Mitral annular calcification (MAC) is a common condition in elderly subjects that sometimes causes degenerative mitral valvular diseases. To investigate the early histopathogenesis of MAC, we examined 180 consecutive autopsies of elderly subjects. After a macroscopic and radiological examination, 5-mm-thick serial tissue blocks obtained from the mitral annulus were examined in all MAC cases. Five cases without MAC were also studied using histology, immunostaining, electron microscopy, analytical electron microscopy and the TUNEL method. The incidence of MAC in females (23%) was higher than that in males (15%). Most MAC was located at the posterior cusp (91%). The mitral annulus showed signs of microscopic calcification and lipid-deposition in some degenerated areas in all of the cases without MAC. The interstitial cells were positive for vimentin and partially positive for smooth muscle actin, indicating the myofibroblastic differentiation. Ultrastructural studies showed an abundance of cellular degradation products and foci of calcium- and phosphorus-deposition on these products in the interstitium. Several interstitial cells tested positive for both single-stranded DNA immunostaining and the TUNEL reaction. In conclusion, the microscopic calcification of mitral annulus is an early stage of MAC and caused by calcium-deposition on cellular degradation products, probably released from apoptotic or necrotic interstitial cells.

Key words: mitral annular calcification, aging, apoptosis

Introduction

Mitral annular calcification (MAC) is an incidental, but frequent postmortem finding in elderly people. As the life span of the general population increases in Western and some Asian countries, MAC has become an important pathological entity as one of degenerative valvular diseases. The reported incidence of MAC in patients over the age of 50 years is 8.5%, and MAC is more frequent in women (11.5%) than in men (4.5%). In another study, the incidence of MAC in patients over the age of 60 years was 10% (6.7% in males and 13.3% in females). In very old patients of more than 90 years, the incidence increased to 33%. MAC causes several cardiac diseases, with mitral valvular regurgitation being the most common. In rare cases, MAC may also cause mitral valvular stenosis as well as thrombotic or infective endocarditis.

The pathogenesis of MAC is largely unknown. Since the mitral valve opens and closes 2.5 to 3 billion times in a person’s life, mechanical stress may play a role in the degeneration and functional loss of the valves, such as that seen in MAC. Some authors have ascribed the cause of MAC to infective endocarditis.
while others consider MAC to be a manifestation of generalized atherosclerosis.7-9 Although several studies have investigated the pathology of advanced MAC, the pathological features of early-stage MAC have, to the best of our knowledge, not been examined. We therefore performed a pathological study of MAC to elucidate the early pathogenesis of this condition. We focused on the early stage of MAC because early lesions, as opposed to advanced ones, are more likely to reveal the basic mechanism of the pathological process. Histopathological study of early-stage MAC may help to elucidate the pathogenesis of MAC and provide useful information for preventing the development of this disease in susceptible persons.

Materials and Methods

Pathology

We reviewed 180 formalin-fixed hearts from consecutive autopsies of senile patients performed at the Department of Pathology, Tokyo Metropolitan Geriatric Hospital, between April 1999 and February 2000. The subjects consisted of 98 males and 82 females with an average age at the time of death of 79 years, ranging from 59 to 103 years. The location and length of all mitral annular calcifications were macroscopically examined. The extent of MAC was divided into mild (1-9 mm), moderate (10-19 mm) and severe degrees (longer than 20 mm), according to the length of MAC.

In all 34 MAC cases, paraffin blocks were prepared from 5-mm-thick serial tissue sections obtained from the entire mitral valvular annulus. The calcified tissues were decalcified by immersing the specimens in 10% EDTA solution for one day. The paraffin sections were stained with hematoxylin-eosin, Azan-Mallory, elastica van-Gieson, oil red O, Congo red, and von Kossa stains.

