Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis

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CALCIFIC AORTIC VALVE STENOSIS was traditionally considered to be a degenerative disorder, involving the passive accumulation of calcium on aortic valve leaflets. However, several studies have suggested that aortic valve calcification is an active biological process and that inflammation is an important component of that process (10, 21). Recent studies have shown that when cultured human aortic valve interstitial cells (HAVICs) are exposed to the proinflammatory cytokines TNF-α and IL-1β in vitro, they exhibit osteoblast-like phenotypic changes (12, 13). Other studies have shown that TNF-α induces bone morphogenetic protein-2 (BMP-2) in vascular cells (5) and that it promotes the calcification of bovine aortic smooth muscle cells (29). Together, these studies suggest that proinflammatory cytokines in the environment surrounding HAVICs may promote the calcification process.

Little is known about the mechanisms by which HAVICs respond to extracellular proinflammatory stimuli. Toll-like receptors (TLRs), particularly TLR2 and TLR4, are known to regulate the expression of various proinflammatory cytokines in other cell types (18, 19). Both TLR2 and TLR4 are implicated in the pathogenesis of atherosclerosis (17, 22, 31), and aortic valve stenosis and atherosclerosis are known to have similar pathological changes (20, 21). However, it is unknown whether HAVICs express functional TLR2 and TLR4. Assessing the expression and function of these receptors in HAVICs may provide insight into the complex pathophysiology of inflammation-associated calcification.

The purpose of this study is to determine whether HAVICs express functional TLR2 and TLR4, to characterize the inflammatory response elicited by TLR2 and TLR4 agonists, and to determine whether TLR2 and TLR4 agonists upregulate factors associated with osteogenesis, such as osteopontin, BMP-2, and Runx2.

MATERIALS AND METHODS

Chemicals and reagents. M199 medium and human serum albumin were purchased from Cambrex Bio Science Walkersville (Walkersville, MD). Antibodies against human TLR2, TLR4, and ICAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against human vimentin, α-smooth muscle actin, osteopontin were purchased from Abcam (Cambridge, MA), and antibody against human BMP-2 was purchased from Novus Biologicals (Littleton, CO). All other reagents were purchased from Sigma Chemical (St Louis, MO).

Cell isolation and culture. Aortic valve leaflets were collected from the explanted hearts of five patients undergoing heart transplantation at the University of Colorado Hospital or the Denver Veteran Affairs Medical Center. This study was approved by the Colorado Multiple Institutional Review Board, and all patients gave written informed consent.

On gross examination, the aortic valve leaflets were thin, and their surfaces were smooth. Microscopic examination of hematoxylin and eosin-stained cryosections confirmed that no leukocyte infiltration was detected in the specimens.

HAVICs were isolated following a modification of a previously described method (16). Valve leaflets were rinsed in Earle’s balanced salt solution (EBSS) and then digested in collagenase (2.5 mg/ml in M199 medium) for 30 min at 37°C. After removing endothelial cells by vortex, the leaflets were further digested with a milder solution of collagenase medium (0.8 mg/ml) for 3 h at 37°C. After vortexing and
aspirating repeatedly to break up the tissue mass, the cell suspension was spun at 500 g for 2 min to remove any remaining tissue. The supernatant was transferred into a fresh tube and spun again at 1100 g for 8 min at 4°C. The cells were resuspended in full medium (M199 with penicillin G, streptomycin, amphotericin B, and 10% FBS), plated onto a 75-cm² flask, and cultured in a cell culture incubator supplied with 5% CO₂. When the cells reached 70–90% confluence, they were subcultured on plates and chamber slides. Cells from passages 3–7 were used for these experiments.

Cells were stimulated with peptidoglycan (PGN, Staphylococcus aureus, 10 μg/ml) or LPS (Escherichia coli 0111:B4, 200 ng/ml) for 10–60 min to assess NF-κB activation (NF-κB phosphorylation and NF-κB intranuclear translocation). Cells were stimulated for 24 h to measure levels of proinflammatory mediators [TNF-α, IL-1β, IL-6, IL-8, IFNγ, monocyte chemotactant protein-1 (MCP-1), and ICAM-1] and factors associated with osteogenic changes (osteopontin, BMP-2, and Runx2).

