

Comparative Study of Calcified Changes in Aortic Valvular Diseases

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Abstract

Calcification of the aortic valve leads to stenosis or regurgitation or both. To clarify the mechanism of heart valve calcification, comparative studies using histological and ultrastructural examinations were performed of calcified aortic valves. These valves were obtained at valve replacement surgery from 11 patients with rheumatic aortic valvular disease (RAVD), 10 patients with degenerative aortic valve disease (DAVD), and 10 patients with congenitally bicuspid aortic valves (CBAV). For electron microscopic study, 5 cases were selected from each group. In RAVD, histological examination revealed calcification in a degenerated amorphous area at the center of fibrous thickened regions and in laminar fibrous thickened areas near the valve surface. In DAVD, calcification was observed mainly in the fibrosa near the valve ring. In CBAV, basic pathological changes were similar to those in DAVD; however, additional severe calcification of the raphe was observed, if the raphe was present. Ultrastructural examinations showed deposition of electron-dense materials in two patterns in all three groups; one pattern was observed in the interfibrillar spaces of collagen fibrils, and the other pattern was widespread macular deposition unrelated to the preexisting structure. In RAVD, microfibril-like fibrillar structures were found in the areas of deposition of electron-dense materials. These findings suggest that newly formed connective tissue degraded and became necrotic because of nutritional deprivation, especially in the thickened central area, causing calcium deposition. In DAVD and CBAV, numerous lipid vacuoles were found in the electron-dense deposition areas similar to lipid deposition in aortic atherosclerosis. Localized calcium deposition in the fibrosa suggests that the stress of valvular motion and pressure load induces sclerotic changes with the degeneration of collagen fibers, providing a core for calcification. In CBAV, the raphe was the main location of calcification, wherein spiraled collagen fibrils were observed. Increasing the hemodynamic load with abnormal structure might influence calcification. The ultrastructural pattern of calcification of the valve is common; however, additional findings suggest that the cause and mechanism are different in each type of heart valve disease.

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Key words: aortic valve stenosis, aortic valve calcification, elastic fiber, collagen fiber

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Introduction

Rheumatic aortic valvular disease (RAVD), degenerative aortic valve disease (DAVD), and congenitally bicuspid aortic valve (CBAV) are the three major causes of aortic valve stenosis. In Japan, the incidence of RAVD has been decreasing, while the number of operations for DAVD has been increasing¹. The incidence of CBAV has been lower in Japan than in Europe or the United States; however, the number of elderly persons undergoing operations for aortic stenosis due to CBAV has recently been increasing^{1,2}. The main histological change in the stenotic valve is severe calcification; however, the mechanism and characteristics of calcification in each type of valvular disease are still unclear. It is important to elucidate the mechanism of calcification to prevent valvular dysfunction. In the present study, histological and ultrastructural examinations of calcified valves were performed to clarify the differences in the calcification patterns among each type of valvular disease.

Materials and Methods

Aortic valves were obtained at valve replacement surgery for aortic stenosis (AS) and/or aortic stenosis with regurgitation (ASR) from 11 patients with RAVD, 10 patients with DAVD (most patients with DAVD were elderly, the severity of sclerosis increased with age, and no clear cause, such as infective endocarditis, rheumatism, and bicuspid valve, was identified), and 10 patients with CBAV (**Table 1a**). All patients gave informed written consent.

Histological Study

Cusps were fixed in 20% formalin, decalcified in 5% formic acid for 4 days to 1 week, and cut vertically toward the valvular ring. Three strips from each cusp were embedded in paraffin, and 3- μ m-thick sections were cut. The serial sections were stained using hematoxylin and eosin (HE), Elastica Masson-Goldner (EMG), and the Alcian blue and periodic acid-Schiff (AB-PAS) method.

