Adult Aortic Valve Interstitial Cells Have Greater Responses to Toll-Like Receptor 4 Stimulation

Xin-Sheng Deng, MD, Xianzhong Meng, MD, PhD, QingChun Zeng, MD, PhD, David Fullerton, MD, Max Mitchell, MD, and James Jaggers, MD

Cardiothoracic Surgery, University of Colorado, Children's Hospital Colorado, and Cardiothoracic Surgery, University of Colorado Anschutz Medical Campus, Aurora, Colorado

Background. **Aortic valve interstitial cells (AVICs) have been implicated in the pathogenesis of calcific aortic valve disease. Signal transducer and activator of transcription 3 (Stat3) possesses antiinflammatory effects. Given that calcification occurs in adult valves, we hypothesized that AVICs from adult valves more likely undergo a proosteogenic phenotypic change than those from pediatric valves and that may be related to different Stat3 activation in the response of those two age groups to toll-like receptor 4 (TLR4).**

Methods. **AVICs from healthy human aortic valve tissues were treated with TLR4 agonist lipopolysaccharide. Cellular levels of TLR4, intercellular adhesion molecule 1, bone morphogenetic protein 2, and alkaline phosphatase, as well as phosphorylation of p-38 mitogen-activated protein kinase (MAPK), nuclear factor-κβ (NF-κβ), and Stat3, were analyzed.**

Results. **Toll-like receptor 4 protein levels were comparable between adult and pediatric AVICs. Adult cells produce markedly higher levels of the above markers after TLR4 stimulation, which is negatively associated with phosphorylation of Stat3. Inhibition of Stat3 enhanced p-38 MAPK and NF-κβ phosphorylation and exaggerated the expression of the above markers in pediatric AVICs after TLR4 stimulation.**

Conclusions. **Adult AVICs exhibit greater inflammatory and osteogenic responses to TLR4 stimulation. The enhanced responses in adult AVICs are at least partly due to lower levels of Stat3 activation in response to TLR4 stimulation relative to pediatric cells. Stat3 functions as a negative regulator of the TLR4 responses in human AVICs. The results suggest that Stat3 activation (tyrosine phosphorylation) may be protective and that TLR4 inhibition could be targeted pharmacologically to treat calcific aortic valve disease.**

(Ann Thorac Surg 2015;99:62–71)

Calcific aortic valve disease (CAVD) may be an active disease process. Mechanisms of inflammation and osteogenesis appear to play important roles in the pathogenesis of the disease [1–4]. For example, inflammation of heart valves due to chronic or recurrent oral bacterial infection [5] or rheumatic heart disease may cause CAVD [6]. Therefore, CAVD may be the result of pathologic inflammatory processes.

The aortic valve interstitial cells (AVICs) are the principal cell type within the aortic valve leaflet and have been implicated in the pathogenesis of CAVD [2, 7]. These cells are metabolically active and responsive to proinflammatory stimulation [2]. Under basal conditions, AVICs have a phenotype similar to myofibroblasts [2]. But proinflammatory stimulation by toll-like receptors 2 and 4 induces an inflammatory phenotype in adult AVICs characterized by the production of intercellular adhesion molecule 1 (ICAM-1) and bone morphogenetic protein 2 (BMP-2) [2]. In adult AVICs, toll-like receptor 4 (TLR4) stimulation activates protein kinases such as p38 mitogen-activated protein kinase (MAPK) and the transcription factor nuclear factor-κβ (NF-κβ) [2]. In turn, activation of NF-κB in adult AVICs is associated with a proinflammatory and proosteogenic phenotype change. Characteristics of this proosteogenic phenotype include the production of bone-forming proteins, osteocalcin and osteopontin, bone-forming transcription factors, and enzymes necessary for bone formation (alkaline phosphatase [ALP]) [8]. Hence, there is a linkage between mechanisms of inflammation and osteogenesis in AVICs. Elucidation of this linkage will lead to a better understanding of the pathogenesis of CAVD.

Stat3 possess both proinflammatory and antiinflammatory characteristics. For example, Stat3 phosphorylation is important for proinflammatory cytokine release by macrophages after lipopolysaccharide (LPS) stimulation [9], but Stat3 also drives an antiinflammatory response in human macrophages [10]. In particular, interleukin-10, the best-studied antiinflammatory cytokine, is mediated through Stat3 activation [11, 12]. Furthermore, a recent study demonstrated that Stat3 seems to have an important role in the protection of inflammation-induced heart damage with advanced age [13]. Therefore, given that calcification occurs in adult valves, we speculated that

Accepted for publication July 9, 2014.

Address correspondence to Dr Jaggers, Pediatric Cardiothoracic Surgery, Children's Hospital Colorado, 13123 E 16th Ave, Aurora, CO 80045; e-mail: james.jaggers@childrenscolorado.org.

