Calcified Rheumatic Valve Neoangiogenesis Is Associated With Vascular Endothelial Growth Factor Expression and Osteoblast-Like Bone Formation

Nalini M. Rajamannan, MD; Thomas B. Nealis, BSc; Malayannan Subramaniam, PhD; Sanjay Pandya, MD; Stuart R. Stock, PhD; Constatine I. Ignatiev, PhD; Thomas J. Sebo, MD, PhD; Todd K. Rosengart, MD; William D. Edwards, MD; Patrick M. McCarthy, MD; Robert O. Bonow, MD; Thomas C. Spelsberg, PhD

Background—Rheumatic heart disease is the most common cause of valvular disease in developing countries. Despite the high prevalence of this disease, the cellular mechanisms are not well known. We hypothesized that rheumatic valve calcification is associated with an osteoblast bone formation and neoangiogenesis.

Methods and Results—To test this hypothesis, we examined human rheumatic valves replaced at surgery (n = 23), normal human valves (n = 20) removed at cardiac transplantation, and degenerative mitral valve leaflets removed during surgical valve repair (n = 15). Microcomputed tomography was used to assess mineralization fronts to reconstruct the extents of mineralization. Immunohistochemistry was used to localize osteopontin protein, α-actin, osteocalcin, vascular endothelial growth factor, von Willebrand factor, and CD68 (human macrophage). Microcomputed tomography demonstrated complex calcification developing within the heavily calcified rheumatic valves, not in the degenerative mitral valves and control valves. Immunohistochemistry localized osteopontin and osteocalcin to areas of smooth muscle cells within microvessels and proliferating myofibroblasts. Vascular endothelial growth factor was present in areas of inflammation and colocalized with the CD68 stain primarily in the calcified rheumatic valves. Alizarin red, osteopontin, and osteocalcin protein expression was upregulated in the calcified rheumatic valves and was present at low levels in the degenerative mitral valves.

Conclusions—These findings support the concept that rheumatic valve calcification is not a random passive process but a regulated, inflammatory cellular process associated with the expression of osteoblast markers and neoangiogenesis.

Key Words: angiogenesis • calcification • cardiovascular diseases • rheumatic heart disease • valves
Methods

Human Calcified Rheumatic Valves and Degenerative Mitral Valves

The use of all human tissue was approved by the Human Subject Protection Offices at the Mayo Clinic (MC) (1732-98) and Northwestern University (1041-002). Coded tissues were collected at the time of surgery and autopsy. We obtained human calcified mitral and aortic rheumatic valves from patients (MC) (n=23; mean age, 72 years; range, 44 to >89 years; male, 13; female, 10) at the time of surgical valve replacement. We compared them to degenerative mitral valve tissue (MC) (n=15; mean age, 72 years; range, 54 to >89 years; male, 14; female, 1) removed at the time of surgical valve repair. Normal control valves were obtained from the time of heart transplantation (MC) (n=20; mean age, 51 years; range, 19 to 56 years; male, 16; female, 4). Upon dissection, tissues were immersed in formalin for paraffin embedding. A total of 6 valves were obtained for microCT analysis from Northwestern University, from which no patient identifiers were obtained.

Immunohistochemistry Analysis

Immunostaining of the valves for demonstration of angiogenesis, cellular proliferation, and bone matrix protein expression was performed on paraffin-embedded tissues using a streptavidin–biotin method. Angiogenic factors, endothelial cells, macrophage cells, proliferating nuclear cell antigen (PCNA), and osteopontin were tested. The following primary antibody was used for angiogenic factor detection: anti–vascular endothelial growth factor (VEGF; Oncogene). Anti-α-actin (Dako, A/S) was used to detect the presence of myofibroblast cells and smooth muscle cells, and anti–von Willebrand factor (Dako, A/S) was used as an endothelial cell marker. Inflammatory markers for human macrophage cells identified were CD68 staining (CD68/HRP; Dako, A/S). PCNA, a DNA polymerase, was used as a marker for cellular proliferation (Dako). Osteopontin and osteocalcin, glycosylated phosphoproteins important in skeletal bone mineralization, were detected with anti–von Willebrand factor (Abcam).