Electron Microscopic Study

Five cases that showed typical calcification on histological examination were selected from each group for electron microscopic study (**Table 1b**). After deformalin with distilled water, pieces of cusp were refixed with 2% osmic acid in 0.01 M phosphate-buffered saline (pH 7.4 for 2 hours at room temperature), washed with 0.01 M phosphate-buffered saline, dehydrated, and embedded in Epok 812 (Nagase, Tokyo, Japan). For ultrathin sections, the peripheral zone of calcification was selected from semithin sections that had been stained with 1% toluidine blue; they were then stained with uranyl acetate and lead citrate and with tannic acid according to the method of Kajikawa et al³. These sections were examined with an electron microscope (H7000, Hitachi, Tokyo, Japan).

Results

Macroscopic Findings

In RAVD, cusps showed a fish-mouth appearance with marked fibrous thickening, commissure adhesion, and diffuse calcification (**Fig. 1a**). The degree of fibrous thickening and calcification of cusps varied among the 11 cases, and there was no obvious difference among the three cusps in each case.

In DAVD, thickening with calcification of cusps was observed at the valvular ring area of the sinus of Valsalva. The free edge of the cusp usually remained intact (**Fig. 1b**). The degree of calcification of the cusp varied among the 10 cases and no obvious difference was found among the three cusps for each case.

In CBAV, the location of the calcification was similar to that in DAVD, and in five cases with a raphe, severe calcification was observed at the raphe (**Fig. 1c**).

Histological Findings

In normal aortic valves, from the ventricular (inflow) surface, three main layers are observed: the elastic layer, spongiosa, and fibrosa⁴.

In RAVD, the layered structure of cusps was destroyed and obscured. The cusps showed diffused

Table 1a Cases for histological study

Group	n	Sex		Age (years)		Diagnosis	
				Range	Mean		
RAVD	11	M 6	F 5	48–81	70	AS 6	ASR 5
DAVD	10	M 3	F 7	66–79	72	AS 10	
CBAV	10	M 5	F 5	19–77	61	AS 10	

Diagnosis:AS, aortic stenosis;ASR, aortic stenosis and regurgitation.

Table 1b Selected cases for ultrastructural study

Group	n	Sex		Age (years)		Diagnosis	
				Range	Mean		
RAVD	5	M 2	F 3	68–75	73	ASR 5	
DAVD	5	M 1	F 4	66–79	74	AS 5	
CBAV	5	M 1	F 4	19–77	59	AS 5	

Diagnosis: AS, aortic stenosis; ASR, aortic stenosis and regurgitation.

fibrous thickening and calcification. Calcification was found mainly at the central area of thickening where the tissue showed amorphous degradation. Calcification was also found near the surface where the fibrous thickening showed a laminar structure of elastic fibers (**Fig. 2a**).

In DAVD, the histological layered structure of cusps was retained, and calcification was usually localized in the fibrosa (**Fig. 2b**). Calcium deposits protruded toward the aortic surface like a knob. The fibrosa neighboring the calcification showed a hyalinous change in collagen fibers, sometimes with cholesterol crystals. A few foamy macrophages were found at the surface of the fibrosa, but an aggregation of these cells, which are usually observed in aortic atheroma, was not found.

In CBAV, the histological layered structure of cusps was observed in some areas; however, fibrous thickening and calcification were more severe than in DAVD. Calcification was present mainly in the fibrosa but sometimes extended from it to the laminar fibrous thickening lesion of the cusp surface. Five of the 10 cases had a raphe, which is fibrous tissue protruding to the aortic surface. Calcification and disarray of the collagen fibers were found at the raphe (**Fig. 2c**).

Electron Microscopic Findings

Deposition of electron-dense materials showed two patterns in all the three groups (**Fig. 3a**). One pattern was found in interfibrillar spaces of collagen fibrils (**Fig. 3b**), and the other pattern was widespread macular deposition unrelated to the preexisting structure (**Fig. 3c**). Needle-shaped crystals of hydroxyapatite were also observed in all groups.

In RAVD, microfibril-like fibrillar structures, approximately 10 nm in diameter, were found in the electron-dense deposition area of calcified materials at the laminar fibrous thickening regions and at fibrous thickened regions (**Fig. 4**).

