xiaoping Yang, PHD, David A. Fullerton, MD, Xin Su, MD, Lihua Ao, BS, Joseph C. Cleveland, JR, MD, Xianzhong Meng, MD, PHD

Denver, Colorado

Objectives Our aim was to determine whether aortic valve interstitial cells (AVICs) and pulmonary valve interstitial cells (PVICs) differ in expression of toll-like receptor (TLR)2 and TLR4, response to TLR agonists, and osteogenic phenotypic changes.

Background Calcific stenosis occurs frequently in aortic valves but rarely in pulmonary valves. Studies have implicated AVICs in the inflammation associated with calcification and progression to stenosis. We previously reported that human AVICs express functional TLR2 and TLR4 and that stimulation of these receptors induces pro-osteogenic factor expression.

Methods Human aortic and pulmonary valve leaflets from the same heart were collected and interstitial cells isolated.

Results Aortic valves express more TLR2 and TLR4, in both tissue and isolated interstitial cells, than pulmonary valves. After stimulation with TLR2 and TLR4 agonists, AVICs express higher levels of pro-inflammatory and pro-osteogenic mediators (bone morphogenetic protein [BMP]-2, runt-related transcription factor 2) and greater osteogenic phenotypic changes (alkaline phosphatase [ALP] activity, calcified nodule formation) than PVICs. Silencing TLR2 and TLR4 in AVICs reduced BMP-2 expression and ALP activity to PVIC levels. ALP activity in AVICs induced by TLR2 and TLR4 agonists was abolished by BMP antagonism with Noggin and mimicked by stimulation with recombinant BMP-2. AVICs isolated from stenotic valves had greater expression of TLR2 and TLR4 and a greater BMP-2 response than AVICs from normal valves.

Conclusions Greater expression of TLR2 and TLR4 and greater pro-inflammatory and pro-osteogenic responses to TLR2 and TLR4 agonists in AVICs than PVICs are associated with osteogenic phenotypic changes. These innate immune receptors may play a critical role in aortic valve calcification and stenosis. (J Am Coll Cardiol 2009;53:491–500) © 2009 by the American College of Cardiology Foundation

Aortic valve calcification, which affects 27% of the U.S. population older than age 60 years, often progresses to calcific aortic stenosis (1). Despite its poor prognosis, the only therapy currently available for severe, symptomatic aortic stenosis is valve replacement. Several mechanisms for heart valve calcification have been proposed, including matrix remodeling, lipid accumulation in the valve, and dysfunction of the renin-angiotensin system (2). Clinical trials of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are underway, but none has yet shown a significant benefit (3).

Several studies have demonstrated an association between inflammation and aortic valve calcification (4). Macrophages, T-lymphocytes, and pro-inflammatory mediators such as interleukin-1, transforming growth factor-beta, and tumor necrosis factor-alpha have all been found in calcified human heart valves (2). But it is not yet clear that inflammation causes valve calcification or what molecular mechanisms may be involved. We recently found that human aortic valve interstitial cells (AVICs) express functional toll-like receptor (TLR)2 and TLR4 (5), important mediators of the innate immune response and inflammation.

Valve interstitial cells (VICs), the major cellular components of heart valve leaflets, have the dual ability to secrete

From the Division of Cardiothoracic Surgery, Department of Surgery, University of Colorado Denver, Denver, Colorado. Supported by American Heart Association grant 0850148Z and the Academic Enrichment Fund of University of Colorado Denver.

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Abbreviations and Acronyms

ALP = alkaline phosphatase
AVIC = aortic valve interstitial cell
BMP = bone morphogenetic protein
PVIC = pulmonary valve interstitial cell
Runx2 = runt-related transcription factor 2
TLR = toll-like receptor
VIC = valve interstitial cell

matrix components and to maintain valvular contractile function (6). AVICs stimulated with TLR agonists up-regulate bone morphogenetic protein (BMP)-2 and runt-related transcription factor 2 (Runx2) (5), which have both been found in calcified valve leaflets (7,8). In addition, AVICs stimulated with BMP-2 up-regulate alkaline phosphatase (ALP) (9), and formation of calcified nodules in VICs is dependent on ALP activity (10). But the mechanisms by which AVICs up-regulate these pro-osteogenic factors are unclear. Because calcification occurs frequently in aortic valves but rarely in pulmonary valves, comparing pro-osteogenic signaling in AVICs and pulmonary valve interstitial cells (PVICs) may provide mechanistic insights.

