Original Article

The Expression of Groups IIE and V Phospholipase A₂ is Associated with an Increased Expression of Osteogenic Molecules in Human Calcified Aortic Valves

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Aim: Eicosanoids play various pathogenic roles in aortic valve calcification. Eicosanoids are derived from the arachidonic acid generated by phospholipase A₂ (PLA₂). We therefore sought to determine whether PLA₂s are expressed in human aortic valves and, if so, whether the expression of PLA₂s is related to the expression of osteogenic molecules in these tissues.

Methods: Histological and gene expression analyses of 38 non-rheumatic aortic valves obtained at the time of cardiac valve replacement surgery were conducted. Moreover, gene expression analyses were performed using valve interstitial cells (VICs) obtained from human aortic valves.

Results: Among the PLA₂s examined, the degree of immunoreactivity for PLA₂s-IIE and -V was found to significantly correlate with the grade of calcification in the aortic valves. The degree of immunoreactivity and gene expression levels of PLA₂s-IIE and -V significantly correlated with those of bone morphogenetic protein (BMP)-2, osteopontin and alkaline phosphatase (ALP). In addition, immunoreactivity for cyclooxygenase (COX)-1, COX-2 and 5-lipoxygenase, downstream enzymes of PLA₂ in the arachidonic acid cascade, was co-localized with that for PLA₂s-IIE and -V in cells expressing α -smooth muscle actin and macrophages expressing CD68. Furthermore, in the *in vitro* experiments using cultured VICs, the mRNA expression levels of BMP-2, osteopontin and ALP were suppressed by the inhibition of the expression of PLA₂s-IIE or -V with specific siRNAs.

Conclusions: The expression of PLA₂s-IIE and -V correlates with the development of calcification as well as the expression of pro-osteogenic molecules in human aortic valves, and inhibiting the expression of PLA₂s-IIE and -V suppresses the induction of osteogenic molecules in cultured cells. Therefore, PLA₂s-IIE and -V may play a role in the pathogenesis of valve calcification.

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Key words: Phospholipase A₂, Aortic valve calcification, Osteogenic molecules

Introduction

Eicosanoids and fatty acids play important roles in osteogenesis¹⁾, and a recent report showed that eicosanoids contribute to the genesis of human aortic

valve calcification²⁾. These lipid mediators are produced by the hydrolysis of membrane phospholipids via the catalytic activity of phospholipase A₂ (PLA₂)³⁻⁸⁾. However, the role of PLA₂ in the pathogenesis of aortic valve calcification remains unknown.

A number of PLA₂ subtypes have been identified and classified into different families based on their biochemical features and primary structures³⁻⁸⁾. For example, human PLA₂ enzymes are classified into the following categories: intracellular cytosolic PLA₂s (cPLA₂, group IV PLA₂), Ca²⁺-independent PLA₂s

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(iPLA₂, group VI PLA₂), PAF acetylhydrolases (groups VII and VIII PLA₂s), lysosomal PLA₂ (group XV PLA₂), adipose-specific PLA₂ (group XVI PLA₂) and secretory PLA₂s (groups IB, IIA, IID, IIE, IIF, III, V, X and XII)³⁻⁸.

The mechanisms underlying the development of aortic valve calcification are complex and only partially understood⁹⁻¹⁴. Previous reports have suggested that the transformation of valve interstitial cells (VICs) into osteoblast-like bone-forming cells is a critical step in the onset of valve calcification^{9,10}. The calcification process is mediated by bone morphogenetic proteins (BMPs)-2/4, members of the transforming growth factor- β superfamily¹⁰⁻¹². BMPs stimulate osteoblast-like cells to induce the production of runt-related transcription factor (Runx)2/Cbfa1, the master osteoblast transcription factor that upregulates the expression of calcification-related proteins, including osteopontin and osteocalcin^{10,12}. BMPs also promote Wnt signaling, leading to the upregulation of the expression of alkaline phosphatase (ALP), which is necessary for the initiation of calcification¹⁰⁻¹². Therefore, these osteogenic molecules play key roles in the pathogenesis of valve calcification.

Aim

This study aimed to determine which PLA₂s are expressed in human aortic valves and whether the expression of PLA₂s is related to the expression of calcification-related proteins in these tissues.

Methods

Valve Samples

The histological and gene expression analyses included a total of 38 non-rheumatic aortic valves obtained from 28 patients with isolated aortic stenosis (AS) and 10 patients with aortic regurgitation (AR) who underwent cardiac valve replacement surgery at Yamanashi University Hospital. Patients with endocarditis, renal insufficiency, chronic inflammatory diseases or malignancy were excluded from this study. The clinical characteristics of the enrolled subjects are shown in **Table 1**. Non-rheumatic aortic valves derived from an additional 10 patients were used to isolate VICs. Written informed consent was obtained from all participants prior to the start of the study, and the study protocol was approved by the ethics committee at our institution.