The mitral annulus was defined as a fibrous structure to which the left atrial myocardium, the left ventricular myocardium and the fibrous core of the mitral valve were attached, according to a standard textbook.10

Pathological assessment of atherosclerosis

The atherosclerosis in the large arteries was semiquantitatively scored by gross inspection on a scale of 0 to 8 according to the ratio of the atheroma-occupied area to the entire surface area: negligible (0 point, ratio = 0 to 1/20), minimal (2 points, 1/20 to 1/6), mild (4 points, 1/6 to 1/3), moderate (6 points, 1/3 to 2/3), and severe (8 points, 2/3 to 1). The Pathological Atherosclerotic Index (PAI) was defined as the average value of the atherosclerotic scores in eight large arteries, including the common carotid artery, subclavian artery, aorta, splenic artery, superior mesenteric artery, common iliac artery, external iliac artery, and left femoral artery.

The severity of coronary arteriosclerosis was represented by the coronary stenotic index (CSI), as previously reported.11

Soft X-ray radiology

After the macroscopic examination of the mitral valvular annulus, soft X-ray radiological examinations were performed using a Softex apparatus (model CMB-2, Softex Co. Ltd., Ebina, Kanagawa, Japan). The operating conditions were optimized as follows: tube voltage, 50 kVp; tube current, 3 mA; exposure time, 90 seconds. Fuji industrial films IXFR (Fuji Photo Film Co. Ltd., Tokyo) were used to obtain the X-ray images.

Electron Microscopy

Fresh specimens of mitral valvular annulus were collected from 5 recent autopsies of subjects without MAC. These subjects consisted of 3 males and 2 females, with an average age of 83 years. Two fixation methods were utilized: the conventional method and Mizuhiara’s microwave method.12 The Mizuhiara method enables the details of the extracellular matrix and cytoskeleton to be well preserved. The conventional Karnovsky fixation solution was composed of 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (0.1 mole/L, pH 7.4). The microwave fixation solution was composed of 2% paraformaldehyde, 0.5% glutaraldehyde solution, 0.1% tannic acid, and 2 mmole/L EGTA in cacodylate buffer (0.1 mole/L, pH 7.2). Ultrathin sections from epoxy resin-embedded blocks were routinely made and observed using a transmission electron microscope (H-7500, Hitachi High-Technologies Corp., Tokyo).

Analytical Electron Microscopy

Based on a method described in a previous report,13 the samples were spatter-coated with osmium using an osmium plasma coater (NL-OPC80N, NIPPON LASER & ELECTRONICS LAB, Nagoya, Japan), examined using a scanning electron microscope (S-4500, HITACHI Ltd., Hitachinaka, Japan), and analyzed using an energy dispersal-type X-ray analyzer (EMAX-7000, HORIBA Ltd., Kyoto, Japan).
Immunohistochemistry
The immunohistochemical study was performed using a two-step method that utilizes Dextran polymer technology (EnVision system, K5027, DakoCytomation). The paraffin sections were deparaffinized, hydrated, and treated with 0.3% H₂O₂ for 10 minutes to inactivate the endogenous peroxidase activity. Anti-vimentin (L1843, DakoCytomation, Kyoto), anti-desmin (#412911, Nichirei Corp., Tokyo), anti-alpha-smooth muscle actin (M0851, 1A4, DakoCytomation), anti-CD68 (N1576, PG-M1, DakoCytomation), anti-single stranded DNA (A4506, DakoCytomation), and anti-von Willebrand factor (M0616, F8/86, DakoCytomation) were used as they primary antibodies. For the anti-CD68 and anti-von Willebrand factor antibodies, the slides were predigested with 0.1% trypsin solution at 37°C for 20 minutes to retrieve the antigens.