We hypothesize that differential TLR expression and differential response to TLR agonists distinguish AVICs from PVICs and are associated with a pro-osteogenic phenotype. In this study we will examine whether: 1) human aortic and pulmonary valves, both tissue and isolated interstitial cells, express different levels of TLR2 and TLR4; 2) stimulation of TLR2 and TLR4 results in differential expression of pro-inflammatory and pro-osteogenic factors in AVICs and PVICs; 3) reducing cellular TLR2 and TLR4 levels via silencing influences the pro-osteogenic response in AVICs; 4) BMP-2 links TLR2 and TLR4 to osteogenic phenotypic changes; and 5) TLR2, TLR4, and BMP-2 expression differ in AVICs isolated from normal versus stenotic valves.

Methods

Cell isolation and culture. Normal aortic and pulmonary valves were collected from 4 explanted hearts of heart transplant recipients and 2 unusable donor hearts (from 4 males and 2 males, mean age 58.3 ± 6.6 years). Explanted hearts were from patients of the University of Colorado Hospital or the Denver Veterans Administration Medical Center with cardiomyopathy but no history of heart valve disease. Stenotic aortic valves were collected from 3 patients who underwent valve replacement surgery for calcific aortic stenosis (1 male/2 female patients, mean age 54.7 ± 16.2 years). All patients gave written informed consent, and this study was approved by the Colorado Multiple Institutional Review Board.

After harvest, aortic and pulmonary valve leaflets were processed as previously described (5). All stenotic valves had overt calcium deposits, and interstitial cells were isolated from soft tissue adjacent to calcium deposits.

AVICs and PVICs were isolated and cultured as previously described (5). Cells from passages 3 to 7 at approximately 90% confluence were used for all experiments. For certain experiments, cells were cultured in conditioning medium (growth medium with 10 mmol/l beta-glycerophosphate, 10 nmol/l vitamin D_3, and 10 nmol/l dexamethasone), a modification of the osteogenic medium used by Osman et al. (9). The medium was changed every 3 days. All comparative experiments on AVICs and PVICs used cells isolated from the same heart. Cells were treated with peptidoglycan (*Staphylococcus aureus*, 10 μg/ml), lipopolysaccharide (*Escherichia coli* 0111:B4, 200 ng/ml), recombinant human BMP-2 (100 ng/ml, R&D Systems, Minneapolis, Minnesota), and/or Noggin (470 ng/ml, R&D Systems) for the time course indicated. Unless otherwise stated, reagents were purchased from Sigma (St. Louis, Missouri).

Immunoblotting. Protein samples were separated on 4% to 20% minigels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California). Proteins were detected using primary antibodies to TLR2 and TLR4 (Imgenex, San Diego, California), BMP-2 (ProSci, Poway, California), BMP-4 (Assay Designs, Ann Arbor, Michigan), or Runx2 (Novus Biologicals, Littleton, Colorado) and corresponding peroxidase-linked secondary antibodies (Cell Signaling Technology, Danvers, Massachusetts). Blots were developed with enhanced chemiluminescence reagent and a computerized densitometer (Molecular Dynamics, Sunnyvale, California) was used to measure band density.

Flow cytometry. Cell-surface expression of TLR2 and TLR4 was detected using phycoerythrin-conjugated TLR2 and TLR4 antibodies (Imgenex) as previously described (11).

Table 1 Nucleotide Sequences of TLR2, TLR4, and Scrambled siRNA

	siRNA Pool
TLR2	
Sense	5′-AAAUCGAGAGCUGCGAUAUU-3′
Antisense	5′-PUAUCGCAGCUCUCAGAUUUUU-3′
Sense	5′-AGGUAAAGUGGAAACGUUAUU-3′
Antisense	5′-PUAACGUUUCCACUUUACCUUU-3′
Sense	5′-UGUUUGGAACUGCGAGAUAUU-3′
Antisense	5′-PUAUCUCGCAGUUCCAAACAUU-3′
Sense	5′-AGUAGGAAUGCAAUAACUAUU-3′
Antisense	5′-PUAGUUAUUGCAUUCCUACUUU-3′
TLR4	
Sense	5′-UGGUGGAAGUUGAACGAAUUU-3′
Antisense	5′-PAUUCGUUCAACUUCCACCAUU-3′
Sense	5′-GUUUAGAAGUCCAUCGUUUUU-3′
Antisense	5′-PAAACGAUGGACUUCUAAACUU-3′
Sense	5′-CAUUGAAGAAUUCCGAUUAUU-3′
Antisense	5′-PUAAUCGGAAUUCUUCAAUGUU-3′
Sense	5′-GGAAAAUGGUGUAGCCGUUUU-3′
Antisense	5′-PAACGGCUACACCAUUUUCCUU-3′
Scrambled siRNA	
Sense	5′-UAGCGACUAAACACAUCAAUU-3′
Antisense	5′-PUUGAUGUGUUUAGUCGCUAUU-3′

siRNA = small interfering ribonucleic acid; TLR = toll-like receptor.

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