

Valvular Heart Disease

Neoangiogenesis, T-Lymphocyte Infiltration, and Heat Shock Protein-60 Are Biological Hallmarks of an Immunomediated Inflammatory Process in End-Stage Calcified Aortic Valve Stenosis

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OBJECTIVES	We investigated the main biomolecular features in the evolution of aortic stenosis, focusing on advanced lesions.
BACKGROUND	“Degenerative” aortic valve stenosis shares risk factors and inflammatory similarities with atherosclerosis.
METHODS	We compared nonrheumatic stenotic aortic valves from 26 patients undergoing surgical valve replacement (group A) and 14 surgical control patients (group B). We performed semiquantitative histological and immunohistochemical analyses on valve leaflets to measure inflammation, sclerosis, calcium, neoangiogenesis, and intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression. We assessed heat shock protein 60 (<i>hsp60</i>) gene expression as an index of cellular stress and C-reactive protein, erythrocyte sedimentation rate, and fibrinogen as systemic inflammatory markers.
RESULTS	In group A valves, we found a prevalence of calcium nodules surrounded by activated inflammatory infiltrates, neovessels, and abundant ICAM-1, VCAM-1, and <i>hsp60</i> gene expression. Specimens from group B were negative for all of these markers, except 2 of 14 positivity for <i>hsp60</i> . The presence of active inflammatory infiltrates correlated with an abundance of thin neovessels ($p < 0.01$) and <i>hsp60</i> gene expression ($p = 0.01$), whereas neoangiogenesis correlated with inflammation ($p = 0.04$), calcium ($p = 0.01$), and <i>hsp60</i> gene expression ($p = 0.04$).
CONCLUSIONS	“Degenerative” aortic valve stenosis appears to be a chronic inflammatory process associated with atherosclerotic risk factors. The coexistence of neoangiogenesis, T-lymphocyte infiltration, adhesion molecules, and <i>hsp60</i> gene expression indicates an active immunomediated process in the final phases of the disease. (J Am Coll Cardiol 2004;43:1670–6) © 2004 by the American College of Cardiology Foundation

Nonrheumatic aortic valve stenosis is the most common valve disease in Europe and North America (1). Here, aortic sclerosis is present in about 26% of the population 65 years of age and older, whereas stenosis is evident in 2% to 7% of this age group (1). Calcified aortic valve stenosis increases in prevalence with age; it has a progressive course and is the most common reason for valve replacement (2). Several studies have demonstrated cardiovascular risk factors in common with atherosclerosis, including hyperlipidemia, hypertension, and diabetes (1,3,4), and similar pathological features, including endothelial damage, lipid deposition, and inflammatory infiltrates, making early aortic valve lesions appear similar to atheromas (5–7). Further evidence exists that progressive calcified aortic stenosis is an “active”

biological process, similar to vascular calcification (8), sustained by mechanical stress and inflammation, and associated with the synthesis of extracellular matrix proteins, such as metalloproteinases (9), tenascin-C (10), osteopontin (11), and bone sialoprotein (12).

More recently, heterotopic ossification, associated with inflammation and neoangiogenesis, has been described, suggesting an unexpected process of tissue repair in end-stage calcified heart valves (12). On the basis of a previous study (7), we hypothesized that chronic valve inflammation is triggered by local injury and sustained by a cellular immune response mediated by heat shock proteins, activated T-lymphocytes, and adhesion molecules. These events are similar to those occurring in atherosclerotic lesions (13,14), where inflammatory infiltrates, neoangiogenesis, and endothelial activation represent a route for leukocyte recruitment and tissue infiltration, thus maintaining a chronic, inflammation-promoting plaque evolution (15,16).

In this study we investigated inflammatory features in end-stage aortic valve lesions from human surgical samples,

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Abbreviations and Acronyms

CRP	= C-reactive protein
DNA	= deoxyribonucleic acid
ESR	= erythrocyte sedimentation rate
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
HE	= hematoxylin-eosin
<i>hsp60</i>	= heat shock protein-60
ICAM-1	= intercellular adhesion molecule-1
LDL	= low-density lipoprotein
mRNA	= messenger ribonucleic acid
PBS	= phosphate-buffered saline
PCR	= polymerase chain reaction
RT	= reverse transcription
VCAM-1	= vascular cell adhesion molecule-1
WVG	= Weigert-van Gieson

using histological, immunohistochemical, and molecular biology procedures. We also measured and compared systemic lipid and inflammatory markers in these patients and in a control group.