TUNEL method
An in situ cell death detection kit (POD #1684817, Roche Diagnostics K.K., Tokyo) was used for the TdT-mediated dUTP nick end labeling (TUNEL) to detect apoptotic figures in the interstitial cells. The kit was used according to the manufacturer’s instructions. At the time of autopsy, small fragments of the mitral valvular annulus were taken and immediately fixed with 10% formalin, stored overnight, and processed into paraffin blocks. The paraffin sections were deparaffinized, hydrated and treated with H₂O₂ solution for 5 minutes, then immersed in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 minutes. The slides were then reacted with freshly prepared TUNEL solution (TdT and nucleotide mixture) at 37°C for 60 minutes and incubated with anti-fluorescein antibody Fab fragments at 37°C for 30 minutes. The peroxidase-positive structures were visualized using diaminobenzidine tetrahydrochloride.

Ethical considerations
Written informed consent was obtained from the family of the patients at the time of the autopsy. The use of autopsy materials for medical education and research is generally permitted by the Act of Postmortem Examinations of Japan.

Statistical analysis
Chi-square test was used to study the correlation between MAC and degenerative mitral regurgitation or calcific aortic valvular sclerosis. The average values of PAI or CSI were compared by two-sided Student t-test. The probability values less than 0.05 were considered significant.

Results
Incidence of mitral annular calcification
Among the 180 cases, mitral annular calcification was present in 34 cases (19%). The incidence of MAC was higher in females (23%) than in males (15%). Table 1 shows the age-dependent increase in the tendency to develop MAC, especially in females. The incidence of MAC was higher in females than that in males in the 80s and 90s, however the differences were not significant.

Location and length of mitral annular calcification
The mitral annulus around the origin of the posterior cusp was affected in 31 of the 34 MAC cases (91%). The mitral annulus of both anterior and posterior cusps was affected in two cases. The posterior commissure was involved in one case.

Three patterns of MAC origin and progression were recognized using low-power histological images of the regions affected by MAC. In the protruding type, the calcified nodules originated at the hinge of the cusps, grew downward, and protruded between the ventricular wall and the cusp (11 cases). In the invasive type, the nodules were characterized by a direct invasion toward the adjacent left ventricular myocardium (11 cases). Two advanced cases of this type also had infective endocarditis complications. In the valvular type, the calcification originated at the hinge and/or at the fibrous layer of the cusp and extended along the mitral valvular cusp, to form one or more movable masses (12 cases). The maximum length and thick-

| Table 1. Incidence of mitral annular calcification in elderly autopsy cases. |
| Age (yr) | Male | Female |
| Case | MAC* (%) | Case | MAC* (%) |
| 50-69 | 16 | 2 (2,00) | 9 | 0 (0,00) |
| 70-79 | 35 | 5 (3,1,1) | 32 | 4 (3,1,0) |
| 80-89 | 35 | 5 (1,1,3) | 26 | 8 (1,5,2) |
| 90-99 | 12 | 3 (0,0) | 15 | 7 (4,2,1) |
| Total | 98 | 15 (9,2,4) | 82 | 19 (8,8,3) |

* The figures in parentheses indicate the numbers of cases with mild, moderate, and severe degree of MAC. MAC: mitral annular calcification
ness of the MAC were 50 mm and 20 mm, respectively. The cases were composed of 17 cases with mild MAC, 10 cases with moderate MAC, and 7 cases with severe MAC. Moderate or severe MAC were especially prevalent in the elderly patients over 80 years of age, as shown in Table 1.

Clinicopathological correlations with cardiac valvular diseases and other systemic diseases

Among the 34 MAC cases, 13 cases exhibited degenerative mitral valvular diseases, including 12 cases with mild to moderate mitral regurgitation, as shown in Table II. All 7 cases with severe MAC showed mitral regurgitation. The incidence of mitral regurgitation in cases with MAC was significantly higher than that in cases without MAC \((p<0.0005)\).

Calcific aortic valvular stenosis was a frequent complication in the MAC cases. 12 of the 34 MAC cases exhibited concomitant calcific aortic valvular sclerosis. The incidence of calcific aortic valvular stenosis in cases with MAC was significantly higher than that in cases without MAC \((p<0.0005)\).

The papillary muscle was involved by old or acute myocardial infarction in 7 cases without MAC and one case with MAC. One case without MAC and another case with MAC suffered from rheumatic mitral valvular disease. No calcification was, however, observed in the chordae and papillary muscle.

