High-mobility group box-1 protein induces osteogenic phenotype changes in aortic valve interstitial cells

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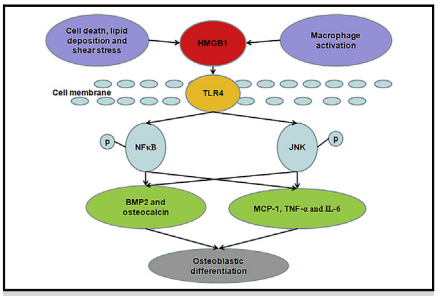
ABSTRACT

Objectives: Calcific aortic valve (AV) disease is known to be an inflammation-related process. High-mobility group box-1 (HMGB1) protein and Toll-like receptor 4 (TLR4) have been reported to participate in several inflammatory diseases. The purpose of the present study was to determine whether the HMGB1-TLR4 axis is involved in calcific AV disease, and to evaluate the effect of HMGB1, and its potential mechanisms, on the pro-osteogenic phenotype change of valvular interstitial cells (VICs).

Methods: Expression of HMGB1 and TLR4 in human calcific AVs was evaluated using immunohistochemical staining and immunoblotting. Cultured VICs were used as an in vitro model. The VICs were stimulated with HMGB1 for analysis, with versus without TLR4 small interfering ribonucleic acid (siRNA), c-Jun N-terminal kinase mitogen-activated protein kinase (JNK MAPK), and nuclear factor kappa-B (NF-κB) inhibitors.

Results: Enhanced accumulation of HMGB1 and TLR4 was observed in calcific valves. Moreover, we found that HMGB1 induced high levels of pro-inflammatory cytokine production and promoted the osteoblastic differentiation and calcification of VICs. In addition, HMGB1 induced phosphorylation of JNK MAPK and NF-κB. However, these effects were markedly suppressed by siRNA silencing of TLR4. In addition, blockade of JNK MAPK and NF-κB phosphorylation prohibited HMGB1-induced production of pro-osteogenic factors, and mineralization of VICs.

Conclusions: The HMGB1 protein may promote osteoblastic differentiation and calcification of VICs, through the TLR4-JNK-NF-κB signaling pathway. (J Thorac Cardiovasc Surg 2016;151:255-62)



The HMGB1 protein may promote the osteoblastic differentiation of VICs, through the TLR4 pathway.

Central Message

The HMGB1 protein promotes AV calcification through a pro-inflammatory effect, and might be a new target for therapeutic intervention.

Perspective

The identification of TLR4 signaling as being downstream of HMGB1 provides novel insight into the function of the HMGB1-TLR4 pathway in the pathogenesis of AV calcification in vitro. Identification of the causal relationship between HMGB1 and calcific AV disease might reveal that HMGB1 might serve as a target to predict and prevent calcific AV disease.

See Editorial Commentary page 263.

Calcific aortic valve (AV) disease is a common health problem associated with high mortality and morbidity in aging societies in which the average age is increasing.[1] Historically, calcific AV disease was considered to be a passive process. Recent studies indicate that calcific AV disease is an active disease that has several pathogeneses that are similar to vascular calcification, such as inflammation, osteogenesis, and mineralization.[1,2] Currently, the only effective therapy is valve replacement or implantation, yet not everyone is appropriate for or can tolerate this surgery.[3]

The high-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that has cytokine, chemokine, and growth-factor activity, orchestrating the inflammatory and immune response in mammals. This protein has been reported to be involved in the pathogenesis of vascular atherosclerosis and inflammation changes,[4-7] and it facilitates the assembly of nuclear proteins and participates in deoxyribonucleic acid (DNA) replication, recombination, transcription, and repair.[8] However,

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Abbreviations and Acronyms

AV = aortic valve
BMP2 = bone morphogenetic protein 2
HMGB1 = high-mobility group box-1 protein
JNK MAPK = c-Jun N-terminal kinase mitogen-activated protein kinase
NF-κB = nuclear factor kappa-B
siRNA = small interfering ribonucleic acid
TLR = Toll-like receptor
TNF-α = tumor necrosis factor-α
VIC = valvular interstitial cell

whether HMGB1 plays a role in calcific AV disease is unknown.

Pro-osteogenic differentiation of valvular interstitial cells (VICs) is considered to be a key cellular mechanism in the development of AV calcification. Studies indicate that human VICs that are isolated from calcific AV leaflets express more Toll-like receptor 4 (TLR4); and activating TLR4 results in the osteoblastic differentiation of VICs.[9] In addition, HMGB1 is one of the important agonists of TLR4.[10-14] However, little is known about the association between HMGB1 and TLR4 in the pathogenesis of AV calcification.