METHODS

We examined specimens of aortic valve leaflets obtained from 26 patients who had undergone aortic valve replacement for calcified aortic stenosis (group A), and 14 nonstenotic aortic valve samples as controls (group B). The control cases were collected from surgical patients affected by aortic

valve insufficiency due to aneurysms on the ascending aorta, with a diagnosis based on preoperative Doppler echocardiography, cardiac catheterization, or both. Relevant clinical data from patients are listed in Table 1.

Diagnosis of aortic valve stenosis was confirmed on inspection of the valve during surgery. To ensure that leaflets obtained were representative of the entire valve and of non-rheumatic aortic stenosis, exclusion criteria were: 1) any evidence of post-rheumatic endocarditis, and 2) significant aortic regurgitation or other coexistent valvular disease, with the exception of calcifications of the mitral ring. The research protocol was approved by the local ethics committee.

Histological analysis. All samples were fixed in 10% buffered formalin for 24 h, decalcified overnight with formic acid, and processed for routine paraffin embedding. Valve samples were excised vertically through the valve cusps near the center of each leaflet. Sections 5- μ m-thick were obtained from paraffin-embedded samples and stained with both hematoxylin-eosin (HE) and Weigert-van Gieson (WVG).

Histological sections were analyzed semiquantitatively according to the following scoring system: for inflammatory cells (0, 1+, 2+, 3+: absence, minimal infiltrates, infiltrates present in aggregates, or widespread infiltrates, respectively); for sclerosis (0, 1+, 2+, 3+: absence, some presence, predominant, and complete, respectively); for calcium (0, 1+, 2+, 3+: absence, isolated areas, multiple areas with

Table 1. Demographic and Clinical Data of Surgical Patients Undergoing Aortic Valve Replacement

	Group A	Group B	p Value
Number	26	14	
Age (yrs) (mean \pm SD)	71.6 \pm 5.2	65.4 \pm 4	0.001
Gender (M/F)	15/11	12/2	0.075
Smokers	16/26	2/14	0.004
Hypertension	20/26	7/14	0.083
Diabetes	3/26	1/14	0.709
Duration of symptoms (months)	35.8 \pm 39.2	17.5 \pm 14	0.495
Coronary artery disease	11/26	4/14	0.279
Clinical coronary heart disease	8/26	1/14	0.088
Carotid atherosclerosis	24/26	2/14	< 0.0001
Clinical cerebrovascular disease	5/26	0/14	0.186
Peripheral arterial disease	3/26	0/14	0.186
Angina	11/26	1/14	0.021
Dyspnea	23/26	9/14	0.068
Syncope	7/26	0/14	0.033
Hypertriglyceridemia (>150 mg/dl)	3/26	2/14	0.876
Hypercholesterolemia (>200 mg/dl)	15/26	3/14	0.014
LDL cholesterol >100 mg/dl	20/26	4/14	0.001
LDL cholesterol (mg/dl) (mean \pm SD)	132.7 \pm 34.2	94.7 \pm 21.4	0.001
HDL cholesterol (mg/dl) (mean \pm SD)	60 \pm 10.2	55.5 \pm 18.2	0.310
Triglycerides (mg/dl) (mean \pm SD)	136.6 \pm 62.2	112 \pm 54.6	0.164
Total cholesterol (mg/dl) (mean \pm SD)	223.8 \pm 46.4	166.2 \pm 34	< 0.0001
Erythrocyte sedimentation rate (mm/h)	33.4 \pm 26.7	11 \pm 2.7	< 0.0001
C-reactive protein (mg/dl) (mean \pm SD)	17 \pm 20	4 \pm 1.3	0.010
Fibrinogen (mg/dl) (mean \pm SD)	550.6 \pm 182.3	339 \pm 76	< 0.0001
Valvular area (cm ²) (mean \pm SD)	0.69 \pm 0.15	—	—
Mean transvalvular gradient (mm Hg) (mean \pm SD)	57 \pm 14	—	—
Left ventricular ejection fraction (mean \pm SD)	52 \pm 10	51.9 \pm 6.3	0.633
Ascending aorta diameter (mean \pm SD)	36 \pm 4	49 \pm 8	< 0.0001

