Full Paper

Contribution of Bone Morphogenetic Protein-2 to Aortic Valve Calcification in Aged Rat

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Abstract. Although aging is well established as an important risk factor for aortic stenosis, the mechanism of age-related aortic valve calcification is yet unknown. Here, we investigated this mechanism in tissue and cellular levels using middle-aged rats. Aortic valve specimens were obtained by dissecting from 9-week-old (young) and 30-week-old (aged) male Wistar rats. In the aged rats, the main risk factors for aortic stenosis in plasma were still in the normal range; however, their number of calcified specimens was significantly increased in comparison with the young rats. Aortic valve interstitial cells (AVICs) obtained from explants of aortic valve specimens were cultured for 14 days after reaching confluence. Spontaneous calcification, the expressions of calcigenic genes, that is, BMP-2, alkaline phosphatase (ALP), and osterix (osteogenic transcription factor) and ALP enzyme activity in AVICs from aged rats were enhanced in comparison with those from young rats. However, neither typical calcification inducing reagents (dexamethasone, β -glycerophosphate, and high concentration of phosphate) nor tumor necrosis factor- α (an inflammatory cytokine) accelerated the spontaneous calcification of AVICs from aged rats. These results suggest that aortic valve calcification progresses with age partly through an activation of the BMP-2 pathway.

Keywords: calcification, aortic valve, BMP-2, aging

Introduction

Aortic valve stenosis (AS) is the most common form of acquired valvular disease in the elderly (1). Since the most viable treatment for patients with severe AS is aortic valve replacement, early prevention of AS is very important (2). Generally, a normal aortic valve area in adult human is approximately 3 cm². In the diagnostic standard of AS, the aortic valve area of AS is smaller than 2 cm². People who have an aortic valve area larger than 2 cm² may be in the preliminary stage of AS, and they often have some mild calcification in the aortic valve that has not yet caused significant heart problems. Sell and Scully reported an age-dependent progression of aortic valve sclerosis (3). Clinical care prohibiting the progression of

aortic valve calcification from the early stage of aortic valve sclerosis has become an urgent challenge. However, effective drug therapy to inhibit the aortic valve calcification has not yet been developed (4, 5).

Aortic valve calcification is an actively regulated process associated with a differentiation of myofibroblasts to the osteoblast-like phenotype (6). The osteoblast-specific transcription factor, Runt-related gene 2 (Runx2) and bone morphogenetic marker, alkaline phosphatase (ALP) have been overexpressed in calcified aortic valve, and they are believed to play an important role in the process of aortic valve calcification (6). Many trigger factors for valve calcification have been characterized, and most of them are cytokines such as tumor necrosis factor- α (TNF- α), TGF- β , and interleukins released from macrophages and T-lymphocytes activated by inflammation (7 – 9). They are potent osteogenic morphogens and contribute to the calcification process to induce bone formation. Various molecular biological studies using

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aortic valve interstitial cells (AVICs) have also suggested that the differentiated osteoblast-like cells contribute to aortic valve calcification through various mechanisms such as Smad, Wnt/ β -catenin, p38MAPK, and ERK1/2 signaling (10–12).

Knowledge about pathophysiological process involved in aortic valve calcification causing AS has accumulated, but there is little information about the detailed mechanism of the age-related aortic valve calcification. Therefore, we investigated whether aortic valve calcification occurs in healthy middle-aged rats. The data in this study clearly demonstrated that even in physiologically normal middle-aged rats (30 weeks of age), aortic valve calcification occurred in both tissue and cellular levels. We further investigated the molecular mechanism underlying aortic valve calcification using rat AVICs and demonstrated that this calcification in aortic valves may be partly due to the process promoted by BMP-2.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo). Fetal bovine serum (FBS, BiofluidsTM) and Penicillin & Streptomycin (GibcoTM) were obtained from Invitrogen Co. (Carlsbad, CA, USA). Dexamethasone, β -glycerophosphate, and other analytical grade of reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Reagents for reverse transcription reactions (Random Primer, 10 mM dNTP Mix, 5 $\times$ First-strand Buffer, 0.1 M DTT, M-MLV RT, and DNase/RNase-free distilled water) were purchased from Invitrogen Co. Ribonuclease inhibitor was obtained from TaKaRa Bio Inc. (Shiga). All primers used for quantitative real-time PCR were obtained from Fasmac Co., Ltd. (Kanagawa). Power SYBR[®] Green PCR Master Mix was supplied by Applied Biosystems (Foster City, CA, USA). All chemicals used were of the highest purity commercially available. All solutions were freshly made at sufficiently high concentrations so only very small aliquots had to be added to the culture medium.