One male case with MAC had suffered from chronic renal failure and had received hemodialysis for 4 years. Three cases with MAC had atrial fibrillation. No statistical differences were observed between the incidence of the accompanying diseases in the MAC cases and the general incidence of those diseases in our autopsy series. Accompanying diseases included hypertension, diabetes mellitus, hyperlipidemia, ischemic heart disease, cerebrovascular disease, dementia and osteoporosis, all of which frequently occur in elderly people.

Atherosclerosis and mitral annular calcification

The degree of systemic atherosclerosis was mild and moderate in most cases with the average values (± S.D.) of PAI, being 3.9 (±1.5). The PAI was significantly higher in the cases with severe MAC than that in cases without MAC or with mild MAC, as shown in Table II. The coronary arteriosclerosis was assessed among 34 cases with MAC. One coronary vessel was affected in 7 cases (21%), two vessels in 8 cases (24%) and three vessels in 4 cases (12%). These incidences were not higher than those appeared in a previous report from our hospital, in which the incidence of one vessel disease was 22%, that of two vessels, 16% and that of three vessels, 16%. There was no significant correlation between the CSI and the severity of MAC among 34 cases with MAC, as shown in Table 2.

Histopathology of mitral annular calcification

The calcified nodules or nodes in the posterior cusp of the mitral annulus were best recognized when viewed upward after cutting the tendinous cords attached to the cusp and lifting the cusp. On micro-
Fig. 1. Histopathological and immunohistochemical pictures of the mitral annulus at the posterior cusp from a case without mitral annular calcification. 

a: Low-power view of the mitral annulus, indicated by the dotted lines, and surrounding structures. Note the lack of basophilic calcification (H.E. staining). Figures 1a to 1e were taken from the same locus of the mitral annulus, indicated by the blue square in Fig. 1a, at the same magnification in serial sections. 

b: Close-up view of the mitral annulus. The homogenous eosinophilic staining of the matrix is apparent. Several interstitial cells are scattered throughout the view (H.E. staining). 

c: Lipid-deposition in the matrix is weakly positive for Oil red O staining. The cytoplasm of some interstitial cells is also positive (red arrows and inset). 

d: Microscopic calcification of the matrix of the mitral annulus. Numerous fine granular or globular substances are visible (von Kossa staining). 

e: The interstitial cells are strongly positive for vimentin immunostaining. 

f: A number of interstitial cells are positive for the TUNEL reaction.
Fig. 2. The endocardium and fibrous layer of the mitral valvular cusp, derived from cases without mitral annular calcification. a: The thickening of the endocardium and the degeneration of the mitral annulus are visible. b: Same field as shown in Fig. 2a. Several CD68-positive macrophages are scattered throughout the thick endocardium. The subcellular granules, probably released from necrotic macrophages, are also positive for CD68. c: The deep interface of the degenerated mitral annulus (right side) and the surrounding tissue. von-Willebrand factor-positive dilated capillaries are present in the surrounding tissue but absent in the mitral annulus.

Fig. 3. Ultrastructural figures of microscopic calcification in the matrix of the mitral annulus originated from a case without mitral annular calcification. a: The interstitium contains large amounts of membranous vacuolar structures of various sizes (cellular degradation products). b: Some vacuoles show electron-dense spicules on their surfaces. The vacuoles contain granular or membranous degenerated materials. c: Some vacuoles contain needle-like structures (hydroxyapatite crystals) embedded in electron-dense materials.
scopic examination, the calcified deposits were composed of fragments of calcified and necrotic tissue, embedded in a degenerative collagenous matrix. The surrounding granulation tissue occasionally exhibited the proliferation of small blood vessels and the infiltration of inflammatory cells, such as lymphocytes, plasma cells and proliferating small osteoclast-like mesenchymal cells. The MAC contained foci of ossification, with the formation of mature lamellar bones in three cases.