HDL = high-density lipoprotein; LDL = low-density lipoprotein.

large amounts of calcium, or widespread infiltration, respectively); and for neoangiogenesis (0, 1+, 2+, 3+: absence, isolated neovessels, minimal aggregates, or abundant neovessels, respectively).

Immunohistochemical analysis. Immunohistochemical analysis was performed on all samples to detect lymphocytes (LCA; DAKO, Denmark; dilution 1:400), T-lymphocytes (UCHL1; DAKO; dilution 1:200), vascular cell adhesion molecule-1 (VCAM-1) (CD106; Novocastra Laboratories, United Kingdom; dilution 1:75), and intercellular adhesion molecule-1 (ICAM-1) (CD54; Novocastra Laboratories; dilution 1:25). Sections mounted on slides were blocked with 3% H₂O₂, washed with phosphate-buffered saline (PBS), incubated for 60 min with the primary antibody, and rewashed with PBS. A biotin-labeled secondary antibody was applied for 30 min, followed by a streptavidin-biotin-peroxidase conjugate (ABC Elite, Vector Laboratories, Burlingame, California). A standard peroxidase enzyme substrate, 3,3'-diaminobenzidine, was used to yield a black reaction product. Sections were counterstained with hematoxylin. The expression of VCAM-1 and ICAM-1 was graded on a semiquantitative scale, ranging from 0 (no expression) to 3 (most intense expression).

Semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR). We detected the expression of heat shock protein 60 (*hsp60*) by a semiquantitative evaluation of its specific messenger ribonucleic acid (mRNA). We examined samples of both aortic degenerative valves and control samples for *hsp60* mRNA. One leaflet from each sample was immediately frozen in liquid nitrogen and stored at -80°C; total RNA was extracted using the Tripure Isolation Reagent (Roche Molecular Biochemical, Mannheim, Germany) following the manufacturer's instructions (17). To avoid the contamination of genomic deoxyribonucleic acid (DNA), the RNA was treated with DNase RQ1 (Promega, Madison, Wisconsin); 300 ng were used for RT, using oligo(dT)₁₂₋₁₈ (Pharmacia, Uppsala, Sweden). Both RNasin and M-MLV RT were obtained from Promega (Madison, Wisconsin). The RT was performed at 42°C for 1 h. To verify the absence of DNA contamination, a parallel reaction without RT was performed as a control. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reference gene) was co-amplified with the *hsp60* target gene, in a multiplex PCR using two couples of specific primers (17). The PCR products were separated on a 6% polyacrylamide gel and silver-stained. The density of the GAPDH and *hsp60* bands were analyzed using the National Institutes of Health Image 1.60 program.

Serum lipids and inflammatory markers. Peripheral venous blood was assayed for total cholesterol, triglycerides, high-density lipoprotein cholesterol (using an enzymatic temporized end point method), low-density lipoprotein (LDL) cholesterol (calculated by Friedewald's equation), erythrocyte sedimentation rate (ESR) (using Westergren's method), C-reactive protein (CRP) (using a high-sensitivity

spectrophotometric method), and fibrinogen (using a coagulation method).

Statistical analysis. For comparisons between groups the nonparametric Kruskal-Wallis test was used for quantitative parameters and the chi-square test in contingency tables for qualitative parameters. Pearson's correlation coefficient was used to evaluate links between quantitative variables. Statistical significance was set at $p < 0.05$.

RESULTS

Clinical findings. The clinical findings in patients affected by degenerative aortic valve stenosis differed from control patients in some atherosclerosis risk factors, as summarized in Table 1.