In DAVD and CBAV, circular materials that had a membrane-like structure (**Fig. 5a**) and irregular vacuoles that circular or orthopedic or both (**Fig. 5b**) were found in the area of calcification.

In CBAV, ultrastructural findings were similar to those in DAVD. Some collagen fibrils showed a flower-like appearance on cross sections and a spiral appearance on longitudinal sections (**Fig. 6a, b**). Electron-dense calcified materials were deposited around these altered collagen fibrils. Disarray of the collagen fibrils was also found. These findings were observed particularly at the calcified areas in the raphe but sometimes were also found in the fibrosa or the fibrous thickening region.

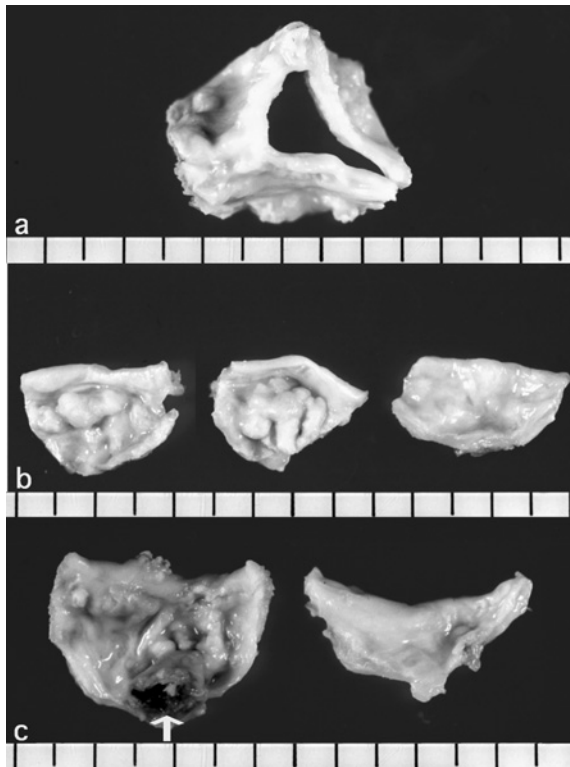


Fig. 1 Macroscopic findings

- a:** RAVD. Valves show severe fibrous thickening with adhesion of the commissure and calcification.
- b:** DAVD. Calcification spread from the valvular ring side of the aortic side to the entire cusp.
- c:** CBAV. Calcification is severe on the valvular ring side, especially the raphe region (→), of the aortic side. The whole cusp shows calcification and fibrous thickening.

Discussion

To clarify the mechanism of calcification of heart valves, calcified valves resulting from RAVD, DAVD, or CBAV were investigated by histological and ultrastructural examinations.

Patterns of Calcium Deposition in Aortic Valves

On ultrastructural examination, two patterns of calcium deposition were commonly observed among the three groups. One pattern was deposition in the interfibrillar spaces of collagen fibrils, and the other pattern was widespread macular deposition unrelated to the preexisting structure. These patterns are observed in the calcified regions of aortic atherosclerosis⁵, dermatomyositis⁶, porcine valve xenografts implanted in humans⁷, and mitral



Fig. 2 Histological findings (Upper side is the aortic side in all figures). **a:** RAVD. Calcified deposits (*) are found in the fibrous thickening area of the fibrosa. There are neovascularizations in the spongiosa. Sometimes calcification is found in the laminar fibrous thickening area of the elastic layer (EMG stain). **b:** DAVD. In many cases, calcification (→) was localized to the valvular ring side of the fibrosa (HE stain). **c:** CBAV. The raphe shows calcification (*) and the disarrangement of collagen fibers (→) (EMG stain).

