Bone Formation and Inflammation in Cardiac Valves

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Background—For nearly a century, the mechanical failure of calcified heart valves was attributed to a passive degenerative process. Recently, several case reports described bone formation in surgically excised heart valves and suggested an unexpected process of tissue repair.

Methods and Results—We studied the prevalence and pathology of heterotopic ossification in 347 surgically excised heart valves (256 aortic, 91 mitral) in 324 consecutive patients (182 men, 142 women; mean age 68 years) who underwent cardiac valve replacement surgery between 1994 and 1998. The valves were examined microscopically to determine the prevalence and features of bone formation and remodeling. Two hundred eighty-eight valves (83%) had dystrophic calcification. Mature lamellar bone with hematopoietic elements and active bone remodeling were present in 36 valves (13%) with dystrophic calcification. Endochondral bone formation, similar to that seen in normal fracture repair, was identified in 4 valves. Microfractures were present in 92% of all valves with ossification. Neoaangiogenesis was found in all valves with ossification. Bone morphogenetic proteins 2 and 4 (BMP 2/4), potent osteogenic morphogens, were expressed by myofibroblasts and preosteoblasts in areas adjacent to B- and T-lymphocyte infiltration in valves where ossification was identified. Mast cells were present in calcified and ossified valves and were especially prominent in atheromatous regions.

Conclusions—Heterotopic ossification consisting of mature lamellar bone formation and active bone remodeling is a relatively common and unexpected finding in end-stage valvular heart disease and may be associated with repair of pathological microfractures in calcified cardiac valves. (Circulation. 2001;103:1522-1528.)

Key Words: pathology ■ valves ■ inflammation ■ risk factors ■ stenosis

The valves of the human heart are supple, flow-regulating membranes inside a complex multichambered pump. Severe disorders of cardiac valves, if uncorrected, can lead to heart failure and death. Cardiac valve replacement is performed in more than 70 000 patients annually in the United States. Dystrophic calcification, first described by Monckeberry nearly a century ago,1 is the most common pathological finding in operatively excised heart valves. Recent studies identified bone proteins in diseased heart valves,2-4 and several case reports described bone in calcified cardiac valves.5-7 In this report, we describe microfractures, endochondral ossification, mature lamellar bone formation, and active bone remodeling in a large series of end-stage diseased heart valves and thus document an unexpected but common process of fracture repair and tissue remodeling.

Methods

Patients

Three hundred forty-seven aortic and mitral valves were operatively excised owing to valvular stenosis or regurgitation from 324 consecutive patients (age range 21 to 94 years) during cardiac valve-replacement surgery between 1994 and 1998 at the University of Pennsylvania Health System. Patients with endocarditis were excluded by study design. Clinical records were reviewed for cardiovascular risk factors, as well as a history of peripheral vascular disease, rheumatic fever, or hyperparathyroidism. Hypercholesterolemia was defined as a total cholesterol level >200 mg/dL and hypertension as a systolic blood pressure >140 mm Hg. The specific medications were available for patients undergoing surgery in 1998. Coronary artery atherosclerotic disease was determined on angiography and defined as the presence of an atherosclerotic lesion >30% luminal stenosis. This study was approved by the Institutional Review Board for Human Studies at the University of Pennsylvania.

Pathological Examination of Cardiac Valves

Hematoxylin-and-eosin–stained slides for each of the valve specimens were retrieved in retrospective fashion from the files of the Department of Surgical Pathology and examined independently by 3 of the authors (F.G., C.R., and R.Z.). There were 2 sections routinely performed on each valve leaflet. To allow for sectioning of the valve specimens and to minimize tissue artifact, the formalin-fixed specimens underwent a brief period of limited demineralization with a 10% formic acid solution. This limited demineralization does not affect bone matrix proteins or cellular elements such as osteoblasts or...
mol, the cases were analyzed microscopically for the presence of atheromatous plaque formation, calcification, cartilage formation, bone formation, osteoclastic activity, and inflammatory cell infiltration. Both intraobserver and interobserver variation were 3%. All foci of ossification were confirmed by polarized light microscopy.