**Immunofluorescent staining.** Immunofluorescent staining was applied to characterize cell isolates and detect and localize TLRs and NF-κB as described previously (25). Cryosections (5-μm thick) of aortic valve tissue were prepared with a cryostat (Minotome Plus; IEC, Needham Heights, MA) and collected on poly-L-lysine-coated slides. Sections were treated with a mixture of 70% acetone and 30% methanol for 5 min and then fixed with 4% paraformaldehyde for 20 min. Sections were washed with PBS, blocked with 10% normal donkey serum for 30 min, and incubated for 1 h with a monoclonal antibody against human TLR2 or TLR4 (5 μg/ml in PBS containing 1% BSA). All incubations were performed at room temperature. After washing with PBS, sections were incubated with indocarbocyanine (Cy3)-conjugated donkey anti-mouse IgG (1:300 dilution with PBS containing 1% BSA). To assess the specificity of the immunostaining, adjacent sections were incubated with nonimmune mouse IgG (5 μg/ml in PBS containing 1% BSA) instead of the primary antibody and then processed identically. The cell surface was counterstained with Alexa 488-conjugated wheat germ agglutinin (WGA; imaged on the green channel), and the nucleus was counterstained with bisbenzimide (imaged on the blue channel). Microscopic observation and photography were performed with a Leica DMRXA confocal microscope (Leica Mikroskopie und Systeme, Wetzlar, Germany) equipped with Slidebook software (Intelligent Imaging Innovations, Denver, CO).

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**Fig. 1.** A and B: Toll-like receptor (TLR) expression in human aortic valve tissue. Aortic valve leaflets from explanted hearts of heart transplant recipients were examined for TLR2 and TLR4 by immunoblotting and immunofluorescent staining. A: a representative gel of aortic valve tissue homogenate from 2 patients shows comparable levels of TLR2 and TLR4. B: micrographs show TLR2 immunoreactivity in longitudinal section and TLR4 immunoreactivity in cross-cutting section. TLR immunoreactivity (red) is localized to the interstitial cells (arrows). N, nucleus (blue); C, collagen fiber (green). Scale bar = 30 μm.
Immunofluorescent staining of cells was performed on chamber slides. After permeabilizing in a methanol-acetone mixture and fixing in 4% paraformaldehyde, cells were washed with PBS and blocked with 10% normal serum (in PBS). Cells were then incubated with primary antibodies (typically 1:100–1:500 dilutions) overnight at 4°C. After washing with PBS, cells were incubated with fluorescently tagged secondary antibodies specific for the primary antibodies. In most cases, bisbenzimide was used to stain nuclei (4,6-diamidino-2-phenylindole, imaged on the blue channel), and WGA was used to stain cell membranes (labeled with Alexa 488 and imaged on the green channel). Proteins of interest (such as TLRs) were imaged using the red (Cy3) channel. Microscopy was performed as described above.

Immunoblotting. Immunoblotting was used to analyze TLRs, phospho-NF-κB p65, ICAM-1, osteopontin, BMP-2, and Runx2. Aortic valve homogenate was prepared in eight parts of homogenate buffer containing PBS (pH 7.5), 0.1% Triton X-100, and a protease inhibitor cocktail. Cells were lysed in a lysis buffer containing PBS (pH 7.5), 1% Triton X-100, and a protease inhibitor cocktail. Cells were centrifuged at 1,000 g, 4°C, for 15 min with an Eppendorf centrifuge (model 5417R; Brinkmann Instruments, Westbury, NY). The supernatant was collected, and protein concentrations were analyzed. The supernatant was mixed with an equal volume of sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol).

Protein samples were run on gradient (4–20%) minigels at 100 V for ~2 h. After transfer, membranes were rinsed with TPBS (PBS containing 0.05% Tween 20) and blocked with 5% nonfat dry milk for 1 h at room temperature. The blocked membranes were incubated in primary antibodies (diluted to 1:1,000–3,000 with TPBS and 5% BSA) at room temperature for 2 h. After washing 2× for 5 min with TPBS, the membrane was incubated with peroxidase-linked secondary antibodies (diluted to 1:5,000 with TPBS and 5% dry milk) at room temperature for 2 h. Following further washes, ECL solution was added for 5 min at room temperature, and then the membrane was exposed on X-ray film. A computerized densitometer (Molecular Dynamics, Sunnyvale, CA) was used to measure the density of the bands.