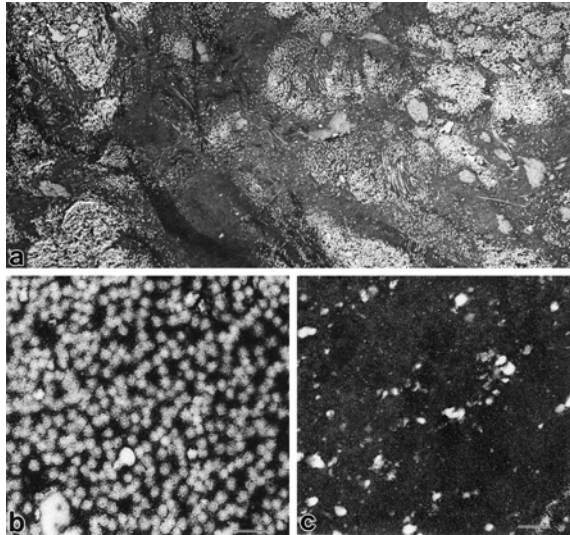


Fig. 3 Electron microscopic findings in all three groups

- a:** Deposition of electron-dense materials is found in the interfibrillar spaces of collagen fibrils and also show widespread macular deposition unrelated to the preexisting structure (uranyl acetate and lead citrate stain, bar = 1 μ m).
- b:** High-magnification view of deposition of electron-dense materials in the interfibrillar spaces of collagen fibrils (uranyl acetate and lead citrate stain, bar = 250 nm).
- c:** High-magnification view of the widespread macular deposition of electron-dense materials (uranyl acetate and lead citrate stain, bar = 250 nm)

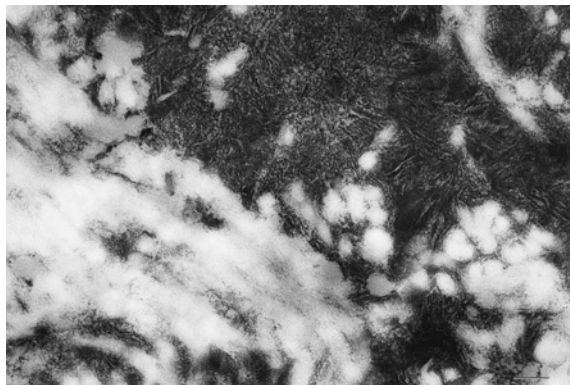


Fig. 4 Electron microscopic findings in RAVD
Microfibril-like fibrillar structures are found in the electron-dense materials deposition areas (uranyl acetate and lead citrate stain, bar = 100 nm).

valve allografts implanted in sheep⁸. Calcium deposition in the interfibrillar spaces of collagen fibrils indicates that apatite crystals occurred around

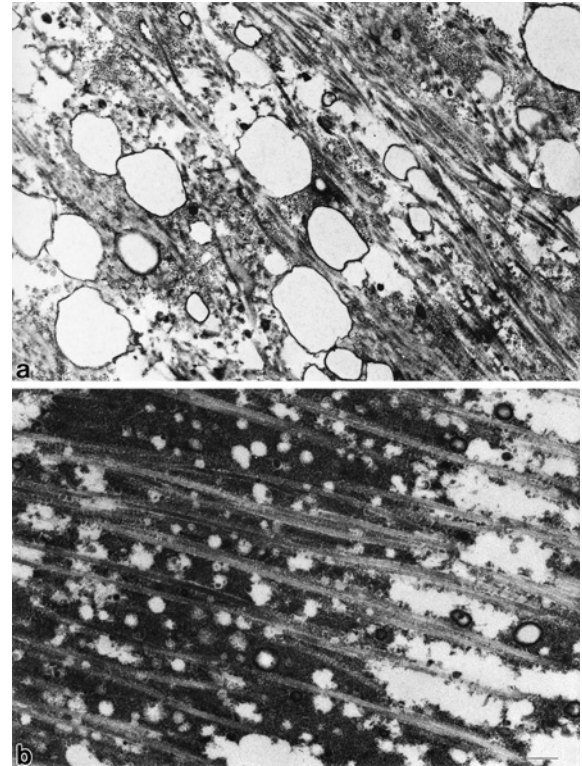


Fig. 5 Electron microscopic findings in DAVD and CBAV

Numerous vacuoles of lipid are found in the calcified area and in the interfibrillar spaces of collagen (a: uranyl acetate and lead citrate stain, bar = 450 nm; b: uranyl acetate and lead citrate stain, bar = 200 nm).

collagen fibrils. Widespread macular deposition unrelated to the preexisting structure indicates that several irregular calcified masses of apatite crystals developed on fibrous tissues. Ectopic dystrophic calcification is mineralization on tissue that is injured or necrotic or both when calcium and phosphorus levels in serum are normal⁹. In normal cells, calcium channels on the cell membrane maintain low intracellular calcium concentrations. The calcium that flows inside a cell is transported outside by the calcium-pump, utilizing adenosine 5'-triphosphate (ATP) for energy.