Histological Analysis

The aortic valve cusps were fixed with 10% form-

Table 1. Clinical characteristics of the patients

	Total patients (n = 38)
Age (years)	70.7 $\pm$ 12.2
Male (n)/Female (n)	16/22
BMI (kg/m ²)	21.3 $\pm$ 3.1
Hypertension (n, %)	25 (65.8)
Diabetes mellitus (n, %)	7 (18.4)
Coronary artery disease (n, %)	9 (23.7)
Smoking (n, %)	14 (36.8)
LDL-C (mg/dL)	126.7 $\pm$ 37.1
HDL-C (mg/dL)	38.5 $\pm$ 11.4
CRP (mg/dL)	0.33 $\pm$ 1.24
BNP (pg/mL)	471 $\pm$ 587
Statin use (n, %)	11 (28.9)
LVEF (%)	59.0 $\pm$ 17.4
Peak trans-aortic gradients (mmHg)	72.7 $\pm$ 25.8

The data are expressed as the mean $\pm$ SD or number (%) of patients. BMI = body mass index, LDL-C = low-density lipoprotein cholesterol, HDL = high-density lipoprotein, CRP = C-reactive protein, BNP = brain natriuretic peptide, LVEF = left ventricular ejection fraction on echocardiography. The peak transaortic gradients were calculated only in patients with aortic stenosis using pulsed and continuous wave Doppler echocardiography.

aldehyde and decalcified in Plank-Rychlo solution (a mixture of 7.0 g of aluminum chloride, 5.0 mL of formic acid, 8.5 mL of 37% hydrochloric acid and 100 mL of distilled water). Thereafter, the samples were embedded in paraffin and cut into 4- μ m sections. For immunostaining, endogenous peroxidase was inactivated by incubating the samples with 0.3% hydrogen peroxide for 30 minutes. After washing, antigen retrieval was performed by heating the sections for 10 minutes at 95°C in Target Retrieval Solution (Dako, Copenhagen, Denmark). The sections were subsequently blocked with 5% skim milk and incubated with the primary antibody (listed in **Supplemental Table 1**), followed by incubation with the secondary antibodies conjugated with horseradish peroxidase labeled polymers (EnVision System, Dako, Copenhagen, Denmark or Histofine Simple Stain, Nichirei Bioscience, Tokyo, Japan). The sections consequently developed a brown color after treatment with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate (Vector Laboratories, Burlingame, USA) and were finally counterstained with hematoxylin¹⁵. The specificity of anti-PLA₂ antibodies for immunoreactivity was confirmed using an absorption test, as shown in **Supplemental Fig. 1** and our previous report¹⁵. Some sections of each sample were incubated with normal rabbit, mouse or goat IgG instead

of the primary antibody, as negative controls. For double immunofluorescence staining, the sections were incubated with the indicated primary antibodies followed by the secondary antibody with Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, Calsbad, CA, USA)¹⁵. The nuclei were then stained with 4', 6'-diamidino-2-phenylidole (DAPI) (Vector Laboratories, Burlingame, CA, USA), and images were acquired using an Olympus FluoView 1000 confocal microscope (Olympus, Tokyo, Japan) and processed using the FV10-ASW software program, version 4.0 (Olympus). In some sections, calcification was visualized using Alizarin red S staining (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Measurement of the Number of Cells and Areas Immunopositive for PLA₂s and Osteogenic Molecules

The number of cells exhibiting immunoreactivity to each PLA₂ antibody was calculated in 10 fields per section under high-power magnification (x 400). The selection of fields for the cell count was determined based on a ratio of 2:4:4 for fibrosa, spongiosa and ventricularis layers, respectively, in all valves examined; the fields in each layer were randomly selected. The number of cells immunopositive for each PLA₂ antibody was expressed as the percentage of nucleated cells. The size of the area stained positively for each PLA₂ antibody was measured under low-power magnification (x 40) and expressed as a percentage of the total area of each section. One section from each sample was selected for measurement of the extent of immunopositive cells and areas. When multiple valve samples were analyzed in one patient, the average values were calculated and used for that patient. For the area measurements, images were captured using an Olympus BX41 microscope (Olympus, Tokyo, Japan) and Olympus DP21 camera (Olympus) under low-power magnification (x 40), and a computer-assisted image analysis was performed using the Photoshop CS5 software package (Adobe Systems, San Jose, CA, USA). In this study, the inter- and intra-observer variability for repeated measurements of PLA₂-IIE-immunopositive areas (including cells), expressed as the percentage of the total area of the sections in 10 valves, was $0.32 \pm 0.05\%$ and $0.29 \pm 0.06\%$, respectively, indicating a high level of reproducibility.

Degree of Aortic Valve Calcification

Based on a previous report¹⁶, the degree of aortic valve calcification and structural damage in the samples was graded using histological sections stained with hematoxylin-eosin, as follows: grade I, in which

the overall structural integrity of the valve cusp was retained; grade II, in which thickening of the valve cusp indicated the early development of calcified nodules; grade III, in which the valve cusp was grossly thickened and distorted with many calcified nodules; grade IV, in which the valve cusp was grossly distorted with alterations in most of the ordinary structural components.