Osteoclasts were identified as multinucleated cells that were present within resorption spaces (Howship’s lacunae). Osteoblasts were characterized as either polygonal cells actively forming matrix (active osteoblasts) or thinned and flattened cells on the bone surface without matrix production (quiescent osteoblasts). Dystrophic calcification was defined as an amorphous aggregate of basophilic crystalline material. In the decalcified sections, this is represented as a basophilic staining of the background proteins. Atherosclerotic plaque was noted as lipid-laden macrophages (foamy) embedded within a myxofibromatous matrix and smooth muscle proliferation with or without associated calcification. Neangiogenesis or new blood vessel formation was defined as an increased number and concentration of thin and delicate vascular spaces in the areas of the atherosclerotic plaque formation. Ossified foci were recorded as any area of the atherosclerotic plaque that demonstrated bone cells with osteoid matrix. Mature lamellar bone was defined as tissue that demonstrated a regular layered pattern of collagen when examined with polarized light. Immature woven bone was defined as an ossified focus consisting of a disorganized pattern of collagen formation similar to “basket weave” when examined with polarized light. Endochondral ossification is the process of bone formation arising from a preexisting cartilage framework as opposed to intramembranous bone formation, in which the bone arises de novo without the benefit of an underlying cartilage skeleton. Microfractures were identified as microseparations of the isolated bone islands (active osteoblasts) or thinned and flattened cells on the bone surface. Osteoid matrix. Mature lamellar bone was defined as tissue that demonstrated a regular layered pattern of collagen when examined with polarized light. Immature woven bone was defined as an ossified focus consisting of a disorganized pattern of collagen formation similar to “basket weave” when examined with polarized light. Immature woven bone was defined as an ossified focus consisting of a disorganized pattern of collagen formation similar to “basket weave” when examined with polarized light.

Immunohistochemical Evaluation of Cardiac Valves
To examine the subtype of lymphocytic infiltration, 30 paraffin-embedded valves were sectioned at a thickness of 5 μm. The cases were chosen in a randomized fashion to attempt to exclude observer bias. These slides were stained immunohistochemically for lymphocyte common antigen (Dako USA) at dilution 1:150, CD20 (B-cell marker; Dako USA) at dilution 1:150, and CD3 (T-cell marker; Dako USA) at dilution 1:110. The sections were stained and processed according to standard protocols in conjunction with the appropriate positive (normal lymph node) and negative (lack of primary antibody) controls. Investigation for the presence of bone morphogenetic protein (BMP) required frozen tissue, and therefore 10 valves were harvested prospectively at the time of surgery and frozen in liquid nitrogen. These cases were chosen from 10 randomly identified surgical procedures. Frozen sections were cut at a thickness of 4 μm and stained immunohistochemically according to standard protocols with a monoclonal antibody to human BMP-2 (recognizing BMP-2 or BMP-4; Genetics Institute, Cambridge, Mass). Human osteosarcoma (either frozen or embedded in paraffin) was used as a positive control. Negative controls included human liver and tissue stained without the primary antibody. Mast cells were identified as cells with large central nuclei when stained by chloracetate esterase (because of the presence of chymotrypsin-like serine esterase activity), as per a previously published method.

Statistical Analysis
Analyses were performed to investigate possible associations between cardiovascular risk factors and the presence of bone tissue within valves. Univariate χ² analyses were performed to investigate associations with dichotomous predictors. Values of potential continuous predictors were expressed as means, and comparisons were made with the Student’s t test. Statistical significance was established at P<0.05.

Results
Patient Demographics
There were 182 men and 142 women (mean age 68±11.4 years). Forty-eight percent had hypertension, 28% had hypercholesterolemia, and 20% had diabetes mellitus. Fifty-four percent of patients had smoked cigarettes at some point in their life (17% were current smokers) (Table). Atherosclerosis of the coronary arteries was reported in 49% of patients, atherosclerosis of the carotid arteries in 9%, and atherosclerosis of the lower-extremity arteries in 9%. A history of rheumatic fever was reported in 18% of patients. None had hyperparathyroidism. Twenty-three patients had 2 valves replaced during the same operation. The mean age of patients with an ossified valve (71.6±8.2 years) was higher than that of those without valve ossification (67.5±11.6 years) but was not statistically different (P=0.1) (Table).

Pathological Examination of Cardiac Valves
A total of 347 valves (256 aortic and 91 mitral) were examined. Three hundred five valves (88%) contained atherosclerotic plaque (Figure 1). Two hundred eighty-eight valves (83%) had dystrophic calcification. Mature lamellar segments of bone (osteoblasts) were sectioned at a thickness of 5 μm. The cases were analyzed microscopically for the presence of atheromatous plaque formation, calcification, cartilage formation, bone formation, osteoclastic activity, and inflammatory cell infiltration. Both intraobserver and interobserver variation were 3%. All foci of ossification were confirmed by polarized light microscopy.

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bone with hematopoietic elements and active bone remodeling was identified in 35 patients (36 valves: 33 aortic, 2 mitral, and 1 both) who had calcification of the valve (13%) (Figure 1). Ninety-five percent of ossified foci (34 valves) contained mature lamellar bone, and 5% (2 valves) contained immature woven bone. In the 36 valves in which bone was identified, 7 contained active osteoblasts, 37 had quiescent osteoblasts, and 13 had osteoclasts. Endochondral ossification was found in 4 valves (Figure 1). Microfractures were present in 92% of all valves with ossification. Fracture callus with robust endochondral ossification was identified in an aortic valve that had sustained a microfracture through an area of dystrophic calcification on 1 face of the valve leaflet (Figure 1). The collagen in all ossified plaques appeared as delicate bundles of fibers with a random orientation and exhibited a mature lamellar or immature woven bone pattern. This resulted in a nodular aggregate of cells, matrix, and debris that did not communicate with the endothelial surfaces. Neoangiogenesis was found in all valves with ossification (Figure 2).