Cytokine assay. Cell culture medium was collected, and cytokine levels were determined using immunoassays as described previously (25, 27).

Statistical analysis. Data are presented as means ± SE. ANOVA with a post hoc Bonferroni/Dunn test was performed to analyze differences between experimental groups. Statistical significance was accepted within a 95% confidence limit.

RESULTS

TLR2 and TLR4 are detected and localized in human aortic valves. We examined the expression and distribution of TLR2 and TLR4 in human aortic valve leaflets. Using immunoblotting, we detected both TLR2 and TLR4 in tissue homogenate (Fig. 1A). Using immunofluorescent staining, the two receptor proteins were localized to the interstitial cells between collagen fibers (Fig. 1B). In sections incubated with nonimmune mouse IgG, no immunoreactivity was observed (data not shown).

HAVICs express TLR2 and TLR4 receptors. Cardiac valve interstitial cells are not yet well-characterized (4, 15). However, when HAVICs are cultured, they adopt a myofibroblast-like phenotype (14, 16, 30). Myofibroblasts are fibroblast-like cells that express both vimentin and α-smooth muscle actin. To identify myofibroblasts in cell isolates, we applied double immunofluorescent staining to detect those two proteins. As shown in Fig. 2, all cells are immunoreactive to an antibody against vimentin, and ~90% of cells are positively labeled by an antibody against α-smooth muscle actin. Thus myofibroblasts constitute the vast majority of cells in our cultured HAVICs.

We applied antibodies against von Willebrand factor, sarcomeric myosin heavy chain, and CD68 to determine whether the cultured HAVICs were contaminated by endothelial cells, smooth muscle cells, or macrophages. No signal for von Willebrand factor, sarcomeric myosin heavy chain, or CD68 was detected (data not shown), indicating that endothelial cells, smooth muscle cells, and macrophages were not detected in the isolates.

Expression of TLR2 and TLR4 in HAVICs was demonstrated by immunoblotting. Figure 3, A and B shows represen-
tative immunoblots for TLR2 and TLR4 in freshly isolated cells (Fig. 3A) and in cultured cells (Fig. 3B). TLR2 and TLR4 are detected in each preparation, and the levels of the two receptors in freshly isolated cells and in cultured cells are comparable. The effects of receptor agonists on receptor protein levels were assessed in cultured cells. Although the levels of TLR4 are not influenced by stimulation with either LPS or PGN, LPS stimulation for 24 h increased TLR2 levels (Fig. 3B).

We applied immunostaining to assess the expression of TLR2 and TLR4 and to localize these receptors in cultured HAVICs. Using specific antibodies, we observed that all cells have detectable levels of TLR2 and TLR4 (Fig. 3C). Both TLR2 and TLR4 were distributed on the cell membrane and in the cytoplasm. In resting cells, however, TLR4 was primarily localized in the cytoplasm. This pattern of TLR4 distribution is similar to that previously described for human coronary artery endothelial cells (7).

TLR2 and TLR4 mediate proinflammatory signaling in cultured HAVICs. To determine whether the TLR2 and TLR4 detected in cultured HAVICs are functional, we assessed the cellular response to the receptor agonists, PGN and LPS, respectively, using NF-κB activation as a readout. Stimulation of cells with PGN and LPS resulted in rapid phosphorylation of NF-κB p65 (Fig. 4A). Stimulation of cells with PGN and LPS also caused NF-κB p65 intranuclear translocation (Fig. 4B).

TLR2 and TLR4 regulate production of proinflammatory mediators in cultured HAVICs. To determine the role of TLR2 and TLR4 in the cellular inflammatory response, we stimulated cultured HAVICs with PGN and LPS and measured cytokine levels in the media 24 h later. Under basal conditions, cultured HAVICs released low levels of IL-6 and IL-8. However, treatment with PGN or LPS significantly increased the release of IL-6 and IL-8 (Table 1). Interestingly, neither PGN nor LPS induced the release of TNF-α, IL-1β, or IFNγ, and the levels of these mediators in cell lysate were not elevated following stimulation with PGN or LPS (data not shown). Only LPS stimulation induced the release of MCP-1 (Table 1).