Cultures of Valve Interstitial Cells (VICs)

VICs were isolated from 10 patients, including seven with AS and three with AR (mean age, 63.7 ± 15.3 years). The degree of valve calcification was grade I-III in all cases¹⁶. The valve tissues were digested with 0.2% type I collagenase solution (Worthington Biochemical Lakewood, NJ, USA) for 30 minutes at 37°C, after which both surfaces were lightly scraped to remove endothelial cells. The tissues were then minced and further dissociated with 0.2% type I collagenase solution for three hours at 37°C. The resulting cell suspension was centrifuged, and the pellets were resuspended in culture medium and plated on culture plates. The culture medium consisted of DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA), 100 U/mL of penicillin G, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B (Sigma-Aldrich, St. Louis, MO, USA). Cells at passage three or four were used for the experiments. More than 95% of the cells expressed α -smooth muscle actin (SMA) and vimentin based on immunocytochemistry, whereas less than 0.5% of the cells expressed CD31 or CD68. The cells obtained from each patient were maintained as independent cultures.

mRNA Expression

In a subgroup of 21 patients, total RNA was extracted from fresh samples of aortic valves using the Qiagen RNeasy kit and DNase I (Qiagen, Tokyo, Japan). The mRNA expression levels were measured according to a two-step real-time PCR assay using SYBR Green I chemistry and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) with the primers listed in **Supplemental Table 2**.

Experiments using Cultures of VICs Treated with siRNA

Silencer select pre-designed small interfering RNA (siRNA) specific for PLA₂-IIE or -V was purchased from Ambion (Carlsbad, CA, USA). The sequence of the sense siRNA was CAACCGCAAAU-AUGCCCAU for PLA₂-IIE and GAGGCUUGCUG-GACCUGAAA for PLA₂-V. The sequence of the nega-

tive control siRNA (Cat. No. 4390843) was not provided. The cultured VICs were transfected with 10 nmol/L of siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). After washing, the VICs were cultured in osteogenic medium containing culture medium with 50 μ g/mL of ascorbate-2-phosphate and 10 mmol/L of β -glycerol phosphate (Sigma-Aldrich, St. Louis, MO, USA) or control medium for seven days. Both the control and osteogenic media contained 10% FBS. The cells were subsequently used to measure the mRNA expression levels of osteogenic molecules in the cell homogenates and the eicosanoid concentrations in the cultured medium. ELISA kits for measurement of the concentrations of prostaglandin E₂ (PGE₂) and cysteinyl leukotrienes (CysLTs) were purchased from Cayman Chemical (Ann Arbor, USA). In a separate experiment, VICs treated with siRNA for each PLA₂ were incubated with the osteogenic medium supplemented with 10% FBS in the presence of vehicle or arachidonic acid (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 100 μ mol/L for seven days, after which the mRNA expression levels of osteogenic molecules in the VICs were measured.

Clinical Parameters

The fasting serum levels of low-density lipoprotein-cholesterol (LDL-C) were determined. In addition, the serum high-sensitivity C-reactive protein (CRP) levels were assayed using rate nephelometry (Dade Behring, Tokyo, Japan), and the plasma levels of brain natriuretic peptide (BNP) were measured using an immunoradiometric assay (Shionogi Pharmaceutical LTD, Osaka, Japan).

Statistical Analysis

The data are expressed as either the mean $\pm$ SD or frequency (%). Student's *t*-test was used to compare variables between two groups, and a one-way analysis of variance with Scheffe's post hoc analysis was used for comparisons between three groups. The correlation between two continuous variables was determined using a linear regression analysis. The correlation between the grade of aortic valve calcification and the level of immunoreactivity for PLA₂ or the osteogenic molecules was determined using Spearman's rank correlation test. All probability values presented are two-tailed, with statistical significance being inferred at a *p* value of <0.05 . All statistical analyses were carried out using the STATA version 10.0 software program (StataCorp, College Station, TX, USA).

Results

Valve Samples

The samples were classified according to the degree of calcification and morphology¹⁶⁾, as follows: seven in grade I (all from AR patients), three in grade II (one AS patient and two AR patients), 14 in grade III (13 AS patients and one AR patient) and 14 in grade IV (all AS patients). The total number of nucleated cells was 32-157 (mean, 19 ± 25)/high-power field ($\times 400$). The area examined in the sections ranged from 7.9 mm²/section to 58.8 mm²/section (mean, 19 ± 12 mm²/section).

Immunohistochemical Staining of PLA₂s and Osteogenic Molecules in the Valve Samples

We examined the levels of immunoreactivity for PLA₂s-IB, -IIA, -IID, -IIE, -IIF, -III, -IVA, -V, -VI, -VII, -X and -XIIA in all samples. The specificity of anti-PLA₂ antibodies for immunoreactivity was confirmed using an absorption test, as shown in **Supplemental Fig. 1** and our previous report¹⁵⁾. Consequently, immunoreactivity for PLA₂s-IB and -III and ALP was observed in nucleated cells, but not the extracellular space (**Fig. 1**). PLA₂s-IID, -IIF and -IVA and Runx2 were also detected in the nucleated cells (data not shown). In contrast, immunoreactivity for PLA₂s-IIE and -V, BMP-2 and osteopontin was observed in both nucleated cells and the extracellular space (**Fig. 1**), and PLA₂s-VI, -VII, -X and -XIIA and osteocalcin were noted in both nucleated cells and the extracellular space (data not shown). Meanwhile, PLA₂-IIA immunoreactivity was found primarily in the extracellular space, being detected in only a few cells (**Fig. 1**).