Microscopic calcification of the mitral annulus in the cases without MAC

Focal degeneration of the mitral annulus was seen in all five cases without MAC (Figs., 1a and 1b); this degeneration included the loss of interstitial cells, the homogenous staining of collagen fibers, the formation of thick collagen bundles, and the accumulation of mucinous substances in the matrix. Some degenerated areas were weakly positive for Oil red O staining, even after paraffin-embedding (Fig. 1c), but were negative for Congo red staining. Some interstitial cells were also positive for Oil red O staining. Fine, granular microscopic calcification was demonstrated by von-Kossa staining in some of the degenerated areas seen in all five cases without MAC (Fig. 1d). Both the microscopic and macroscopic calcification occurred in the fibrous core of the mitral annulus, not in the left atrium, nor left ventricle, and the same kind of microscopic calcification was observed around the calcified foci of minimal MAC lesions. Within the areas of microscopic calcification, large, basophilic von-Kossa-positive globules up to 7 \( \mu \text{m} \) in diameter were visible on the hematoxylin-eosin sections. Immunostaining showed that the interstitial cells were positive for vimentin (Fig. 1e). A few interstitial cells were positive for alpha-smooth muscle actin. The interstitial cells were completely negative for desmin. Many of the interstitial cells were positive for single-stranded DNA, as shown by immunostaining and the TUNEL reaction (Fig. 1f). A few CD68-positive macrophages were seen scattered at the periphery of the mitral annulus and, in some cases, frequently in the thickened endocardium (Figs., 2a and 2b). Immunostaining with anti-von Willebrand factor revealed very few capillaries in the mitral annulus (Fig. 2c).

Electron microscopy studies showed numerous cellular degradation products in the interstitium (Fig. 3a). Membranous structures of various size, often containing amorphous or membranous structures, were seen. Some of the membranous structures exhibited an increase in electron density at their periphery as well as needle-like hydroxypatite crystals (Figs., 3b and 3c). Interstitial cells in various states were seen: some active (proliferative) interstitial cells exhibited an abundance of intermediate filaments, many rough endoplasmic reticulum structures, and a few Golgi apparatus, whereas some quiescent (resting) cells exhibited a poorly developed rough endoplasmic reticulum, many secondary lysosomes, and some lipid droplets (Fig. 4a). Some interstitial cells contained microfilaments with dense bodies beneath their cell membranes (Fig. 4b). Thus, the electron microscopic features of the interstitial cells were compatible with those of myofibrob-
lasts. Calcification of the collagen fibrils or elastic fibers was not observed. X-ray microanalysis revealed that the main elementary components of the globules in the degenerated areas were calcium and phosphate (Figs., 5a to 5d).

Discussion

Several reports on the clinicopathological features of MAC have been made. These reports have described a high incidence in elderly female patients, the frequent involvement of the posterior cusp, and the coexistence of calcific aortic valvular stenosis.\textsuperscript{1,5,8} Sugiura et al.
examined 600 consecutive autopsy cases performed at our hospital during 1972 and 1973 and reported that the incidences of MAC in males in their 70s and 80s were 7.5% and 7.0%, respectively, while those in females in their 70s and 80s were 9.9% and 16.4%, respectively. The incidence of MAC in the present study was higher than these previously reported results. The Framingham Study, a longitudinal community-based cohort study, reported similar figures regarding the prevalence of MAC. In all of the previous reports, the prevalence of MAC tended to increase with age and was higher among females than among males. The major pathologic features of MAC observed in the present study were similar to the results described in previous reports.