METHODS

Chemicals and Reagents

The following antibodies were used: HMGB1, osteocalcin, and bone morphogenetic protein (BMP)2 (Abcam, Cambridge, Mass); TLR4 (Santa Cruz Biotechnology, Santa Cruz, Calif); and phosphorylated and total NF-κB and JNK MAPK (Cell Signaling Technology, Danvers, Mass). Beta-actin was used as a normalization for total and cytosolic protein determination. Recombinant HMGB1 (R&D Systems, Minneapolis, Minn) and collagenase and other reagents (Sigma-Aldrich, St Louis, Mo) were used as well.

Calcific Aortic Valve Collection

This study complied with the Declaration of Helsinki and was approved by the review boards of Union Hospital and Tongji Medical College; all patients provided written informed consent. Calcific AV leaflets were obtained intraoperatively from 15 patients undergoing AV replacement in Union Hospital to treat severe AV calcification. The thin and "normal" AV leaflets were collected from 10 age-matched patients undergoing Bentall surgery to treat acute aortic dissection. Demographic information for patients is summarized in Table 1. The tissue samples were kept frozen in liquid nitrogen until use. All valves were tricuspid.

Cell Culture and Treatment

Valvular interstitial cells were isolated from patients undergoing Bentall surgery for acute aortic dissection, using the collagenase I digestion method previously described.[15] Briefly, isolated leaflets were digested in essential medium containing 1 mg/ml of collagenase type I at 37°C for 30 minutes. After removal of endothelial cells by vortexing, the leaflets were further digested with a fresh solution of 1 mg/ml of collagenase medium for 4 to 6 hours at 37°C. After vortexing and repeated aspirating to break up the tissue mass, the suspension was spun at 1000 rpm for 10 minutes to precipitate cells. Cells were resuspended and cultured in essential medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum, in an incubator with 5% CO_2, at 37°C. Cells of passages 2 to 5 were used for all experiments. When they had grown to 70% to 90% confluence, the VICs were stimulated with HMGB1 in the presence of the condition medium: 10 mmol of β-glycerophosphate; 100 nmol of dexamethasone; and 50 μg/ml ascorbic acid (all 3 from Sigma-Aldrich, St Louis, Mo). If needed, pharmacologic reagents, including 10 μmol/L SP600125 and 10 μmol/L BAY 11-7082 (both from Beyotime, Nantong, People's Republic of China), were added 1 hour before the addition of HMGB1. Cells from 3 patients were used for each intervention.

Real-Time Polymerase Chain Reaction RNA Analysis

Real-time polymerase chain reaction analysis was used to detect expression of messenger RNAs encoding interleukin-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1. The RNA was isolated and reversely transcribed to complementary DNA (deoxyribonucleic acid), as previously described.[15] Real-time polymerase chain reaction assays were carried out using an SYBR Premix Ex Taq (Takara Bio Inc, Otsu, Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, Calif). Primers were as follows: interleukin-6 (F: 5′-ATC AGG AGA CCT GCT TGA TG-3′; R: 5′-TGG TGG CTT TGT CTG GAT TC-3′); tumor necrosis factor-α (F: 5′-CCA ATG GCA GAG TGG GTA TG-3′; R: 5′ -TGA AGA GGA CCT GGG AGT AG-3′); monocyte chemoattractant protein-1 (F: 5′-GTC ACC AGC AGC AAG TGT C-3′; R: 5′-CCA GGT GGC TTA TGG AGT C-3′); and β-actin (F: 5′-GAC CTG ACC GAC TAC CTC-3′; R: 5′-GCT TCT CCT TGA TGT CCC-3′). Results were normalized to β-actin expression and analyzed by the comparative C_T (real-time polymerase chain reaction data) method.

Silencing Toll-like Receptor 4

To knock down TLR4, cells (60%-80% confluence) in 6-well plates were incubated with a mixture of siRNA (50 nmol/L), and control cells were treated with scrambled siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) and Opti-MEM (Life Technologies, Carlsbad, Calif) according to manufacturer instructions. The medium was changed 4 to 6 hours after transfection; 72 hours later, the cells were harvested for protein expression analysis.

Alizarin Red Staining

For mineralization experiments, aortic VICs were seeded in 24-well plates. After 70% confluence was achieved, cells were incubated, per indicated interventions, in essential medium supplemented with 5% fetal bovine serum, 2 mmol/L β-glycerophosphate, 100 nmol/L dexamethasone, and 50 μg/ml ascorbic acid for 14 days. Alizarin red staining for calcium deposits was performed as described previously.[16] Briefly, cell monolayers were washed twice with phosphate-buffered saline and fixed for 15 minutes in 4% paraformaldehyde. After incubation with 0.2% alizarin red solution (pH: 4.2) for 30 minutes, excessive dye was removed, by washing with distilled water. To quantify the staining, cell monolayers were bleached with 10% acetic acid at 75°C. Supernatant was spectrophotometrically analyzed at 450 nm.

Immunohistochemistry

Immunohistochemical detection of HMGB1 protein in AV leaflets was performed by using the biotin-linked peroxidase technique. Paraffin

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