Immunoreactivity for PLA₂s and Osteogenic Molecules

Based on the expression pattern of each PLA₂, we determined the percentage of nucleated cells immunopositive for PLA₂-IB, -IID, -IIF, -III and IVA, Runx2 and ALP among the total number of nucleated cells in each section as a parameter of the expression of these molecules in the samples. When immunoreactivity was found in both nucleated cells and the extracellular space, the immunopositivity of the cells was hardly discriminated from that of the extracellular space. Therefore, the percentage of the area immunopositive for PLA₂-IIE, -V, -VI, -VII, -X and -XIIA, BMP-2, osteocalcin and osteopontin among the total area of the section was determined as a parameter of the expression of these molecules in the samples. In addition, the percentage of areas immunopositive for

Fig. 1. Representative histological and immunohistochemical staining of each PLA₂ and osteogenic molecule in the aortic valves. (A) Histology of the aortic valves based on hematoxylin and eosin (HE) staining. (B-I) Immunohistochemical staining of each of PLA₂ and osteogenic molecule. Each PLA₂ and osteogenic molecule was visualized using 3,3'-diaminobenzidine tetrahydrochloride (brown). The asterisks in panels A-H indicate calcified areas. The broken lines in panels A-H indicate the boundaries of the calcified areas. The inset (blue rectangle) at higher magnification corresponds to the rectangular space (red) in panels B and I. Adjacent sections were used for A-H. OPN indicates osteopontin. ALP indicates alkaline phosphatase. The sections are representative of three patients. Scale bars=200 μ m

PLA₂-IIA among the total area was analyzed. Consequently, the rate of immunoreactivity for PLA₂-IB and -III was 45-73% and 61-96% of nucleated cells, respectively, while that of Runx2 and ALP was 4.3-66.0% and 0.4-14.2% of nucleated cells, respectively, and that of PLA₂s-IIA, -IIE and -V was 1.4-35.3%, 0.7-35.9% and 0.3-16.8% of all areas in the samples, respectively. Similarly, the frequency of the expression of BMP-2, osteocalcin and osteopontin was 0.3-10.9%, 0.2-6.1% and 0.1-18.5% of the areas in the samples, respectively. However, the degree of immunoreactivity for the other PLA₂s examined was very faint. Specifically, we observed the following expression levels: PLA₂-IID, 1.3% of all nucleated cells;

PLA₂-IIF, 1.1% of all nucleated cells; and, PLA₂-IVA, 1.6% of all nucleated cells. In addition, the following data were obtained: PLA₂-VI, 0.07% of the area of the samples; PLA₂-VII, 0.10% of the area of the samples; PLA₂-X, 0.05% of the area of the samples; and, PLA₂-XIIA, 0.03% of the area of the samples. Hence, the data for PLA₂s-IID, -IIF, -IVA, -VI, -VII, -X and -XIIA were excluded from the further analyses due to the low frequency of their expression. The negative control staining showed no immunoreactivity for any type of PLA₂ (data not shown).

Table 2. Correlations between immunoreactivity for each PLA₂ and the expression of the pro-osteogenic molecules

	IB	IIA	IIE	III	V
BMP-2	0.0001	0.157	0.550**	0.209	0.481**
Runx2	0.215	0.167	0.300	0.225	0.342*
Osteopontin	0.154	-0.019	0.399*	0.212	0.387*
Osteocalcin	-0.330	0.020	0.172	0.099	0.060
ALP	0.215	-0.092	0.440**	0.205	0.383*

BMP=bone morphogenetic protein, Runx=runt-related transcription factor, ALP=alkaline phosphatase

* $p < 0.05$, ** $p < 0.01$.

Table 3. Correlations between the gene expression level of each PLA₂ and that of the pro-osteogenic molecules

	IB	IIA	IIE	III	V
BMP-2	0.377	0.189	0.511**	0.129	0.673**
Runx2	-0.028	-0.173	0.039	-0.199	0.322
Osteopontin	-0.081	-0.080	0.462*	-0.182	0.489*
Osteocalcin	-0.204	-0.278	0.149	0.073	0.251
ALP	0.102	0.298	0.497*	0.030	0.656**

* $p < 0.05$, ** $p < 0.01$.

Correlations between the Expression of PLA₂ and Grade of Calcification or Expression of Osteogenic Molecules

Spearman's rank correlation test showed that the grade of valve calcification had a significant and positive correlation with the degree of immunoreactivity for PLA₂-IIE and -V ($\rho = 0.66$ and 0.43 , respectively, $p < 0.01$). In addition, the grade of calcification exhibited significant and positive correlations with the expression levels of BMP-2, osteocalcin, osteopontin and ALP ($\rho = 0.53$, 0.41 , 0.51 and 0.57 , respectively, $p < 0.05$), but not that of Runx2 ($\rho = 0.16$, $p = 0.4$). Meanwhile, the immunohistochemical analysis showed that the degree of PLA₂-IIE or -V immunoreactivity positively correlated with the expression levels of BMP-2, osteopontin and ALP (**Table 2**), while the extent of immunoreactivity for PLA₂-V, but not PLA₂-IIE, demonstrated a significant correlation with that for Runx2 (**Table 2**). Next, we determined whether the gene expression levels of the PLA₂s in the valves correlated with those of the osteogenic molecules. Consequently, the RT-PCR studies showed that the mRNA expression levels of PLA₂-IIE or -V positively correlated with those of BMP-2, osteopontin and ALP (**Table 3**), whereas the degree of immunoreactivity and mRNA expression of PLA₂-IB, -IIA and -III showed no significant associations with the grade of calcification (data not shown), degree of immunoreactivity or mRNA expression of the osteogenic mole-

