Stenotic aortic valves have dysfunctional mechanisms of anti-inflammation: Implications for aortic stenosis

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Objective: Aortic stenosis is an inflammatory disease, associated with increased tissue levels of interleukin-1 beta. We hypothesized that the antagonist of interleukin-1 beta, interleukin-1 receptor antagonist, is deficient in aortic valves and that its production by aortic valve interstitial cells is less in cells from stenotic valves than from controls.

Methods: Valve leaflets from stenotic aortic valves (n = 6) and from valves from hearts explanted at the time of cardiac transplantation (n = 6) were studied by immunostaining for interleukin-1 receptor antagonist. Aortic valve interstitial cells were isolated from valves, and receptor antagonist levels were determined from cell lysates (enzyme-linked immunosorbent assay). Osteogenic phenotype changes in valve cells stimulated by toll-like receptors 2 and 4 were determined by immunoblotting for bone morphogenetic protein-2 after treatment with and without interleukin-1 receptor antagonist (100 μg/mL). Statistics were by analysis of variance.

Results: Interleukin-1 receptor antagonist was abundant in nonstenotic aortic valve leaflets and virtually absent in leaflets from stenotic valves. Aortic valve interstitial cells from grossly normal leaflets produced significantly more receptor antagonist at baseline and in response to toll-like receptor 2 and 4 stimulation, than did cells from diseased valves ($P < 0.05$). Interleukin-1 receptor antagonist was able to significantly attenuate toll-like receptor 2, but not toll-like receptor 4, stimulated bone morphogenetic protein-2 production in aortic valve interstitial cells ($P < .05$).

Conclusions: Interleukin-1 receptor antagonist–mediated mechanisms of anti-inflammation are dysfunctional in stenotic valves. We conclude that such impaired mechanisms of anti-inflammation may contribute to the pathogenesis of aortic stenosis. (J Thorac Cardiovasc Surg 2011;141:481-6)

Calcific aortic stenosis has traditionally been considered a ''degenerative'' process with passive accumulation of calcium on the aortic valve leaflets. However, recent studies now suggest that calcific aortic stenosis may in fact be an active disease process; mechanisms of inflammation and osteogenesis appear to play important roles in the pathogenesis of aortic stenosis.[1-6] As such, aortic stenosis may be an inflammatory disease.

The aortic valve interstitial cell (AVIC) has been implicated in the pathogenesis of aortic stenosis.[7] In response to proinflammatory stimulation via toll-like receptors (TLRs) 2 and 4, the phenotype of human AVICs changes from that of a myofibroblast to that of a bone-forming–like cell.[7-10] Characteristics of this osteogenic phenotype include an increased expression of the potent bone-forming protein, bone morphogenetic protein-2 (BMP-2), the osteogenic transcription factor, Runx2, and an increased expression and activity of alkaline phosphatase.[7-10]

The net inflammatory state of any tissue is determined by the relative balance of proinflammatory and anti-inflammatory mechanisms.[11] In response to proinflammatory stimulation, a deficiency of anti-inflammatory mechanisms will lead to unopposed actions of proinflammatory mechanisms. Such an imbalance of proinflammatory and anti-inflammatory mechanisms has been implicated in the pathogenesis of many inflammatory diseases. Specifically, increased tissue levels of interleukin-1 beta (IL-1β) relative to its anti-inflammatory antagonist, interleukin-1 receptor antagonist (IL-1RA), have been implicated in the pathogenesis of rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and other inflammatory diseases.[12]

IL-1β has recently been implicated in the pathogenesis of aortic stenosis as well.[13] Produced by circulating mononuclear cells, the proinflammatory actions of IL-1β are mediated by the membrane-bound IL-1 receptor (IL-1R).[14] The specific antagonist of IL-1β is the anti-inflammatory cytokine, IL-1RA, which blocks the actions of IL-1β at its receptor.[12] The balance between IL-1 and IL-1RA in a given tissue may determine the development of inflammatory disease; a deficiency of the anti-inflammatory actions of IL-1RA relative to IL-1β leads to inflammation and tissue

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Abbreviations and Acronyms

AVIC = aortic valve interstitial cell
BMP-2 = bone morphogenetic protein-2
ELISA = enzyme-linked immunosorbent assay
IL-1β = interleukin-1 beta
IL-1R = IL-1 receptor
IL-1RA = interleukin-1 receptor antagonist
LPS = lipopolysaccharide
PBS = phosphate-buffered saline
PGN = peptidoglycan
TLR = toll-like receptor

destruction.[12] Given that aortic stenosis appears to be an inflammatory disease and that a deficiency of IL-1RA has been implicated in the pathogenesis of many inflammatory diseases, we hypothesized that a deficiency of IL-1RA may contribute to the pathogenesis of aortic stenosis as well. We further hypothesized that the source of IL-1RA in aortic valve leaflets is the AVIC.