To further determine the role of TLR2 and TLR4 in the cellular inflammatory response, we performed immunoblotting to examine ICAM-1 expression. As shown in Fig. 5, cultured HAVICs express a low level of ICAM-1 before stimulation. ICAM-1 levels increased following stimulation with either PGN or LPS, LPS-stimulated cells had much higher ICAM-1 levels than PGN-treated cells.

TLR2 and TLR4 regulate production of BMP-2 and Runx2 in cultured HAVICs. To explore the role of TLR2 and TLR4 in the expression of factors involved in osteogenesis, we examined cellular levels of osteopontin, BMP-2, and Runx2 following stimulation of these receptors. Figure 6 shows the results of

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Fig. 3. A–C: expression of TLR2 and TLR4 in HAVICs. A: representative immunoblots show that both TLR2 and TLR4 are present in freshly isolated HAVICs. B: representative immunoblots and densitometry data (mean of 2 separate experiments) show the expression of TLR2 and TLR4 in cultured cells with or without peptidoglycan (PGN) or LPS stimulation. TLR4 levels are not influenced by stimulation with either LPS or PGN. LPS stimulation for 24 h causes TLR2 levels to increase. C: representative micrographs show TLR2 and TLR4 distribution in cultured HAVICs. TLR2 and TLR4 are stained red with specific antibodies. The cells (membrane and cytoplasm) are labeled green, and the nuclei are labeled blue. TLR2 and TLR4 are present on cell membranes and in the cytoplasm. Scale bar = 30 μm.
immunoblotting for cellular osteopontin, BMP-2, and Runx2. Stimulation of TLR2 and TLR4 had no influence on osteopontin levels 24 h following treatment but did increase cellular BMP-2 and Runx2 levels.

DISCUSSION

TLR2 and TLR4 are known to regulate the cellular inflammatory response to gram-positive and gram-negative bacteria (28) as well as to endogenous agents such as heat shock proteins (18, 19). However, it was previously unknown whether HAVICs express TLR2 and TLR4 and whether these innate immune receptors are involved in HAVIC activation. Our results show, for the first time, that the two receptors are present in human aortic valve tissue and that they are present in aortic valve interstitial cells, in particular.

The distinction between HAVICs in tissue and in culture should be emphasized. In normal human cardiac valves, myofibroblasts are only a small percentage of the population of

Table 1. Cytokine levels in the culture media following TLR stimulation

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control, pg/ml</th>
<th>PGN, pg/ml</th>
<th>LPS, pg/ml</th>
</tr>
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<tbody>
<tr>
<td>TNF-α</td>
<td>4.2±1.3</td>
<td>3.4±0.4</td>
<td>4.9±0.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>7.2±1.3</td>
<td>6.5±2.0</td>
<td>8.6±2.9</td>
</tr>
<tr>
<td>IL-6</td>
<td>415±23</td>
<td>1,648±132*</td>
<td>1,396±145*</td>
</tr>
<tr>
<td>IL-8</td>
<td>33±9.1</td>
<td>524±45*</td>
<td>312±51*</td>
</tr>
<tr>
<td>INFγ</td>
<td>52±7.6</td>
<td>65±4.1</td>
<td>44±11</td>
</tr>
<tr>
<td>MCP-1</td>
<td>385±64</td>
<td>446±76</td>
<td>814±77*</td>
</tr>
</tbody>
</table>

n = 5; *P < 0.05 vs. control. TLR, Toll-like receptor; MCP-1, monocyte chemoattractant protein-1; PGN, peptidoglycan.
valve interstitial cells (6, 24). However, in culture, a high percentage of valve interstitial cells exhibit a myofibroblast-like phenotype (14, 16, 30). This may reflect the fact that myofibroblast-like valve interstitial cells, which express \( \alpha \)-smooth muscle actin, better adhere to the culture plate (2). Nevertheless, despite this phenotypic difference, we found that freshly isolated HAVICs and cells that had been passaged multiple times had comparable levels of TLR2 and TLR4. It seems that culturing HAVICs did not markedly influence the levels of these two receptors. However, the cellular response observed in culture may have certain limitations.