**Examination for Inflammatory Cells**

Lymphocytic infiltration was found in 80 valves and occurred in 2 patterns (Figure 3): (1) perivascular lymphocytic aggregate tightly surrounding small to medium vessels and (2) diffuse streaming of lymphocytes into the surrounding tissue. Both patterns were composed of small B and T lymphocytes admixed with plasma cells. Mast cells were identified in calcified valves and were associated with the presence of bone (data not shown).

**Immunohistochemical Evaluation of Valve Ossification**

Ten valves were examined for the presence of BMP. Immunohistochemical staining of surgically explanted aortic valves revealed the presence of BMP 2/4 in areas of ossification (Figure 3).

**Association Between Cardiovascular Disease and Valve Calcification or Ossification**

Patients with calcified valves had an increased prevalence of coronary artery disease (52% versus 38%; \( P=0.047 \)), peripheral arterial disease (10% versus 2%; \( P=0.044 \)), hypercholesterolemia (59% versus 27%; \( P=0.0002 \)), and hypertension (52% versus 30%; \( P=0.003 \)) compared with those without valve calcification on univariate analysis. There was no statistical difference between the number of men (n=25,
13.7%) and women (n=11, 7.7%) or for a particular racial background with calcification of valves (Table).

Additional analyses revealed no significant associations between valvular bone tissue and other concurrent cardiovascular diseases or risk factors. Also, there were no statistical sex or racial differences for patients with valvular bone formation. Stratification by valve origin (ie, rheumatic, bicuspid, or degenerative) failed to reveal any hidden associations. There was no relationship between history of valvuloplasty and subsequent valve ossification. Because of the limited number of calcified mitral valves (n=37), a meaningful statistical analysis for only the mitral valves was not possible. When the mitral valves were excluded from the data set, there was no significant change in results.

Statins and Ossification
Twenty-nine of the 323 patients enrolled in the study were taking an HMG-CoA reductase inhibitor (statin) drug. Of the 29 patients taking a statin drug, 26 (90%) also had valve calcification. The prevalence of valve calcification in those patients not receiving statins, however, was similar (89%, P=NS). Only 1 (4%) of the 26 patients taking statins had valve ossification. Although the prevalence of valve ossification (35 patients, 12%) was higher among the 297 patients not receiving statins, this difference was not statistically significant.

Discussion
The major finding of our study is that heterotopic ossification occurs commonly in end-stage valvular heart disease. Dys trophy calcification is a passive process in degenerating connective tissues, whereas heterotopic ossification is an active process of abnormal tissue repair. In all cases in which heterotopic ossification was noted, there were features of active bone remodeling with osteoblastic bone formation and osteoclastic bone resorption. The majority of ossified valves contained mature lamellar bone, but 4 valves also exhibited earlier stages of endochondral osteogenesis similar to that seen in the growth plate, in fracture healing or in fibrodys-
plasia ossificans progressiva (FOP). Ossified areas stained positively for the extracellular bone matrix proteins BMP 2 and BMP 4, which are potent osteogenic morphogens. Prior studies also reported the presence of bone proteins such as osteopontin, osteocalcin, and osteonectin in calcified valves, but this is the first report of the presence of BMPs in ossified valves.

The association of hypertension, hypercholesterolemia, and atherosclerotic disease with cardiac valve calcification, as noted in the present study, is consistent with the findings of previous studies. Because the present study was conducted at a single tertiary-care medical center on the east coast of the United States, it is possible that there were biases with regard to the sample population. For example, there were only 3 Asian patients in the present study. Nevertheless, among the patients studied, there were no significant associations between valvular bone and the cardiovascular covariates. The power of the study was adequate to have detected significant differences that would not likely be due to chance.

The origin of bone cells in ossified valves is unknown. Lymphocytes, monocytes, and mast cells must enter the valve from the circulation in response to endothelial injury. A continuous subendothelial network of smooth muscle pericyte-like cells exists in the human vascular bed, and myofibroblast-like cells are present throughout the fibrosal layer of cardiac valves. Cultured myofibroblasts from cardiac valves undergo phenotypic differentiation into osteoblast-like cells. Evidence for myofibroblast differentiation comes from recent reports suggesting a population of ossifying cells in both the aorta and cardiac valves.