Fig. 2. Colocalization of the areas immunopositive for PLA₂s-IIE and -V and the areas with calcification. (A and C) Areas with calcification stained with Alizarin red S. (B and D) Areas and cells immunopositive for PLA₂-IIE (B) and PLA₂-V (D) (brown, red arrows). Adjacent sections were used for A and B as well as C and D. Scale bars = 20 μ m

cules (**Tables 2 and 3**). **Supplemental Fig. 2** shows the results of agarose gel electrophoresis of the amplified PCR products of PLA₂s-IB, -IIA, -IIE, -III and

Fig. 3. Representative immunofluorescent images of PLA₂s-IIE and -V, α -SMA and CD68 in the aortic valves. Staining for PLA₂s-IIE (A and D, red), PLA₂-V (G and J, red), α -SMA (B and H, green) and CD68 (E and K, green) and double-staining for PLA₂ and α -SMA or CD68 (C, F, I and L). The nuclei were stained with DAPI (C, F, I and L, blue). Immunoreactivity for PLA₂-IIE and -V was co-localized in cells immunopositive for α -SMA or CD68 (arrows). The sections are representative of three patients. Scale bars = 10 μ m

-V and osteogenic molecules in the aortic valves. As indicated in the figure, the areas immunopositive for PLA₂s-IIE and -V were partially localized in the areas of calcification stained with Alizarin red S (Fig. 2).

Co-localization of PLA₂ Immunoreactivity with CD68- and α -SMA-immunopositive Cells as well as Cyclooxygenase-1 (COX-1), COX-2, 5-lipoxygenase (5-LOX) and Osteogenic Molecules

Immunoreactivity for PLA₂-IIE and -V was found to be co-localized in cells immunoreactive to CD68 or α -SMA (Fig. 3). In addition, immunoreactivity for PLA₂s-IIE and -V was co-localized with COX-1,

Fig. 4. Representative immunofluorescent images of PLA₂s-IIE and -V, COX-1, COX-2 and 5-LOX in the aortic valves. Staining for PLA₂-IIE (A, D and G red), PLA₂-V (J, M and P, red), COX-1 (B and K, green), COX-2 (E and N, green) and 5-LOX (H and Q, green) and double-staining for PLA₂ and COX-1, COX-2 or 5-LOX (C, F, I, L, O and R). The nuclei were stained with DAPI (C, F, I, L, O and R, blue). Immunoreactivity for PLA₂-IIE and -V was co-localized with COX-1, COX-2 and 5-LOX (arrows). The sections are representative of three patients. Scale bars = 10 μ m

Fig. 5. Representative immunofluorescent images of PLA₂s-IIE and -V, BMP-2, osteopontin (OPN) and ALP in the aortic valves. Staining for PLA₂-IIE (A, D and G red), PLA₂-V (J, M and P, red), BMP-2 (B and K, green), OPN (E and N, green) and ALP (H and Q, green) and double-staining for PLA₂ and BMP-2, OPN or ALP (C, F, I, L, O and R). The nuclei were stained with DAPI (C, F, I, L, O and R, blue). Immunoreactivity for PLA₂s-IIE and -V was co-localized with BMP-2, OPN and ALP (arrows). The sections are representative of three patients. Scale bars = 10 μ m

Fig. 6. Transfection of siRNA specific for PLA₂-IIE or -V inhibited the gene expression of BMP-2 (A), osteopontin (OPN) (B) and alkaline phosphatase (ALP) (C) and the production of PGE₂ (D) and cysteinyl leukotrienes (CysLTs) (E) in response to osteogenic medium in the cultured VICs. *n* = 10 per experiment. (F and G) Transfection of siRNA specific for PLA₂-IIE (F) or -V (G) suppressed the expression of the respective genes. *n* = 6 per experiment. Open bars: treatment with control siRNA; bars with oblique lines: treatment with siRNA for PLA₂-IIE; closed bars: treatment with siRNA for PLA₂-V. The data are expressed as the mean ± SD. **p* < 0.05, ***p* < 0.01.

Fig. 7. The addition of arachidonic acid reversed the gene expression of BMP-2 (A) and OPN (B) in response to the osteogenic medium in the VICs treated with siRNA specific for PLA₂-IIE or PLA₂-V. In contrast, the addition of arachidonic acid did not reverse the ALP expression (C). Open bars: treatment with vehicle; closed bars: treatment with arachidonic acid (100 μmol/L). The data are expressed as the mean ± SD. *n* = 6 per experiment. **p* < 0.05.