When we stimulated cultured HAVICs with agonists for TLR2 and TLR4, we found that the TLR4 agonist LPS increases cellular TLR2 levels but has no influence on cellular TLR4 levels. This influence of LPS stimulation on TLR2 levels is similar to what was previously observed in pulmonary microvascular endothelial cells when LPS-stimulated neutrophils generated reactive oxygen species that induced TLR2 (8).

Since HAVICs are important in the inflammatory process, we assessed how they function in response to proinflammatory stimuli. In this study, we found that agonists to TLR2 and TLR4 induced NF-\( \kappa \)B activation and the expression/release of multiple proinflammatory cytokines in cultured HAVICs. Both PGN and LPS induced IL-6 and IL-8, suggesting that these mediators may be involved in aortic valve tissue inflammation in response to gram-negative and gram-positive bacterial products. Note that LPS caused a significant increase in MCP-1, whereas PGN had no effect on the level of MCP-1 in the media. This suggests that the TLR2 and TLR4 pathways differently influence the release of this chemokine. Since MCP-1 is important in leukocyte recruitment, the TLR4 pathway may play a major role in inducing leukocyte infiltration into the aortic valve in vivo. In addition, note that LPS induced a more robust increase in ICAM-1 than PGN. This suggests that, in cultured HAVICs, the TLR2 and TLR4 pathways have different influences on the inflammatory response. It also suggests that ICAM-1, together with MCP-1, may be involved in leukocyte recruitment and adhesion to valve tissue following activation of the TLR4 pathway.

Although stimulation by PGN and LPS increased several proinflammatory cytokines, in our experiments, neither PGN nor LPS stimulation increased TNF-\( \alpha \) or IL-1\( \beta \) levels in the cell culture media. We considered the possibility that TNF-\( \alpha \) is produced but not released. We eliminated that possibility when our cell lysate assay found extremely low levels of cell-associated TNF-\( \alpha \) and IL-1\( \beta \) (data not shown). Others observed that a high concentration of TNF-\( \alpha \) is capable of inducing osteoblast-like phenotypic changes in cultured HAVICs (12). It is unclear, however, whether TNF-\( \alpha \) is present at such high concentrations in the environment surrounding aortic valve interstitial cells in vivo. The role of endogenous TNF-\( \alpha \) in the osteogenic changes in aortic valve cells is not yet understood.

We also assessed the expression of BMP-2 and Runx2 from cultured HAVICs after stimulation by the TLR2 and TLR4 agonists PGN and LPS. BMP-2 and Runx2 have important roles in bone formation (3, 9). Increased levels of BMP-2 and Runx2 have been found in calcified human aortic valves, and both BMP-2 and Runx2 have been implicated in the pathogenesis of aortic valve stenosis (1, 11, 26). Indeed, chronic exposure of cultured HAVICs to BMP-2 in vitro causes osteogenic changes (23). In the present study, the expression of BMP-2 and Runx2 is upregulated after stimulation by either PGN or LPS. Although PGN and LPS may have TLR-independent effects that contribute to the induction of pro-osteogenic mediators, our findings suggest that TLR2 and TLR4 have a potential role in the complex pathogenesis of aortic valve stenosis.

Csiszar et al. (5) reported that TNF-\( \alpha \) induces BMP-2 expression in coronary artery endothelial cells. Our study did not address the question of identifying the factor responsible for the induction of BMP-2. However, since the level of TNF-\( \alpha \)
in the media did not increase after stimulation by either PGN or LPS, it seems unlikely that TNF-α was responsible for the effect of PGN or LPS on BMP-2 expression. Nevertheless, the induction of BMP-2 by the TLR2 and TLR4 agonists PGN and LPS in cultured HAVICs suggests that these innate immunity pathways are involved in osteogenesis-associated changes in aortic valve cells.

In summary, HAVICs express TLR2 and TLR4 receptors. Stimulation by the TLR2 and TLR4 agonists PGN and LPS differentially regulates the expression of proinflammatory mediators. Stimulation by PGN and LPS also upregulates the expression of osteogenesis-associated factors BMP-2 and Runx2 in cultured HAVICs. These findings are consistent with the hypothesis that innate immune receptors are involved in the aortic valve tissue inflammatory response and suggest that they might contribute to the pathogenesis of calcific aortic valve stenosis.

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GRANTS

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REFERENCES