Male Wistar rats at 9 and 30 weeks of age were purchased from Clea Japan, Inc. (Tokyo). All experiments were performed according to the Animal Experiment Guidelines of the Laboratory of Animal Experiments for Hirosaki University School of Medicine. The animals were maintained for 1 week at a temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of 57% on a 12-h light / 12-h dark cycle (lights on 08:00–20:00) and had free access to water and food.

Aortic valve isolation and von Kossa staining

Aortic valves were obtained from the young (9-week-old) and aged (30-week-old) Wistar rats. Immediately after dissection from the heart, one specimen from each aortic valve was fixed in 10% buffered formalin for 24 h and then embedded in paraffin. Paraffin-embedded specimens were examined for morphology with hematoxylin-and-eosin staining and for mineralization with von Kossa staining. The chi-square test using contingency tables was performed to compare the number of calcified or non-calcified specimens in young or old group.

Isolation and culture of AVICs

After rat aortic valves were excised from hearts, the specimens were gently cut into 2–3 pieces. Valve pieces were then placed in culture plates and covered with DMEM (9.5 g/l) containing L-glutamine (0.584 g/l), 10 U/l penicillin and 10 $\mu\text{g/l}$ streptomycin, and 10% FBS. On reaching 40%–50% confluence, cells were split into 35-mm dishes by soaking in trypsin-EDTA, followed by rinsing with culture medium, and then they were distributed among the dishes. The cells were cultured at 37°C under 5% CO_2 , and the medium was replaced with fresh medium every 3 days. After passage 4 of AVICs reached 95% confluence, they were further cultured in medium for 7 or 14 days. The change in cell number was measured using Celltac (Nihon Kohden, Tokyo) and defined as the basal cell number at Day 0.

Various calcification inducing reagents, dexamethasone (0.1 μM) + 50 μM ascorbic acid, β -glycerophosphate (1 mM) + 50 μM ascorbic acid, and high concentration of phosphate (2.6 mM), and TNF- α (100 ng/ml) were added to AVICs and cultured for 14 days to stimulate calcification (13).

Alizarin Red S staining of AVICs

After reaching confluence (Day 0), AVICs were cultured in DMEM containing 10% FBS for 7 or 14 days. Calcium precipitation in the AVICs was assessed by Alizarin Red S staining. Briefly, the matrix was washed with phosphate-buffered saline (PBS) and incubated with 10% buffered formalin for 15 min. After three washes with purified water, cultures were treated with Alizarin Red S solution at room temperature for 5 min. The excess Alizarin Red S solution was completely washed away using purified water to allow color development. Alizarin Red S staining was released from cell matrix by incubation in cetyl-pyridinium chloride, and the amount of released dye was quantified by spectrophotometry at 550 nm.

Total RNA preparation and cDNA synthesis

Total RNA was extracted using the Quick Gene RNA cultured cell kit S for AVICs (Fujifilm, Tokyo). After resuspension of the purified total RNA in RNase-free water, aliquots containing 1 μ g of total RNA were used as a template for the reverse transcription reaction (14).

Reverse transcription was carried out in a 14- μ l reaction mixture containing 1 μ g of total RNA and 10 nmol of dNTP Mix. After denaturation at 65°C for 5 min, the reaction mixture was cooled on ice for 2 min and then 200 U of M-MLV reverse transcriptase, 200 nmol of DTT, and 2 U of ribonuclease inhibitor were added to the reaction mixture. Extension was carried out at 37°C for 60 min and then 75°C for 15 min.

Quantitative real-time PCR

For real-time PCR reactions, the total reaction volume was adjusted to 26 μ l and contained 3 μ l of a 1:4 dilution of the first-strand reaction product, 1 μ l of 10 μ M specific forward and reverse primers, 8 μ l of pure water, and 13 μ l of SYBR Green. Amplification and analysis of cDNA fragments were carried out using an ABI Prism 7000 (Applied Biosystems). The primers used for ALP, BMP-2, osterix, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed by Primer Express ver. 2.0[®] (Applied Biosystems) and their sequences are shown in Table 1. The cycling conditions were initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/primer extension at 60°C for 1 min.