Macroscopic findings. All aortic valves examined were tricuspid. Macroscopically, they were noticeably thickened, irregular, and showed multiple, contiguous nodules extending from the base toward the middle portion, with preserved commissures. The control valve group appeared normal, with only slight thickening of the leaflets.

Light microscopy analysis. At HE and WVG staining, we found large amounts of calcification in a nodular form in almost all samples (25 of 26) (Fig. 1a), and dense fibrosis in all of them. In 3 of 26 aortic valve samples we observed cartilage tissue with chondrocytes and areas of ossification, with bone marrow structure (Fig. 1b). Inflammatory infiltrates were present in almost all samples (25 of 26) in small aggregate areas around substantial calcium deposits, or diffused throughout the leaflets, on the aortic or ventricular side of the subendothelial layers. The T-lymphocytes were the most predominant cells in these infiltrates, but monocytes and plasma cells were also present. A correlation was found between valve inflammation and duration of symptoms (chi-square = 4.227; $p = 0.04$). All control samples appeared negative for neoangiogenesis, inflammatory infiltrates, and calcium deposits, consistent with the notion that valve cusps in noninflamed conditions are nonvascular tissues, sufficiently thin to allow complete nutrition by diffusion (Fig. 1c).

Neoangiogenesis was evident in 85% (22 of 26) of aortic valve leaflets examined, with a larger number of neovessels in areas where inflammatory infiltrates were more dense (2+ or 3+). We observed a high number of neovessels with thin walls (thickness <10 μ m) (Fig. 2a) in areas rich in lymphocyte infiltrates, and neovessels with thick walls (thickness >30 μ m) in both inflammatory and fibrotic areas (Fig. 2b). Correlations were found between total neoangiogenesis and amounts of inflammatory cells (chi-square = 4.129; $p = 0.042$); between neoangiogenesis and calcium (chi-square = 5.643; $p = 0.01$); and between thin vessel walls and abundance of inflammatory cells (chi-square = 6.686; $p = 0.01$).

Immunohistochemistry. Endothelial cells of neovessels were positive for ICAM-1 and VCAM-1, with different degrees of expression (Figs. 2c and 2d), whereas only