Cells of bioprosthetic valves or allografts which are nonviable or injured or both of cannot make use of ATP for energy. The cell membrane is disrupted, and calcium extrusion from the cell is impaired¹⁰. Then, calcium is deposited as calcific crystals by reacting with phosphorus in the tissue^{10,11}. In human valves, the metabolic disorder of calcium resulting

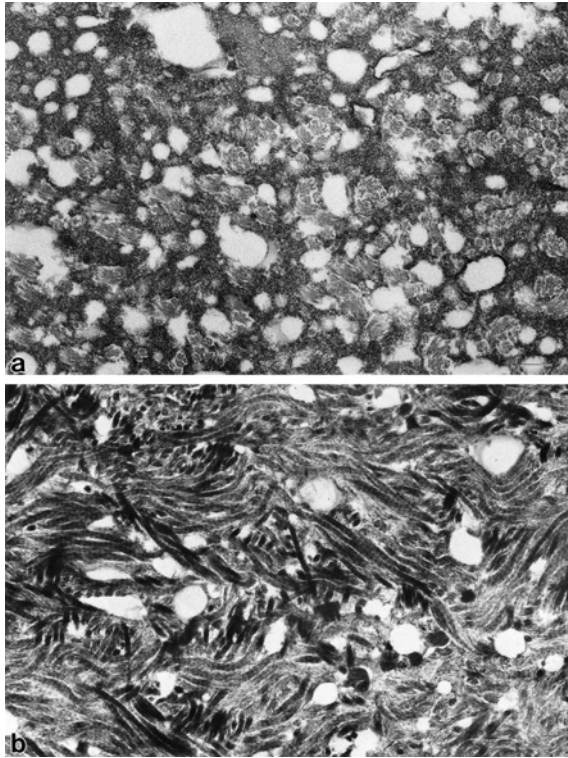


Fig. 6 Electron microscopic findings in CBAV
a: Collagen fibrils show a flower-like appearance and are surrounded by calcium deposits (uranyl acetate and lead citrate stain, bar = 200 nm).
b: Some collagen fibrils show spiral appearance and the disarray of collagen fibers on longitudinal sections (Kajikawa stain, bar = 300 nm).

from the degeneration/necrosis of fibrous components and cells by valvular disease may cause progression of valvular calcification, such as the calcification process of bioprosthetic valves.

Calcification in RAVD

The layered structure of the aortic valve was destroyed. The structure became indistinct owing to rheumatic inflammation, and the central core of the destroyed region showed degeneration. The laminar fibrous thickening formed by chronic inflammation and repair processes under the stimulation of blood flow was found. Nourishment for the cardiac valve is supplied directly by blood circulating in the heart. When cusps become thick, nourishment cannot be supplied to the central part of the cusp. Calcium metabolic derangements caused by anoxia and nutrient deficiency would lead to calcification in RAVD^{10,12,13}. Ultrastructural examination showed microfibril-like fibrillar structures in the calcified

area. Fine filamentous materials have been reported in superficial dystrophic cutaneous calcification¹⁴ and acute myocardial infarction¹⁵. Fartasch et al.¹⁴ have found that nucleation of apatite crystals begins around microfibrils of elastic fibers in implanted valves. In the present study, microfibril-like fibrillar structures were observed in the calcification area of the laminar fibrous thickening regions and in fibrous regions. In the valves of RAVD, inflammation and repair occur repeatedly. One cause of calcification is that collagen fibers denatured by repeated inflammation become microfibril-like fibrillar structures¹⁶ where calcification materials are deposited. In addition, during the neogenesis of elastic fibers caused by the repair process of inflammation, the microfibrils become necrotic before maturation, and calcification materials are deposited. In RAVD, these microfibril-like fibrillar structures may become the foundation for calcium deposition¹⁷.