Amplification of the housekeeping gene G3PDH served as a normalization standard. Real-time PCR data were represented as cycle threshold (Ct) levels and normalized by the individual G3PDH control Ct values. Relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method (15).

ALP activity assay

After the cells were washed twice with PBS, the cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged, and the supernatants were assayed for ALP activity using a commercially available kit (Laboassay[™] ALP; Wako, Tokyo). One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 min. Protein concentrations were determined by Bradford's method. The change in activity was expressed as a ratio to the basal value of young rat at Day 0.

Statistics

All statistical analyses were carried out by KyPlot 5.0 software (Kyenslab, Tokyo). For comparison with the formation of calcium precipitation, the chi-square test for independence was performed. Multiple group comparisons were performed by one-way ANOVA, followed by Tukey's procedure for comparisons of means. All data were expressed as the mean $\pm$ S.E.M., and values of $P < 0.05$ was taken to indicate significant differences.

Results

Aging of rats characterized by various physiological data

Table 2 shows various physiological data for young (9-week-old) and aged (30-week-old) rats. Body weight was significantly increased with age. Although systolic and mean blood pressures of aged rats were significantly higher than those of young rats, these blood pressure levels of aged rats were considered to be in a normal range.

Plasma total levels of main risk factors for aortic stenosis were measured (Table 3). The alkaline phosphatase activity and inorganic phosphate content were significantly decreased in aged rats but considered to be in a normal range. Calcium and indexes of diabetes and atherosclerosis (triglyceride, high density lipoproteins,

Table 1. Primers used for quantitative real-time PCR

Gene symbol	Genbank accession No.	Sequences (5'-3')
ALP	J03572	Forward: TCCGTGGGTCGGATTCCT Reverse: GCCGGCCCAAGAGAGAAA
BMP-2	L20678	Forward: CACCGTGCTCAGCTTCCA Reverse: CGGGAAGTTTTCCCACTCATT
Osterix	AY177399	Forward: TGACTGCCTGCCTAGTGCTACA Reverse: TGGATGCCCCGCCTTGT
Msx2	NM_012982	Forward: TCACCACGTCCCAGCTTCTAG Reverse: CTGCGATGGAGAGGTACTGTTTC
G3PDH	BC059110	Forward: TGCCAAGTATGATGACATCAAGAAG Reverse: AGCCCAGGATGCCCTTAGT

and glucose) showed no significant differences.

Calcification of aortic valve

Figure 1 shows the typical bright field view of an aortic valve specimen of Wistar rats stained with hematoxylin-eosin and von Kossa. Two in 9 aortic valve specimens from young rats were positive on von Kossa staining (Fig. 1A). On the other hand, 22 in 27 of specimens from aged rats were positive (Fig. 1B). There was a significant difference in the numbers of calcified specimens between young and aged rats (Chi-square test, $P < 0.01$).

Calcium precipitation in cultured AVICs

Figure 2A shows the typical Alizarin Red S staining of the AVICs from young and aged rats. Both AVICs were weakly and similarly stained at Day 7. After 14 days of culture, however, the staining of AVICs from aged rats increased 2.29-fold compared with that of young rats,

indicating a progress in calcification (Figure 2: A and B). Although the cell numbers of young and aged AVICs at Day 14 were increased to 2.27 ± 0.23 ($n = 3$)– and 1.91 ± 0.35 ($n = 5$)–fold in comparison with Day 0, respectively, the degree of cell proliferation between AVICs from young and aged rats were not significantly different.

Various calcification-inducing reagents, dexamethasone ($0.1 \mu\text{M}$) + $50 \mu\text{M}$ ascorbic acid, β -glycerophosphate (1 mM) + $50 \mu\text{M}$ ascorbic acid, high concentration of phosphate (2.6 mM), and TNF- α (100 ng/ml) failed to accelerate the calcification of AVICs from not only young but also aged rats for 14 days in spite of significant spontaneous calcification (Fig. 3: A and B).