Published by Elsevier

0003-4975/$36.00
http://dx.doi.org/10.1016/j.athoracsur.2014.07.027

Abbreviations and Acronyms

ALP = alkaline phosphatase
AVICs = aortic valve interstitial cells
BMP-2 = bone morphogenetic protein 2
CAVD = calcific aortic valve disease
ICAM-1 = intercellular adhesion molecule 1
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
NF-κβ = nuclear factor-κβ
shRNA = short hairpin RNA
Stat3 = signal transducer and activator of transcription 3
TLR4 = toll-like receptor 4

adult AVICs have greater inflammatory and osteogenic responses to LPS stimulation than those of pediatric cells, and that this difference is due to lower Stat3 activation. Stat3 may function as a protective and antiinflammatory regulator of TLR4 response to the inflammatory stimulation in AVICs.

Material and Methods

Material

Antibodies against ICAM-1 and TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against BMP-2 was purchased from ProSci (Poway, CA). Antibody against ALP was purchased from ABCAM (Cambridge, MA). Antibodies against phosphorylated p38 MAPK, total p38 MAPK, phosphorylated NF-κB, total NF-κB, phosphorylated Stat3(Y), phosphorylated Stat3(S), total Stat3, glyceraldehyde 3-phosphate dehydrogenase, and β-actin were purchased from Cell Signaling (Beverly, MA). Medium 199 was purchased from Lonza (Walkersville, MD). Lipopolysaccharide (*Escherichia coli* 0111:B4) and all other chemicals were from Sigma-Aldrich Chemical (St. Louis, MO).

Cell Isolation and Treatment

Aortic valve leaflets were collected from the explanted hearts undergoing heart transplantation at the University of Colorado Hospital and Children's Hospital of Colorado (Table 1). All adult patients and children's parents gave informed consent for the use of their own or their children's valves for this study, which was approved by the University of Colorado Denver Institutional Review Board.

The AVICs were isolated and cultured using a previously described method [14], with modification [2]. Briefly, valve leaflets were subjected to sequential digestions with collagenase, and cells were collected by centrifugation. Cells were cultured in M199 growth medium containing penicillin G, streptomycin, amphotericin B, and 10% fetal bovine serum. Cells from passages 2 to 6 were used for this study. Cells were treated when they reached 80% to 90% confluence. The AVICs were stimulated with LPS (200 ng/mL) for 24, 48, and 72 hours to assess changes in inflammatory and proosteogenic markers. Then, the AVICs were stimulated with LPS (200 ng/mL) for 5 to 120 minutes to evaluate NF-κB and p-38 MAPK activation. The AVICs were also stimulated with LPS (200 ng/mL) for 5 to 30 minutes to study Stat3 activation. After pretreating pediatric AVICs with Stat3 inhibitor S3I-201 for 1 hour, LPS (200 ng/mL) was added for 1 hour to see NF-κB and p-38 MAPK changes. Pediatric AVICs were treated with or without Stat3 inhibitor S3I-201 (5 μm) in the presence of LPS (200 ng/mL) for 48 hours to determine inflammatory and proosteogenic marker changes.

Table 1. Patient Demographics, Aortic Valve

Patients	Treatment	Age (years)	Sex	Diagnosis
Pediatric patients				
060107[a]	Replacement	0.1	Female	Stenosis
031208	Transplant	0.5	Male	Cardiomyopathy
070307[a]	Replacement	1	Female	Stenosis
111307	Transplant	3	Female	Cardiomyopathy
051508	Transplant	4	Female	Cardiomyopathy
050909	Transplant	4	Male	Cardiomyopathy
102307	Transplant	13	Male	Cardiomyopathy
120107	Transplant	16	Male	Cardiomyopathy
052308	Transplant	16	Male	Cardiomyopathy
Adult patients				
010709	Transplant	50	Male	Cardiomyopathy
051810	Transplant	60	Male	Cardiomyopathy
082410	Transplant	57	Female	Cardiomyopathy
040907	Transplant	51	Male	Cardiomyopathy
090707	Transplant	48	Male	Cardiomyopathy
022010	Transplant	47	Male	Cardiomyopathy
091106	Transplant	41	Male	Cardiomyopathy

[a] Only used for toll-like receptor-4 distribution assay.

Knockdown of Stat3 With Short Hairpin RNA

Pediatric AVICs were infected using a previously described method [15]. Briefly, cells were treated with control short hairpin RNA (shRNA) or Stat3 shRNA (shStat3) or combined with LPS (200 ng/mL) for 72 hours, and then collected and subjected to immunoblotting analysis.

Immunoblotting

The AVICs in culture were lysed. Cell lysates were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and the proteins were transferred onto polyvinylidene difluoride membranes. After being blocked with 5% skim milk solution, membranes were incubated with primary antibodies, followed by peroxidase-linked secondary antibodies specific to the primary antibodies. Protein bands were revealed using the enhanced chemiluminescence system. Band density was analyzed using the National Institutes of Health Image J software (Wayne Rasband,