After fixation, slides were blocked with 3.0% (vol/vol) H2O2 in methanol for 30 minutes, washed with PBS (wt/wt) for 30 minutes, and incubated in primary antibodies for 16 hours at 4°C. Anti-VEGF required a pretreatment antigen enhancing step of heating slides in 95°C 0.1 mol/L citrate buffer, pH 6.0, for 10 minutes before incubation with the anti-VEGF primary antibody. After primary antibody incubations, slides were incubated with biotinylated secondary antibodies (Dako, A/S) for 60 minutes and streptavidin/HRP complex (Dako, A/S) for 60 minutes at room temperature. Slides treated with primary antibody anti-CD68/HRP complex did not require secondary antibodies and were directly developed. To develop color, slides were incubated in 3-amin-9-ethylcarbazole (Sigma Diagnostics, Inc) as a chromogen with 1% (vol/vol) H2O2 for 15 minutes, followed by counterstaining with hematoxylin acid solution (Sigma Diagnostics, Inc) for 10 minutes and washing with H2O for 3 minutes. To provide a degree of quantification to our analysis, PCNA expression was determined with digital image analysis. The samples were scored semiquantitatively for the expression of the immunostains present within the valve tissue and are listed in the Table. Results are also shown qualitatively in the photomicrographs.

Microcomputed Tomography

After fixing in formalin, the valves were examined with a Scanco microcomputed tomography (MicroCT) system (MicroCT–40) operated at 45 kV. Sampling was with ~8-μm voxels (volume elements), maximum sensitivity (1000 projections, 2048 samples), and 0.3 second per projection integration (n=6 valves total were tested).

Statistical Analysis

Comparison was made among the 3 groups using ANOVA. Scheffe’s method of adjustment was performed for multiple pairwise comparisons. All statistical tests were 2 tailed, and values of P<0.05 was considered significant.

Results

Immunohistochemistry of the Human Rheumatic Valves for Neoangiogenesis Markers

Hematoxylin and Eosin and Masson Trichrome Stains

Special stains for hematoxylin and eosin and Masson trichrome stains are shown in Figure 1-A1 and 1-A2. Both the hematoxylin and eosin and the Masson trichrome demonstrate a marked inflammatory cellular infiltrate within the rheumatic valve lesions. In Figure 1-A2, the Masson trichrome purple staining indicated an increase in collagen synthesis in areas of calcification and mineralization. Furthermore, there were multiple areas of new vessels forming (see arrows) within the valve lesions.

Immunohistochemistry for Vascular Markers

Figure 1-B1 demonstrates the low-power magnification of the α-actin staining smooth muscle cells within the areas of neoangiogenesis in the rheumatic valves. Figure 1-B2 demonstrates the high-power magnification of the α-actin staining, with the arrow pointing to the new vessels. Figures 1-C1 and 1-C2 demonstrate the von Willebrand stain demarcating the endothelial lining of the new vessels forming in the rheumatic valves. Immunohistochemistry for human macrophage cells shown in Figures 1-D1 and 1-D2 confirm the presence of a marked inflammatory cellular infiltrate that has been described in previous studies. Figures 1-E1 and 1-E2 demonstrate the stain for VEGF in the area of angiogenesis and CD68-positive inflammatory cells. The Table indicates that there is upregulation of vascular markers present in the rheumatic valves as compared with the controls.

Immunohistochemistry of Calcified Human Rheumatic Valves for Identification of Bone Matrix Markers

PCNA Staining

Figure 2-A1 shows that PCNA-positive brown-staining cells were detected in the proliferating myofibroblasts. The total density of PCNA-positive cells was 37.64±7.3 cells/mm2 in

Comparison of Rheumatic, Control, and Degenerative Valves

<table>
<thead>
<tr>
<th></th>
<th>α-Actin</th>
<th>Osteopontin</th>
<th>CD68</th>
<th>VEGF</th>
<th>vWF</th>
<th>PCNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5±1</td>
<td>0.5±1</td>
<td>1.5±1.5</td>
<td>1.75±1.75</td>
<td>1.25±1.25</td>
<td>1.26±0.32</td>
</tr>
<tr>
<td>Calcified rheumatic</td>
<td>100±101*</td>
<td>148.5±145*</td>
<td>107.8±89.9*</td>
<td>52.6±41.5*</td>
<td>40±25.1*</td>
<td>37.6±7.3*</td>
</tr>
<tr>
<td>Degenerative mitral</td>
<td>15.0±1</td>
<td>8.4±0.6</td>
<td>11.3±0.7</td>
<td>20±1</td>
<td>15±1.3</td>
<td>4.9±0.4</td>
</tr>
</tbody>
</table>

vWF indicates von Willebrand factor.

*P<0.05, calcified rheumatic valves vs control and degenerative mitral valves.
the rheumatic valves and 1.26±0.32 cells/mm² in control valves (P<0.001), as shown in the Table.