Gene expressions of calcification-regulating proteins in AVICs

The calcigenic gene expressions in AVICs from young rats at Day 0 were defined as the basal expression levels. The gene expressions of BMP-2, ALP, and osterix of

Table 2. Body weights and blood pressures from young (9-week-old, $n = 8$) and aged (30-week-old, $n = 12$) rats

	Body weight (g)	Systolic blood pressure (mmHg)	Mean blood pressure (mmHg)	Diastolic blood pressure (mmHg)
Young	285.0 ± 6.2	114.3 ± 2.2	93.3 ± 2.3	83.2 ± 2.6
Aged	$497.4 \pm 9.5^{***}$	$128.7 \pm 3.1^{**}$	$103.1 \pm 2.4^*$	90.1 ± 2.4

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$: compared with young rats.

Table 3. Plasma total levels of main risk factors for aortic stenosis in young (9-week-old, $n = 8$) and aged (30-week-old, $n = 7$) rats

	Alkaline phosphatase (IU/l)	Plasma Ca (mg/dl)	Inorganic phosphate (mg/dl)	Triglyceride (mg/dl)	High-density lipoprotein (mg/dl)	Glucose (mg/dl)
Young	1897.8 ± 95.3	17.3 ± 0.5	9.4 ± 0.2	130.0 ± 7.3	18.9 ± 1.2	176.5 ± 5.2
Aged	$852.0 \pm 132.9^{**}$	14.9 ± 0.8	$6.0 \pm 0.3^{**}$	98.6 ± 21.0	17.9 ± 2.8	158.0 ± 25.3

** $P < 0.01$, compared with young rats.

Fig. 1. Aortic valve calcification and gene expression in young (9-week-old) and aged (30-week-old) rats. Typical bright field view of hematoxylin-eosin and von Kossa staining of aortic valve specimen from young rats (A) and aged rats (B). The calcification area is indicated with arrows.

Fig. 2. Alizarin Red S staining of cultured AVICs from young and aged rats. AVICs were cultured in DMEM containing 10% FBS. After reaching confluence (Day 0), AVICs were further cultured for 7 (Day 7) or 14 days (Day 14). A: Cultures of AVICs from three young rats (9-week-old) and five aged rats (30-week-old) were stained by Alizarin Red S. Figure shows a typical result. B: Quantification of Alizarin Red S staining at Day 14 via extraction with cetyl-pyridinium chloride. The amount of released dye was quantified by spectrophotometry at 550 nm. Bars represent the mean $\pm$ S.E.M. (* P < 0.05 vs. young rats).

Fig. 3. Calcification of AVICs from young and aged rats in the presence of various calcifying agents. AVICs were cultured in DMEM containing 10% FBS. After reaching confluence (Day 0), AVICs were further cultured for 14 days (Day 14) in the presence of various calcifying agents. A: Cultures of AVICs from young rats (9-week-old, $n = 3$) and aged rats (30-week-old, $n = 3$) were stained by Alizarin Red S. The figure shows a typical result. B: Quantification of Alizarin Red S staining at Day 14. The amount of released dye was quantified by spectrophotometry at 550 nm. White and black bars represent young and aged rats, respectively. a) Absence of reagents. b) Dexamethasone ($0.1 \mu\text{M}$) + $50 \mu\text{M}$ ascorbic acid. c) β -Glycerophosphate (1 mM) + $50 \mu\text{M}$ ascorbic acid. d) High concentration of phosphate (2.6 mM). e) Tumor necrosis factor- α (TNF- α , 100 ng/ml). Bars represent the mean $\pm$ S.E.M.

AVICs from aged rats were significantly and time-dependently upregulated in comparison with those from young rats (Fig. 4). However, the *Msx2* gene expression of AVICs from aged rats at Day 14 (14.65 ± 5.47 fold) was not increased by aging (14.53 ± 3.36 fold in young rats).

ALP activity

To confirm that ALP gene expression reflects the spontaneous calcification of AVICs from aged rats, we further investigated the ALP activity. At Day 0, the basal ALP activity of AVICs from aged rats was not increased by aging (1.01 ± 0.05 fold). AVICs from both groups were accompanied by upregulated ALP activity at Day 7. The activity further increased significantly in AVICs from aged rats at Day 14 (Fig. 5).

Discussion

In the present study, we demonstrated that most of the aortic valve specimens from physiologically normal aged rats (30-week-old) were calcified, and the number of calcified specimens was significantly increased in comparison with the values of young rats (9-week-old). We next investigated the calcification of AVICs. Spontaneous calcification of AVICs from aged rats occurred significantly in comparison with that of AVICs from young rats. Gene expressions of BMP-2 and ALP were significantly accelerated in AVICs from aged rats. These results suggest the contribution of BMP-2 to age-related spontaneous aortic valve calcification.