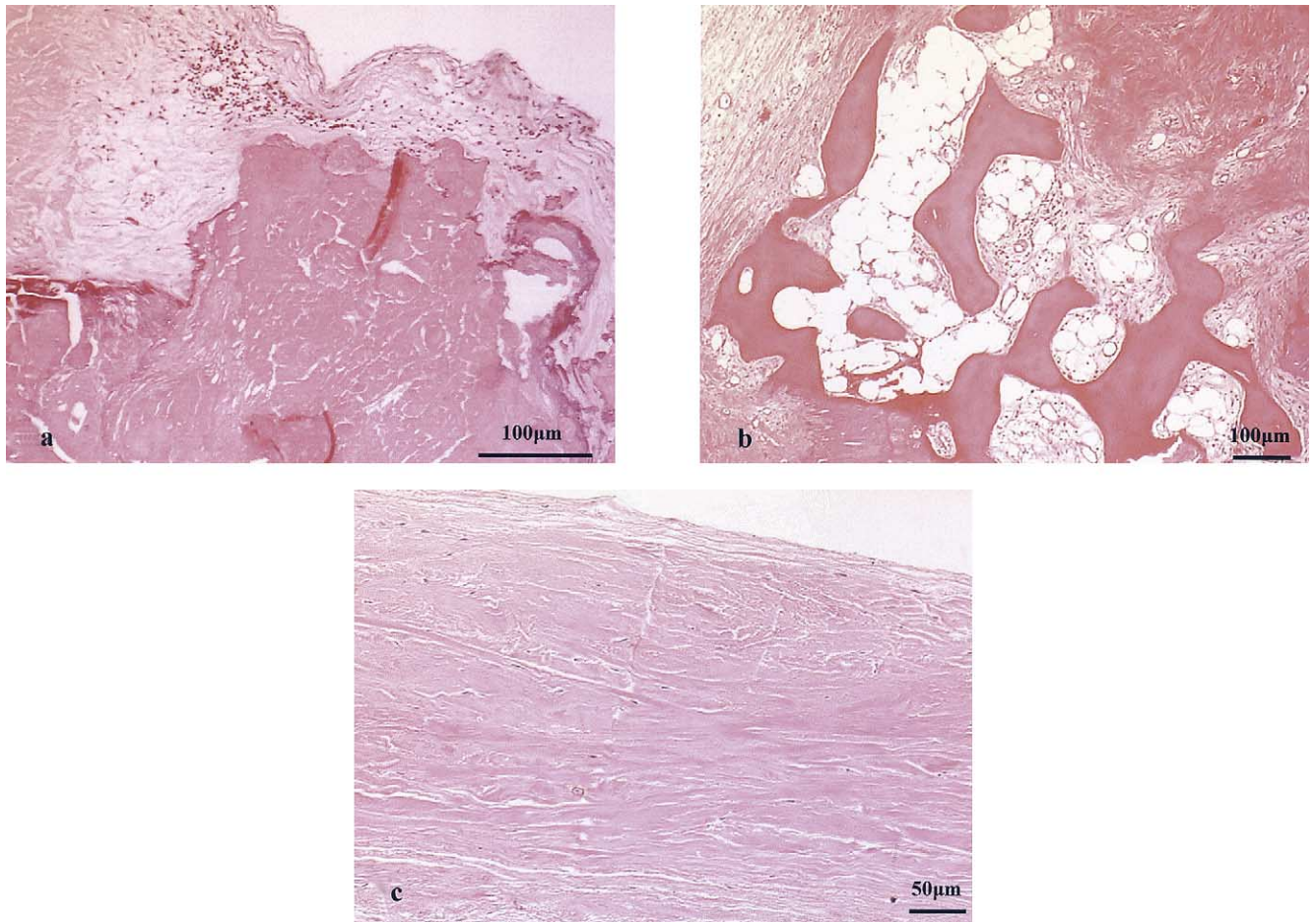


Figure 1. (a) The majority of calcified stenotic valves studied, as in this case, are occupied by calcium deposits (violet area, hematoxylin-eosin [HE]). (b) Case 12: areas with ossification and bone marrow formation were observed in a calcified aortic valve leaflet (HE). (c) Sample of normal aortic valve from control patient showing slight thickening and fibrosis of the valve leaflet (HE).

ICAM-1 expression was observed in inflammatory cells. Endothelial activation was co-localized with inflammatory infiltrates. A lesser degree of adhesion molecule expression was evident on endothelial valve surfaces. A positive correlation was found between ICAM-1 expression and calcium deposits (chi-square = 6.232; $p = 0.044$). In control samples of aortic valves, adhesion molecule expression was negative.

Expression of *hsp60*. In group A, *hsp60* mRNA expression was present at a variable degree of 0.22- to 1.13-fold from the constitutively expressed GAPDH gene (Fig. 3). We found that *hsp60* gene expression is correlated to neo-angiogenesis (chi-square = 4.267; $p = 0.03$) and to T-lymphocyte inflammatory infiltrates (chi-square = 5.745; $p = 0.01$). In group B, *hsp60* mRNA expression proved positive in 2 of 14 of the samples examined.

Serum lipid and inflammatory markers. Serum lipid and inflammatory markers were evaluated in the group of cases (group A) and in the surgical controls (group B), with statistical significance (Table 1). No correlation was found between abundance of inflammatory infiltrates and systemic inflammatory markers.

DISCUSSION

Valve tissue findings. Our main finding is evidence that an active inflammatory process, sustained by neoangiogenesis, occurs in “degenerative” aortic valve stenosis. Inflammatory infiltrates in fibrotic areas are localized around large nodular calcium deposits, the most prominent component of advanced lesions. Inflammatory infiltrates are characterized by abundant T-lymphocytes and neovessels. Calcium deposits, neoangiogenesis, and inflammation appear to be relevant biological features in the final stage of calcified valve lesions in comparison to the early phase of aortic disease, where cholesterol, lipoproteins, and macrophages are prevalent in relation to T-lymphocytes and tissue mineralization (5,6). In contrast, in our control samples, inflammatory cells and neovessels are absent. Thick neovessel walls are associated with fibrosis and sparse inflammatory cells, whereas thin neovessel walls are localized in areas with a prevalence of T-lymphocyte infiltration, showing a significant correlation with the latter. The activated endothelium, expressing VCAM-1 and ICAM-1, represents the main route for tissue leukocyte infiltration and for a self-perpetuating inflammatory process (18).