COX-2 and 5-LOX, downstream enzymes for PLA₂s (Fig. 4), and immunoreactivity for these PLA₂s was colocalized with BMP-2, osteopontin and ALP (Fig. 5).

Cultures of Valve Interstitial Cells (VICs)

VICs are able to transdifferentiate into osteoblast-like cells^{9, 10}. As there were correlations between the expression levels of PLA₂-IIE and -V and those of the osteogenic molecules, we examined the possible roles of PLA₂-IIE and -V in the transdifferentiation of VICs to osteoblast-like cells. Consequently, incubation with an siRNA specific for either PLA₂-IIE or -V inhibited the expression of mRNA of BMP-2, osteopontin and ALP in the VICs in association with reductions in the mRNA expression of the respective PLA₂s (Fig. 6). In addition, transfection with the specific siRNA decreased the concentrations of PGE₂ and CysLTs in the cultured medium (Fig. 6), while the exogenous addition of arachidonic acid to the cultured medium reversed the mRNA expression of BMP-2 and osteopontin, but not ALP, in the VICs transfected

with the specific siRNA (Fig. 7).

Associations between the Clinical Parameters and PLA₂ Immunoreactivity in the Valves

Immunoreactivity for PLA₂-V displayed a significant correlation with levels of CRP (*r* = 0.51, *p* = 0.005) and BNP (*r* = 0.38, *p* = 0.04), but not other clinical parameters, including age, hypertension, diabetes mellitus, smoking and LDL-C. In contrast, immunoreactivity for PLA₂-IIE demonstrated no significant associations with these parameters (data not shown).

Discussion

This study showed that the expression of PLA₂s-IIE and -V is increased in calcified aortic valves and correlates with the extent of calcification. In addition, the expression of PLA₂s was found to correlate with the expression of BMP-2, osteopontin and ALP in human aortic valves, and the downregulation of these sPLA₂s genes attenuated the mRNA expression of

BMP-2, osteopontin and ALP in VICs cultured from human aortic valves. These data indicate that PLA₂s-IIE and -V play a role in the genesis of aortic valve calcification in humans.

COX-1, COX-2 and 5-LOX are downstream enzymes of PLA₂ in the arachidonic acid cascade. Because these molecules were found to be co-localized with PLA₂-IIE and -V in the aortic valves in the present study, eicosanoids may be produced within valve tissue and/or play a role in the development of valve calcification¹¹. In line with the present findings, a previous report showed that the 5-LOX expression is increased in calcified human aortic valves and that the incubation of myofibroblast cultures with LTC₄, a 5-LOX metabolite, induces calcium nodule formation²¹. In cultures of tendon stem cells, serotonergic receptor signals have been shown to stimulate tissue-nonspecific ALP through the actions of eicosanoids produced via the PLA₂ pathway¹⁷. Another report showed that PGE₂ stimulates the osteogenic differentiation of cultured tendon stem cells via BMP-2 induction¹⁸. Taken together, these previous reports^{2, 17, 18} appear to support the hypothesis that eicosanoids produced via the PLA₂ pathway contribute to the process of ectopic osteogenesis in cardiac valves.

In the present study, the protein expression of PLA₂-V, but not PLA₂-IIE, exhibited a significant correlation with that of Runx2, an important transcription factor that upregulates the expression of calcification-related proteins. Although the present study did not elucidate the reason for the lack of correlation between the Runx2 and PLA₂-IIE expression, it is possible that PLA₂-IIE activates the BMP/Wnt signaling pathway for osteogenesis in human aortic valves by bypassing the BMP/Runx pathway.

The present immunofluorescence study showed that PLA₂s-IIE and -V are co-localized in cells expressing α -SMA, a marker of myofibroblasts and smooth muscle cells, and CD68, a marker of macrophages. Moreover, the present immunofluorescence study demonstrated that PLA₂s-IIE and -V are co-localized in cells expressing BMP-2, osteopontin or ALP. Although the source of osteoblasts in valves remains unclear, previous reports have suggested that myofibroblasts and infiltrated macrophages may be precursors of osteoblasts in human valves^{9, 10, 19}. Furthermore, the present study showed that inhibiting the expression of PLA₂-IIE or -V results in the downregulation of pro-osteogenic molecules and a reduction in the production of PGE₂ and CysLTs in isolated VICs. In addition, the exogenous addition of arachidonic acid was shown to reverse the expression of BMP-2 and osteopontin. These data support the possibility

that PLA₂s initiate the process of osteogenesis in aortic valves. Hence, PLA₂s-IIE and -V may play a role in the transition of myofibroblasts to osteoblast-like cells in an autocrine or paracrine manner.

A very recent report showed that the gene expression of PLA₂-VII has a significant correlation with the extent of calcification and structural damage in human aortic valves²⁰. In the current immunohistochemical study, immunoreactivity for PLA₂-VII was very faint in the calcified aortic valves. Therefore, we did not examine the correlation between the expression of PLA₂-VII and the extent of calcification or expression of osteogenic molecules. The above report included control non-calcified aortic valves obtained from patients undergoing heart transplantation and excluded aortic valves with AR²⁰. The difference in the profiles of the valves may therefore explain the discrepancy in results between our and previous reports. PLA₂s have been shown to participate in the pathogenesis of several inflammatory and cardiovascular diseases^{3, 21, 22}. However, it remains unclear whether PLA₂s play a role in the osteogenesis of bone.