Calcified Change in DAVD

Vacuoles that are similar to the fat droplets in arteriosclerosis¹⁸ were found in calcified areas and in the interfibrillar space during ultrastructural examination. This finding suggests that the mechanism of calcification in DAVD is similar to that in arteriosclerosis¹⁹. However, as we reported previously²⁰, smooth muscle cells and aggregation of macrophages are absent in cardiac valves, and the fibrosa is composed of redundant collagen fibers. Because the histological structure of the aortic valve is different from that of arteries and the essential components for arteriosclerosis are not present in the valve, it is thought that there are additional factors relating to calcification in DAVD. Recently, Garg et al. have reported that mutations in the signaling and transcriptional regulator NOTCH1 cause a spectrum of developmental aortic valve anomalies and severe valve calcification in nonsyndromic autosomal-dominant human pedigrees²¹. A genetic factor is thought to be one cause of aortic valve calcification.

The fibrosa is subjected to high aortic pressure during diastole, and the cusps bend near the valvular ring during systole. Localized calcification at

the fibrosa near the ring indicates that the stress of valvular motion and pressure load over many years induce the degeneration of collagen fibers and provide the core for calcification. Thubrikar et al.²² have reported that intramural stress, but not shear stress, plays an important role in accelerating atherosclerosis in valves. The stagnation and turbulent flow of the blood stream, which are similar to the property of low shear stress^{23,24}, also occur in the fibrosa and the basal part of the sinus of Valsalva during systole. It is reasonable that the infiltration of lipids is promoted by the stagnation, high-pressure load, and turbulence of the blood stream, as in arteriosclerosis. In DAVD, calcification is caused by impoverishment of the cusp and sclerotic change with lipids.

Calcified Change in CBAV

Localized calcification in CBAV was similar to that in DAVD. However, the patient was young and the degree of degeneration was severe, compared with those in most cases of DAVD²⁵. In CBAV, hemodynamic factors and physical factors, such as the stress of valvular motion, stimulation of the pressure load, and turbulence, differ from those of the tricuspid aortic valve^{26,27}. The distances between the lateral attachments of a normal aortic valvular cusp along its free margins are curved lines. The extra length allows the cusps to move freely during the opening and closing of the valves. However, the distances between lateral attachments of CBAV along their free margins approach straight lines. If these distances are truly straight lines, the valves cannot open widely enough and cannot cling to the arterial wall during ventricular systole²⁶. This abnormal structure of CBAV causes turbulent flow or swirl flow or both, when the bloodstream through the valve orifice. In CBAV, the fatigue of valvular motion with aging and the abnormal bloodstream lead to damage of collagen fibers, calcification, and fibrous thickening^{28,29}. Ultrastructural findings, such as spiraled and disarranged collagen fibrils, suggest that the alteration of collagen fibers helps trigger calcification in CBAV. Roberts et al.²⁷ and others^{30,31} have reported that calcification advances along the raphe. In our cases with a raphe, severe calcification

was found on the raphe. The raphe is an avascular fibrous mass of connective tissue which protrudes from the aortic surface. Thus, progressive nutritional deprivation, denaturation, and necrosis might occur more rapidly in the raphe. Additionally, a calcification factor may act strongly because the bloodstream is disturbed and becomes intensified by the protruding fibrous structure of the raphe.

In conclusion, the calcification of aortic valves usually results from nutrient deficiency in valves. In RAVD, fibrous thickening and degeneration of the cusp caused by chronic inflammation and the repair process contributes to the calcification of valves. In DAVD, fatigue due to valvular motion and sclerotic changes in the fibrosa causes the calcification of valves. In CBAV, because the stress of valvular motion is greater than that in DAVD, the calcification of valves progresses more rapidly and becomes more severe than in DAVD.

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