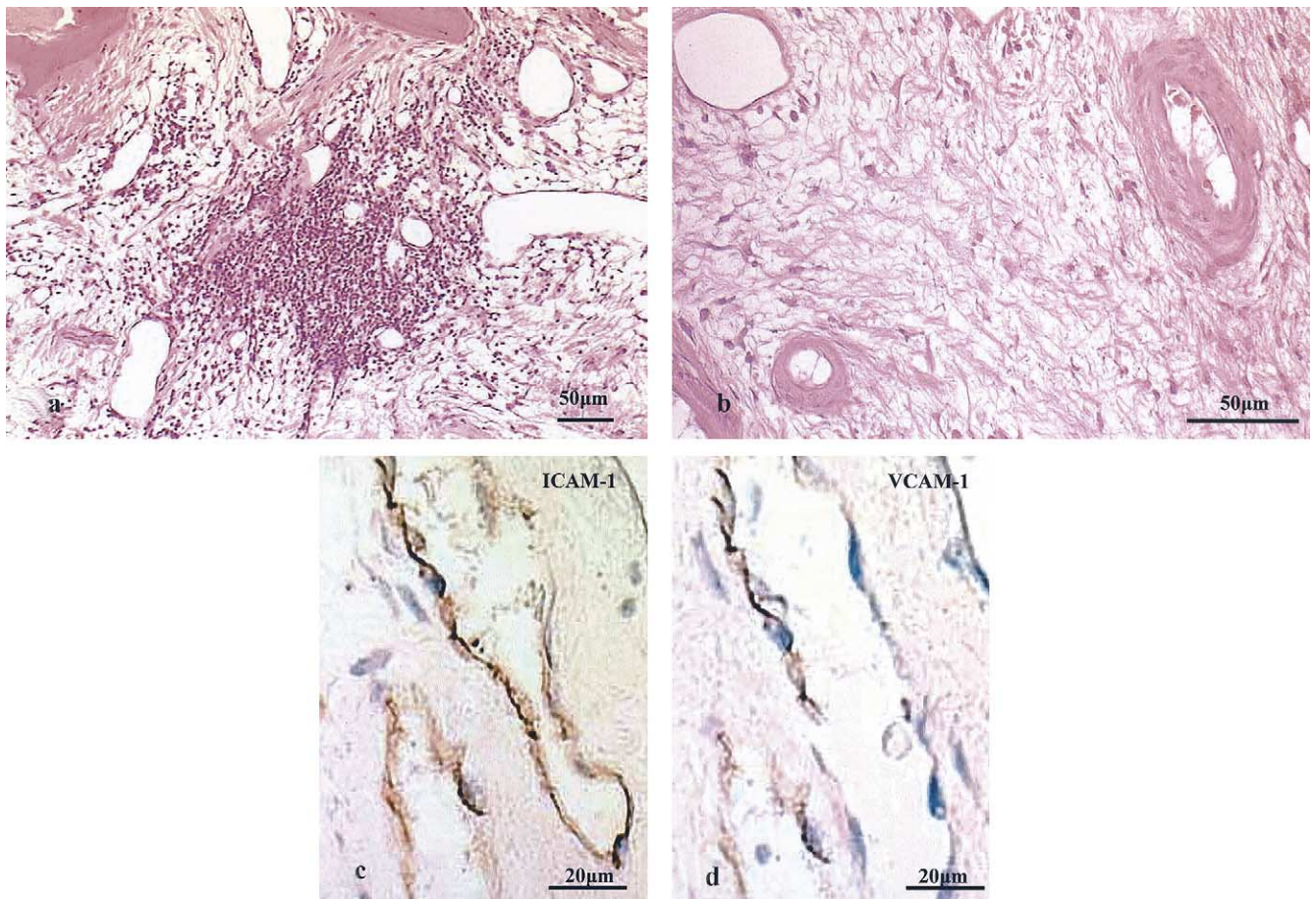


Figure 2. (a) In a calcified aortic valve, abundant inflammatory infiltrates are observed with small thin-walled neovessels, prevalently composed of T-lymphocytes, plasma cells, and macrophages (hematoxylin-eosin [HE]). (b) In a calcified aortic valve, thick-walled neovessels are observed in a fibrotic area devoid of inflammatory infiltrates (HE). (c, d) Immunohistochemical positivity for intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (stained dark brown) is observed in the endothelial cells of thin-walled neovessels 3,3'-diaminobenzidine.

In our study, the correlation between neoangiogenesis and calcium deposits supports the hypothesis that calcification is an active process sustained by neoangiogenesis and vascular endothelial growth factors, rather than a passive process of dystrophic calcification into degenerating connective tissue (10,11). Moreover, three valve samples included endochondral and bone tissue associated with bone marrow, showing neoangiogenesis and activated endothelium in percentages similar to those identified by Mohler et al. (12). We demonstrated *hsp60* gene expression in all calcified stenotic valves, correlating both with inflammatory infiltrates and with neoangiogenesis. Heat shock proteins are expressed by cells under stress conditions (such as inflammation induced by cholesterol and triglyceride-containing lipoprotein particles, viral or bacterial antigen exposure, shear or mechanical stress) (14,19,20). In 2 of 14 cases of control valves, *hsp60* gene expression suggests that it may be primarily generated within the cells as a response to a mechanical injury in degenerative aortic valve disease (14). In contrast, in calcified aortic valves, the process probably begins with endothelial damage induced by known risk factors, which leads to *hsp60* cellular expression and