In nonstenotic and stenotic aortic valve leaflets, the purposes of this study were as follows: (1) to characterize IL-1RA in aortic valve leaflets, (2) to examine the production of IL-1RA by AVICs, and (3) to examine the ability of IL-1RA to prevent the osteogenic phenotype changes of AVICs induced by the proinflammatory activation of TLR-2 and TLR-4. The results of this study demonstrate the following: (1) IL-1RA is deficient in stenotic aortic valve leaflets; (2) production of IL-1RA is significantly reduced in AVICs from stenotic aortic valves; and (3), IL-1RA attenuates the osteogenic phenotypic change of AVICs induced by proinflammatory stimulation by TLR-2. These findings have not previously been described and provide mechanistic insight into the pathogenesis of aortic stenosis.

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MATERIALS AND METHODS

This study was approved by the Colorado Multiple Institutional Review Board of the University of Colorado Health Sciences Center. All patients provided written informed consent.

Cell Isolation and Culture

Stenotic aortic valves were obtained from 6 patients who underwent aortic valve replacement surgery for calcific aortic stenosis (men, aged 54–77 years). Controls were nonstenotic aortic valves obtained from the explanted hearts of patients undergoing heart transplantation at the University of Colorado Hospital (n = 6). The etiology of heart failure was idiopathic dilated cardiomyopathy in all patients (men, aged 38–55 years). None of these patients was a previous ventricular assist device recipient. On gross examination, nonstenotic valve leaflets were thin and pliable, and their surfaces were smooth; they were grossly normal. Microscopic examination of hematoxylin and eosin–stained cryosections confirmed the absence of leukocytic infiltration. All stenotic valves were thick and exhibited overt calcification. Noncalcified tissue was used for cell isolation. Small sections of each valve were frozen in OCT solution (Triangle Biomedical Science, Durham, NC), placed in 10% formaldehyde for histologic analysis, flash frozen for future tissue homogenization, or used for AVIC isolation. Isolation was by collagenase digestion, as previously described,[2] and culture was in growth medium (Medium 199 [Lonza Walkersville, Inc, Walkersville, Md] with amphotericin B, penicillin G, streptomycin, and 10% fetal bovine serum) in an incubator supplied with 5% carbon dioxide. Cells of passages 3 to 6 were grown to 70% to 90% confluence and subcultured to 24-well cell culture plates for experiments. Cells of each patient were maintained as independent cultures.

Chemicals and Reagents

Medium 199 was purchased from Lonza (Walkersville, Md). Rabbit polyclonal antibody against human BMP-2 was obtained from ProSci (Poway, Calif). Mouse affinity purified antibody against human IL-1RA was obtained from eBioscience (San Diego, Calif). Human IL-1RA DuoSet ELISA kit was obtained from R&D Systems (Minneapolis, Minn). Recombinant human IL-1RA was a generous gift from Dr Charles Dinarello (Aurora, Colo). Collagenase and other reagents were purchased from Sigma Chemical Co (St Louis, Mo).

AVIC Treatment

AVICs studied by enzyme-linked immunosorbent assay (ELISA) for IL-1RA were serum-starved for 24 hours after reaching approximately 90% confluence, treated with serum-free medium alone (controls), lipopolysaccharide (LPS, 200 ng/mL), and peptidoglycan (PGN, 10 μg/mL). After 24 hours, the cells were washed once with cold phosphate-buffered saline (PBS) and cell lysates were prepared with 200 μL of mammalian protein extraction reagent (Thermo Scientific, Rockford, Ill), centrifuged for 10 minutes at 10,000 RPM, and supernatant collected. AVICs treated with recombinant IL-1RA (100 μg/mL) were pretreated in serum-free medium alone (controls) or IL-1RA 1 hour before treatment with LPS or PGN (200 ng/mL and 10 μg/mL, respectively). Cells were washed once with cold PBS and lysed with mammalian protein extraction reagent.

Immunohistochemistry

Immunohistochemical detection of IL-1RA in aortic valve leaflets was performed by the biotin-linked peroxidase technique. Cryosections (5 μm) were prepared and dried at room temperature for 2 hours. Sections were fixed with 4% paraformaldehyde in PBS at room temperature for 10 minutes and then washed with PBS. Unless indicated, all incubations were performed at room temperature. Endogenous peroxidase activity was quenched by incubating sections with 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were incubated for 1 hour with 10% goat serum in PBS to block nonspecific binding sites. Sections were then incubated overnight at 4°C with mouse affinity purified antibody against IL-1RA (10 μg/mL in PBS containing 10% goat serum), followed by a 60-minute incubation with biotinylated goat anti-mouse immunoglobulin G (1:200 dilution with PBS containing 10% goat serum) from the Mouse ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, Calif). Subsequently, sections were washed 3 times with PBS and incubated with avidin–biotin–peroxidase complex (1:50 dilution with PBS) for 30 minutes. After thorough washes with PBS, color development was carried out with peroxidase substrate (0.03% H_2O_2 and 0.05% diaminobenzidine in double-distilled H_2O). Sections were counterstained with hematoxylin and eosin.

Cytokine Assay

IL-1RA levels in cell lysates and culture media were determined using a DuoSet ELISA kit per manufacturer's instructions.

Immunoblotting

Cell lysates were separated on 4% to 20% minigels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif). After blocking with 5% fat-free milk solution, BMP-2 was detected using primary antibodies to BMP-2 and then a corresponding peroxidase-linked