The downstream pathways of BMP-2 expression known to enhance ALP expression, which is a typical gene expression marker for calcification, are Smad (16) and Wnt (17) signalings. We further investigated the gene expression of calcification marker proteins relating

Fig. 4. Real-time PCR analysis of ALP mRNAs expression in AVICs from young and aged rats. After reaching confluence (Day 0), AVICs were further cultured in DMEM containing 10% FBS for 7 (Day 7) or 14 (Day 14) days. White and black bars represent the mRNA levels in AVICs from young (9-week-old) and aged (30-week-old) rats, respectively. The BMP-2 (A), ALP (B), and osterix (C) mRNA levels were determined by quantitative real-time PCR and normalized by the corresponding levels of G3PDH mRNA. All ratios were calculated vs. young at Day 0. Bars represent the mean $\pm$ S.E.M. [$n = 3$ (young) and $n = 5$ (aged)]. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. aged rats at Day 14.

Fig. 5. Assay of ALP activity. After reaching confluence (Day 0), AVICs from young (9-week-old) and aged (30-week-old) rats were further cultured for 7 and 14 days. The cells were solubilized with 1% Triton X-100 in 0.9% NaCl, and the supernatants were assayed for ALP activity by using a commercially available kit (Laboassay™ ALP). One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 min. All ratios were calculated vs. young at Day 0. White and black bars represent young and aged rats, respectively. Each value represents the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ vs. aged rats at Day 14.

to two downstream signaling pathway of BMP-2. In AVICs, osterix, a transcription factor on the Smad pathway, but not *Msx2*, a transcription factor on the Wnt pathway, gene expression was increased. Thus, we can hypothesize that age-related aortic valve calcification partly progresses through the BMP-2/Smad pathway. We could not

measure the level of BMP-2 and ALP gene expressions in the tissue level because the small volume of the aortic valve specimen prohibited its total RNA extraction.

It was demonstrated that the spontaneous calcification of AVICs from aged (30-week-old) rats was not affected by the calcigenic stimulations such as dexamethasone, β -glycerophosphate, high concentration of phosphate, and TNF- α (18), which were associated with pathophysiological conditions such as atherosclerosis, cardiovascular inflammation, and kidney dialysis (Figs. 2 and 3). Furthermore, gene expression of ALP, BMP-2, and osterix in AVICs from aged rats were significantly stimulated (Fig. 4) but not amplified in the presence of high concentration of phosphate (K. Seya et al., unpublished data). If AVICs were already differentiated into osteoblast-like cells, age-related spontaneous calcification of AVICs could be enhanced in the presence of these calcification inducing reagents. Thirty-week-old rats are almost equivalent to thirty-year-old humans, namely middle-aged, and yet maintained the main risk factors for aortic stenosis within a normal level. These results suggest that AVICs from aged rats do not yet differentiate into osteoblast-like phenotype and thus they are insensitive to the stimulation by various calcifying reagents. Namely, age-related spontaneous calcification in aortic valve specimens and AVICs at 30 weeks of age may be independent of the pathophysiological conditions.

The remaining important issue is how BMP-2 expression contributes to the age-related aortic valve calcification. BMP-2 is produced by various inflammatory factors like TNF- α , TGF- β , and interleukins (9). However,

TNF- α did not show the acceleration of spontaneous calcification. This result suggests the contribution of factors other than inflammation. One report demonstrates that age-related expression of BMP-2 was physiologically accelerated in horse articular cartilage (19). The aortic valve, especially the annulus area, was physiologically exposed to long-term mechanical stress throughout life, similar to articular cartilage. One possibility is that age-related aortic valve calcification induced by BMP-2 may progress by degrees under long-term mechanical stress until strong invasion of inflammatory stimulations are induced by hypertension, hyperlipidemia, and hypercholesterolemia (20). However, we could not clarify the upstream signaling of BMP-2 expression caused by physiological mechanical stress. The more detailed mechanism of BMP-2 production underlying the age-related aortic valve calcification awaits further investigation.

In conclusion, we demonstrated using physiologically normal aged rats (30-week-old) that BMP-2 may partly contribute to the aged-related calcification that is not affected by various pathophysiological conditions known to induce calcification. Further studies on the upstream of BMP-2 signaling and comparative investigations of the age-related aortic valve calcification may provide new insights into the contribution of aging upon valvular calcification.

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