The results from recent studies on the pathophysiology of heterotopic endochondral ossification in atherosclerotic plaque of arterial walls showed that osteoprogenitor cells resemble microvascular pericytes and that those cells express bone proteins. Additionally, microvascular pericytes express osteoblast markers in vivo and give rise reproducibly to endochondral ossification when cultured in diffusion chambers in vitro. These results are consistent with the observation that microvascular pericytes serve as a reservoir of premature precursor cells capable of giving rise to heterotopic ossification through an endochondral bone formation pathway and are influenced by cytokines and morphogens from circulating immune cells of hematopoietic origin.

Atherosclerosis is an inflammatory disease. The results from the present study are consistent with cardiac valve calcification and ossification also being an inflammatory process. Macrophages and lymphocytes accumulate in areas of dystrophic calcification and ossification. Furthermore, recent evidence suggests that mast cells upregulate angiogenesis and are involved in heterotopic ossification (oral communication, 2000). Mast cells contain metalloproteinases, serine proteinases, chymases, acid hydrolases,
Figure 4. Schematic model of ossification of aortic valve. A, Early lesion of cardiac valve calcification includes endothelial damage, cholesterol deposition, and inflammation. B, Dystrophic calcification ensues with lesion that includes, in addition to cholesterol and inflammatory cells, hydroxyapatite crystal formation. Lamellar bone formation occurs secondary to endochondral process thought to originate from calcifying valve cells (CVC) of myofibroblast origin. MC indicates mast cell; FB, fibroblast; MP and Macro, macrophage; Chond, chondrocyte; Osteo, osteoblast; Endoth, endothelial cell; Cho and Chol, cholesterol; and Alk Phos, alkaline phosphatase.

cathepsins, histamine, heparin, and proangiogenic peptides sequestered in metachromatic granules. Proinflammatory molecules such as tumor necrosis factor-α, prostaglandin D2, and leukotrienes can also be unleashed from mast cells. It is likely that activation of these inflammatory cells leads to release of cytokines, chemokines, growth factors, and hydrolytic enzymes that contribute to angiogenesis, atherosclerotic plaque growth, and ossification of the valve.

Angiogenesis is essential for longitudinal bone growth, and angiogenic factors such as vascular endothelial growth factor (VEGF) are required for endochondral bone formation and fracture healing. The presence of neoangiogenesis in all ossified valves is consistent with the hypothesis that angiogenesis facilitates endochondral bone formation in calcified cardiac valves. Angiogenic growth factors such as fibroblast growth factor, VEGF, and transforming growth factor-β released from mast cells are likely to induce angiogenesis in calcified cardiac valves. Thus, the microenvironement of the damaged cardiac valve, with immune cells of hematopoietic origin in contact with subendothelial microvascular pericyte-like cells, contains the necessary minimal conditions needed to induce heterotopic ossification through an endochondral pathway (Figure 4).35

The retrospective nature of the present study could have resulted in an underestimation of the incidence of ossification. Also, the definition of microfractures is subjective, and there is a remote chance that some of the microfractures could be due to artifact.

For reasons not yet explained, most calcification and ossification observed in diseased heart valves occurs on the aortic side of the aortic valve and on the ventricular side of the mitral valve.36,37 Theories of cardiac valve calcification have implicated abnormal blood flow rheology, which results in abnormal mechanical stress and valvular damage.38 Further work is necessary to better understand the interaction between biophysical and molecular factors that promote microfractures, trigger fracture healing, stimulate heterotopic ossification, and regulate remodeling in damaged heart valves.

BMP-2 stimulates osteoblastic differentiation. Mundy et al recently linked the BMP-2 promoter to a luciferase gene reporter and found after screening >30 000 compounds that HMG-CoA reductase inhibitors (statins) stimulate the BMP-2 promoter. Statins also increased the number of mouse osteoblasts and amount of new bone formed, similar to that seen with recombinant BMP-2 itself. The physiological significance of this effect was confirmed because oral administration of a statin increased the volume of trabecular bone and increased the rate of bone formation in rats.39 Three case-control studies were recently published with results indicating that statins are protective against bone fracture.40–42 However, recently published data43 also indicate that the amount of calcium in coronary arteries is reduced with statins, as noted on electron-beam computed tomography. The results of the present study indicate that statin drugs are not associated with ossification of aortic valves. Perhaps statin drugs will stabilize atherosclerotic cardiac valves and retard calcification and ossification. Prospective studies are needed to determine the effect of statin drugs on calcified cardiac valves.

Recent findings44,45 have led to the identification and cloning of a large family of potent extracellular inhibitors of the BMPs now implicated here in the pathophysiology of bone formation in diseased cardiac valves. The role of these BMP inhibitors in cardiac valve ossification is unknown. The expression, physiology, and pharmacology of BMPs and BMP inhibitors could provide important insight into the pathophysiology and prevention of heterotopic ossification in the heart.44–46

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