**Osteoblast Bone Markers**

Figure 2-A2 shows the immunohistochemistry for osteopontin, a bone matrix protein important in osteoblast mineralization. Figure 2-A2 demonstrates that osteopontin localizes to the neointima of new vessel formation (the arrow) and proliferating myofibroblasts within the rheumatic valves. Figures 2-B1 and 2-B2 demonstrate the alizarin red stain, a marker for mineralization important in skeletal bone formation. Figure 2-B1, the low-power magnification, demonstrates the large area of alizarin red stain in the rheumatic valve. Figures 2-C1 and 2-C2 are the low- and high-power magnifications of the osteocalcin stains. Osteocalcin is an osteoblast-specific marker. The arrow in Figure 2-C2 indicates that osteocalcin is localized to the myofibroblast staining cells. The Table indicates the quantification of the bone matrix markers present in the rheumatic valves compared with the controls.

**MicroCT**

A 3D analysis of the calcified rheumatic valve by MicroCT reveals the depth and extent of calcification within the valve. Figure 2-D1 is a 3D reconstructed slice of the calcified valve lesion demonstrating the complex distribution of mineral within each leaflet. The soft tissue of the valve leaflet is the lighter blue area surrounding the areas of calcification. Figure 2-D2, the MicroCT of the degenerative mitral valve tissue, indicates no evidence of calcification in the valve.

**Immunohistochemistry of the Human Mitral Degenerative Valves for Neoangiogenesis Markers**

**Immunohistochemistry for Vascular Markers**

Figure 3-A1 demonstrates the α-actin–staining myofibroblast cells localized to the atrial surface of the mitral valves (arrow points to the cells). There was no evidence of new vessels forming within the mitral valves. Figure 3-B1 demonstrates the von Willebrand stain showing the endothelial lining along the atrial surface (see arrow) of the mitral valve leaflet. There were no von Willebrand–staining new vessels within the mitral valve leaflets. Immunohistochemistry for human macrophage cells shown in Figure 3-C1 demonstrated very little evidence of macrophage cells in the degenerative mitral valves, indicating a mild amount of inflammation present in these valves. Finally, Figure 3-D1 shows the special stain for VEGF, which was present only in the endothelial lining along the atrial surface of the mitral valves (see arrow). The Table indicates the quantification of the immunostains in the degenerative mitral valves compared with the controls.
Immunohistochemistry of the Human Mitral Degenerative Valves for Identification of Bone Matrix Markers

**PCNA Staining**

As Figure 4-A1 shows, PCNA-positive cells were detected along the surface of the degenerative mitral valves, indicating a mild increase in cellular proliferation.

**Osteoblast Bone Markers**

Figure 4-B1 demonstrates the immunohistochemistry for osteopontin, which is localized along the atrial surface of the mitral valve leaflet. Figure 4-C1, showing the osteocalcin stains, also demonstrates a mild amount of osteocalcin along the surface of atrial surface. Osteocalcin is an osteoblast-specific marker. The arrow in Figure 4-D1 indicates that the protein is localized to the myofibroblast staining cells.

The Table demonstrates the quantification of the new vessels and the level of PCNA expression in the degenerative mitral valves compared with the rheumatic valves and control tissue.

**Discussion**

Calcification of human valves is the most common manifestation of diseased rheumatic heart valves. Until recently, the cellular mechanisms of the calcification process have been thought to be due to a passive accumulation of calcium along the surface of the valve leaflet. We hypothesized that the mechanism for valvular calcification is similar to skeletal bone formation and that calcification occurs in areas of neoangiogenesis, which is stimulated by an active inflammatory process and the release of VEGF. VEGF is well known to play a key role in angiogenesis in pathological inflammatory diseases. Decker et al have suggested that VEGF regulates bone remodeling by attracting endothelial cells and by stimulating osteoblast differentiation. Our findings indicate that VEGF is localized to cells in inflammatory regions of the valve fibrosa, specifically the macrophages and myofibroblasts. Our group has recently demonstrated that an osteoblast phenotype is associated with nonrheumatic, degenerative calcific aortic stenosis. The current data, including the production of osteopontin and osteocalcin proteins (both osteoblast cell products) and proliferating myofibroblast cells synthesizing bone matrix proteins, indicate that a similar osteoblast-like process that occurs in degenerative calcific aortic stenosis develops in the calcification process in rheumatic valves. The cellular differences between the rheumatic and degenerative lesions are the intense inflammatory response.
infiltrate and the demonstration of VEGF in areas of neoangiogenesis found in the rheumatic valves compared with the degenerative aortic stenosis and in the degenerative mitral valves analyzed in the present study.

Although calcification in rheumatic valves has been described in the literature for >200 years, the cellular mechanisms responsible for the calcification have not been previously described. These new observations support the hypothesis that mineralization of rheumatic cardiac valve tissue is similar to skeletal bone formation that is associated with neoangiogenesis and show that studying this disease process will provide important information on the treatment of valvular heart disease.

**Acknowledgments**

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