Although the pathology of advanced MAC has been well described, no reports on the early stage of MAC have been made, to the best of our knowledge. The cases without MAC in the present study produced the following findings: 1) microscopic calcification and lipid-deposition in the mitral annulus were common findings in elderly autopsy cases; 2) microscopic calcification was initiated by calcium-deposition on cellular degradation products, found in abundance in the interstitium; 3) the interstitial cells of the mitral annulus were myofibroblasts; 4) many interstitial cells tested positive for the TUNEL reaction, and 5) a paucity of infiltrating macrophages and a lack of capillaries was observed in the mitral annulus. We assured that these microscopic calcification was the early stage of MAC. The best reasonable way to confirm this assumption is a longitudinal histopathological study of mitral annulus in the same patients who eventually develop MAC, however it is actually impossible to perform due to difficulty of valvular biopsy. The alternative, but less direct method is to speculate the developing process of MAC by studying and comparing the mitral annulus with normal histology to severe MAC as many as possible. We observed that both microscopic and macroscopic calcification occurred in the same locus of mitral valvular annulus, and that microscopic calcification was present around the calcified foci of minimal MAC. Thus, we speculated microscopic calcification was the earliest stage of MAC. The pathological changes showing the microscopic calcification of the mitral annulus closely resembled those that have been reported for aortic valvular calcification and aortic medial calcification. In these reports, the authors emphasized the major role of the apoptosis of interstitial cells in the pathogenesis of calcification. Cellular degradation products released from the apoptotic cells are usually desquamated into the lumen or digested by macrophages and do not result in calcification. The absence of this scavenger system in cartilage and cardiovascular connective tissue seems to cause bone formation or calcification. Our study showed that a number of interstitial cells were positive for both anti-single stranded DNA immunostaining and the TUNEL reaction. A recent study on the aortic media of patients with various kinds of aortic diseases revealed that the smooth muscle cells in this region show no molecular evidence of apoptosis, despite the presence of large numbers of TUNEL-positive smooth muscle cells. The authors indicated that the TUNEL reaction was not specific for apoptosis and only revealed the presence of DNA-strand fragments. Further studies are needed to determine the nature of the DNA fragmentation in the myofibroblasts and smooth muscle cells of cardiovascular connective tissue.

A recent report has proposed another theory stressing the importance of the accumulation of plasma-derived lipoprotein and the activation of macrophages in the early phase of aortic valvular calcification pathogenesis. They described the accumulation of apolipoproteins B, (a), and E as well as the infiltration of macrophages adjacent to the microscopic or gross calcification of the aortic valve. Activated macrophages are known to produce osteopontin, a protein involved in tissue calcification. This theory, however, might not be applicable to MAC, since microscopic calcification always takes place in a deeper region of the mitral annulus and macrophages are not found in the calcified areas.

Prospective studies have shown that patients with MAC were more likely to develop stroke and cardiovascular morbidity and mortality. Factors such as an advanced age, female sex, obesity, hypertension, diabetes mellitus, hypercholesterolemia, cardiomegaly, and arrhythmia were significantly associated with MAC. Since these factors are also known to be associated with atherosclerotic factors, the authors believe that MAC is a manifestation of generalized atherosclerosis. The present study also showed a significant correlation between atherosclerosis and MAC, however, from a pathological point of view, atherosclerosis is an intimal disease and the calcification of the cardiovascular connective tissue is a disease of the cardiac skeleton and aortic media. The pathological basis of the correlation between atherosclerosis and MAC remains to be clarified.
Conclusions

As the proportion of elderly people has been rapidly increasing in both developed and developing countries, the geriatric cardiac diseases, such as mitral annular calcification, calcific aortic valvular stenosis, and senile cardiac amyloidosis, become much more important. The pathogenesis of calcific aortic valvular stenosis has been studied by several researchers, as the disease is occasionally cured by valvular replacements and the surgical materials of the calcified aortic valves are available. Little attention, however, has been paid to MAC. Many unsolved questions could be listed about the pathogenesis of MAC: Are the cellular degradation products deposited in the mitral annular matrix truly derived from dead interstitial cells? If so, what causes the death of interstitial cells? Whether the death of interstitial cells is apoptosis or necrosis? If the microscopic calcification of the mitral annulus is a common finding in the elderly population, what causes the progression of calcification from a microscopic to macroscopic stage?

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