Among secretory PLA₂s, PLA₂s-IIE and -V belong to the same subgroup of I/II/V/X PLA₂s, based on their structure³. This subgroup of PLA₂s has been implicated in the onset of inflammatory responses³. Moreover, PLA₂s-IIE and -V possess a heparan sulfate proteoglycan (HSPG)-binding activity and exert potent hydrolytic effects against the cell surface membrane in either an HSPG-dependent or -independent manner^{3, 23}. HSPG is abundant in valvular tissue and may facilitate the release of PLA₂s-IIE and -V to form arachidonic acid, a source of pro-inflammatory eicosanoids. PLA₂-IIA also displays an HSPG-binding activity²⁴. Although it is unclear why the expression of PLA₂-IIA and pro-osteogenic molecules is not correlated, PLA₂-IIA may exhibit differences in its target membrane and/or substrates compared to PLA₂-IIE or -V³.

This study is associated with several limitations. First, the number of study patients was small, and we cannot rule out the presence of inherent bias in the process of patient selection. Second, there was a relatively high level of subjectivity in the analysis of the proportion of immunopositive cells and areas. Third, some of the secretory PLA₂s may have been secreted into the extracellular space from activated cells, which may have resulted in the underestimation of the degree of cellular immunoreactivity for the secreted PLA₂ types, thus explaining the relatively modest percentage of cells positive for sPLA₂s. Fourth, a majority of the aortic valve samples contained end-stage calcification, with relatively few valve samples exhibiting

normal findings or mild calcification. Consequently, it was difficult to analyze the expression of PLA₂s and the osteogenic markers in progression from mild to severely calcified aortic valves. The preponderance of samples with late-stage aortic valve calcification may also partly explain the lack of significant correlation between the expression of PLA₂s or osteogenic molecules and the clinical parameters. Fifth, the mRNA expression levels of PLA₂-IIE in the aortic valves and cultured VICs was very low. This finding requires careful interpretation with respect to the biological role of PLA₂-IIE in aortic valves and VICs. However, the inhibition of PLA₂-IIE via the specific siRNA-suppressed production of arachidonic acid-derived lipid mediators, as well as the expression of osteogenic molecules in the cultured VIC cells, indicated that PLA₂-IIE may have significant biological effects. Sixth, calcification frequently occurs on the aortic side (fibrosa layer) and the cusp coaptation of the aortic valves^{10, 25}. Therefore, the degree of immunoreactivity for PLA₂s and osteogenic molecules may have been influenced by the selection of regions in the aortic valve samples, although we examined the degree of immunoreactivity in each valve sample based on the same ratio of regions in the fibrosa to that in all layers.

Conclusion

In conclusion, the PLA₂s-IIE and -V expression correlates with the development of calcification and an increased expression of osteogenic markers in human aortic valves, while the inhibition of the expression of PLA₂s suppresses the induction of osteogenic molecules in cultured cells. These data indicate that PLA₂s-IIE and -V play a role in the pathogenesis of valve calcification.

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Conflicts of Interest

The authors have no conflicts of interest or financial disclosures to declare in conjunction with the publication of this work.

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Supplemental Table 1. Primary antibodies used

Antigen	Type of antibody	Dilution	Source	Cat. No.
gIB PLA ₂	Rabbit Polyclonal	1:50	Abcam, Japan	ab103872
gIIA PLA ₂	Mouse Monoclonal	1:200	Cayman Chemicals, USA	160500
gIID PLA ₂	Goat Polyclonal	1:200	Santa Cruz, USA	sc-104296
gIIE PLA ₂	Rabbit Polyclonal	1:500	Biovendor, Czech Republic	RD181064100
gIIF PLA ₂	Goat Polyclonal	1:200	Santa Cruz, USA	sc-164549
gIII PLA ₂	Rabbit Polyclonal	1:400	Lifespan Bioscience, USA	LS-A9486
gIVA PLA ₂	Rabbit Polyclonal	1:200	Santa Cruz, USA	sc-438
gV PLA ₂	Rabbit Polyclonal	1:200	Lifespan Bioscience, USA	LS-B1612
gVI PLA ₂	Goat Polyclonal	1:200	Santa Cruz, USA	sc-14463
gVII PLA ₂	Rabbit Polyclonal	1:100	Novus Biologicals, USA	NBP1-81317
gX PLA ₂	Rabbit Polyclonal	1:100	Shionogi, Japan	—
gXIIA PLA ₂	Rabbit Polyclonal	1:100	ProteinTech Group, USA	16009-1-AP
α -SMA	Mouse Monoclonal	1:400	Abcam, Japan	ab18460-1
CD68	Mouse Monoclonal	1:100	DAKO, Denmark	M0814
BMP-2	Rabbit Polyclonal	1:100	Abcam, Japan	ab14933
Runx2	Rabbit Polyclonal	1:50	Santa Cruz, USA	sc-10758
Osteopontin	Mouse Monoclonal	1:400	Abcam, Japan	ab69498
Osteocalcin	Mouse Monoclonal	1:400	Abcam, Japan	ab13421
ALP	Mouse Monoclonal	1:200	Abcam, Japan	ab17973
Vimentin	Mouse Monoclonal	1:200	DAKO, Denmark	M7020
CD31	Mouse Monoclonal	1:40	DAKO, Denmark	M0823
COX-1	Goat Polyclonal	1:100	Santa Cruz, USA	sc-1754
COX-2	Goat Polyclonal	1:100	Santa Cruz, USA	sc-1747
5-LOX	Rabbit Polyclonal	1:300	Abcam, Japan	ab39347