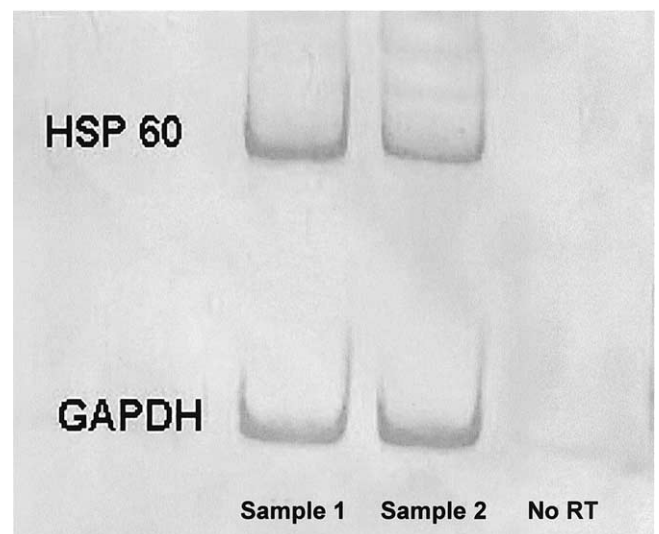


Figure 3. Gel electrophoresis of two samples from patients with calcified aortic valve disease (Cases 13 and 19) after reverse transcription (RT)-polymerase chain reaction. Both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heat shock protein-60 (*hsp60*) indicate their respective amplification products. In the “No RT” lane, retrotranscription after the DNase treatment of the samples was omitted.

subsequent triggering of an autoimmune reaction against local antigens, culminating in stenotic valve disease.

Comparison with previous published reports. Many investigators agree that valve calcification is an active inflammatory process. Collagen (tenascin-C) or bone matrix proteins (osteopontin and osteonectin) produced by activated macrophages and vascular cells (10,21) promote neoangiogenesis (22) and are involved in the regulation of calcium deposition in atherosclerotic plaques (21-23), as well as in cardiac valves (10). In contrast, Mohler et al. (12) suggest that dystrophic calcification is a passive degeneration of connective tissue, whereas heterotopic ossification is an active process of abnormal tissue repair. Our study shows no difference in the correlation between calcium deposits and neoangiogenesis either in calcified or in ossified valve samples. Conversely, the existing correlations among calcification, neoangiogenesis, inflammation, and *hsp60* gene expression, as well as that between neoangiogenesis and inflammation, or neoangiogenesis and calcium, support the hypothesis that global valve mineralization is a biologically active process.

The coexistence of biological features such as tissue mineralization, high prevalence of T-lymphocyte infiltration, neoangiogenesis, endothelial activation, and *hsp60* gene expression may be indicative of a cell-mediated immune mechanism, as previously suggested for the development of atherosclerotic plaques (13,14). The demonstration of T-lymphocyte activation, by detection of interleukin-2 receptor expression in nonrheumatic aortic valve stenosis (7), suggests that an immune reaction against local antigens may be a pathogenic factor for valve calcification and stenosis. No data are currently available showing the coexistence of T-lymphocyte infiltration, neoangiogenesis, endothelial activation, and *hsp60* gene expression as hallmarks of an immunomediated inflammatory process in the end-stage of aortic stenosis. In these advanced phases of the disease, abnormal rheology and mechanical stress overload, exacerbated by loss of aortic wall compliance in elderly patients, may induce additional changes in the microstructure of the aortic leaflet (24).

Clinical and biohumoral features. In this study, patients with calcified aortic valve stenosis frequently exhibited risk factors for atherosclerosis such as smoking and hypercholesterolemia. Systemic hypertension was present in 80% of group A patients and 50% of group B patients. In the latter, hypertension alone appeared insufficient to induce a visible inflammatory tissue response. Moreover, group A patients showed high serum levels of total and LDL cholesterol, and of inflammatory markers such as ESR, CRP, and fibrinogen, compared to group B.

Comparison with the published data. Risk factors for atherosclerosis are probably pathogenetically involved in early valve lesions by inducing endothelial injury (4-6). Recent data by Poggianti et al. (25) demonstrate an association between aortic valve sclerosis and systemic endothelial dysfunction. We confirmed a correlation among smoking,

carotid atherosclerosis, hypercholesterolemia, and high serum LDL cholesterol levels on the one hand, and calcified aortic valve stenosis on the other. Further studies also highlight a new role for modified serum lipoproteins and vascular smooth muscle cells in the development of calcification in atherosclerosis.

Deposition of oxidized LDL in the artery wall increases monocyte-induced vascular calcification (26), stimulates calcification by enhancing osteogenic differentiation of vascular smooth muscle cells (27), and influences the progression of valve calcification and stenosis (28). Similar to atherosclerotic vascular disease, previous studies have shown increased plasma levels of CRP (29) and ICAM-1 (18), systemic markers of inflammation in patients with degenerative aortic valve stenosis. In agreement with Galante et al. (29), CRP plasma levels in patients with calcified valve stenosis are higher, together with ESR and fibrinogen. Moreover, by ultrasound and catheterization we found evidence of diffuse atherosclerotic disease (involving the carotids, and coronary and peripheral arteries) in the same patients, which might further explain the increase of systemic inflammatory markers. This issue requires additional evaluation to confirm whether atherogenesis may indeed be considered an extensive chronic inflammatory process (30).

Study limitations. Limitations to our study lie in the objective difficulties of: 1) analyzing the progression from sclerosis to end-stage aortic valve lesions in order to support our research into similar characteristics between atherosclerosis and aortic valve disease and 2) the lack of surgical patients with similar age, lipid levels, and other risk factors for atherosclerosis without aortic valvular disease, to form an ideal control group.

Conclusions. The end-stage of nonrheumatic aortic valve stenosis, characterized by the high prevalence of tissue mineralization, shows some biological features of an active inflammatory process. The co-existence of T-lymphocytes and neoangiogenesis, adhesion molecule expression in endothelial and inflammatory cells, and *hsp60* gene expression is indicative of a chronic, active immunomediated process. Inflammation and progression of mineralization appear to be sustained by a constant angiogenetic process. The presence of local and systemic inflammation in patients with degenerative aortic valve stenosis, and the similarities of risk factors for atherosclerosis, reinforce the hypothesis that a common inflammatory process is involved in valvular disease and atherosclerosis.

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