PLA₂, phospholipase A₂; SMA, smooth muscle actin; BMP, bone morphogenetic protein; Runx, runt- related transcription factor; ALP, alkaline phosphatase; COX, cyclooxygenase; LOX, lipoxygenase.

Supplemental Fig.1. Absorption test

A 10–20-fold excess volume of proteins or peptides, as listed in **Supplemental Table 3**, was added to the diluted antibodies and mixed well, then kept for two hours at room temperature. Following centrifugation at $10,000 \times g$ for 10 minutes, the supernatant was used as a pre-absorbed antibody. The aortic valve sections were subsequently stained with non-pre-absorbed and pre-absorbed antibodies using the regular staining procedures described in the text. Specific staining was judged to be positive when the signal decreased or disappeared with the pre-absorbed antibody compared with that observed with the non-pre-absorbed antibody.

The immunoreactive signals (red arrows) were abolished or decreased on the immunohistochemical images obtained using pre-absorbed antibodies compared with that observed on the images obtained using the non-pre-absorbed antibodies. Therefore, the immunoreactivity induced using each antibody was specific to each PLA₂.

Scale bars = 50 μ m.

Supplemental Table 2. Sequences of PCR primers

	Forward primers	Reverse primers
gIB PLA ₂	5'- GGATGAACTGGACAAGTGCTG -3'	5'- TTGCTGCTACAGGTGATTGC -3'
gIIA PLA ₂	5'- CAAGGATGCAACGGATCG -3'	5'- TTGCACAGGTGATTCTGCTC -3'
gIIE PLA ₂	5'- ACGACTATGGCTGTTACTGCGG -3'	5'- TTTCCAGTTTGGGCTCACAGCC -3'
gIII PLA ₂	5'- GACACCAGGTTTCAGCAATG -3'	5'- TGTGCCGTACATCCTACACC -3'
gV PLA ₂	5'- CCAATGTTCCGACTGGAGAC -3'	5'- GCACAGCAGGCACACTACAAG -3'
BMP-2	5'- CGGACTGCGGTCTCCTAA -3'	5'- GGAAGCAGCAACGCTAGAAG -3'
Runx2	5'- CTTCACAAATCCTCCCCAAG -3'	5'- ATGCGCCCTAAATCACTGAG -3'
Osteopontin	5'- CGCAGACCTGACATCCAGT -3'	5'- GGCTGTCCCAATCAGAAGG -3'
Osteocalcin	5'- TGAGAGCCCTCACACTCCTC -3'	5'- ACCTTTGCTGGACTCTGCAC -3'
ALP	5'- AAGGACGCTGGGAAATCTG -3'	5'- GGCATCTCGTTGTCTGAGTACC -3'
GAPDH	5'- GAGTCAACGGATTTGGTCGT -3'	5'- TTTGCCATGGGTGGAATC -3'

PLA₂, human phospholipase A₂; BMP, bone morphogenetic protein; Runx, runt-related transcription factor; ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Supplemental Fig.2.

Agarose gel electrophoresis of the amplified PCR products for PLA₂s-IB, -IIA, -IIE, -III and -V and the osteogenic molecules in the aortic valves

Agarose gel electrophoresis of the PCR products amplified at 35 cycles for PLA₂-IB, 25 cycles for PLA₂-IIA, 38 cycles for PLA₂-III, 38 cycles for PLA₂-IIE, 30 cycles for PLA₂-V, 30 cycles for BMP-2, 28 cycles for Runx2, 30 cycles for osteopontin, 33 cycles for osteocalcin, 30 cycles for ALP and 25 cycles for GAPDH from 0.1 μg of total RNA derived from the aortic valves.

Supplemental Table 3. Protein or peptides used for the absorption test

Antigen	Position of amino acid sequence/ length of amino acid of primary molecule	Company, City	Cat. No.	Immunogen for production of antibodies listed in Table 1
gIB PLA ₂	110-140 a.a./148 a.a.	Abgent, San Diego	BP4878b	110-140 a.a.
gIIA PLA ₂	21-144 a.a./144 a.a.	R&B systems, Minneapolis	5374-PL-010	1-144 a.a.
gIIE PLA ₂	1-140 a.a./140 a.a.	Abnova, Walnut	H00030814-P01	20-140 a.a.
gIII PLA ₂	1-509 a.a./509 a.a.	Abnova, Walnut	H00050487-T01	175-225 a.a.
gV PLA ₂	79-94 a.a./138 a.a.	Cayman, Ann Arbor	360512	79-94 a.a.

PLA₂, human phospholipase A₂; a.a., amino acid.