Original Article

Enhanced expression of neopterin in valve tissue of bicuspid aortic stenosis

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Background: Aortic valve stenosis (AS) occurs in bicuspid aortic valve (BAV) patients at a relatively young age compared to tricuspid aortic valve (TAV) patients. However, the underlying cause of this phenomenon remains unknown. Neopterin, which is a by-product of the guanosine triphosphate (GTP) pathway, enhances the oxidative potential of reactive oxygen species. To clarify the role of neopterin in the aortic valve, we immunohistochemically studied the presence of neopterin in aortic valve specimens from patients with AS harboring either TAV or BAV.

Methods: Frozen aortic valve samples were surgically obtained from 68 patients with severe AS with TAV (n=34) and BAV (n=34). Normal aortic valves were obtained from cadavers who died of non-cardiovascular causes as controls (n=9). Samples were immunohistochemically stained with antibodies against smooth muscle cells, macrophages, T lymphocytes, neopterin, and 4-hydroxy-2-nonenal (4-HNE).

Results: Quantitative analysis showed that the percentage of macrophages, 4-HNE- and neopterin-positive macrophage score, and the number of T lymphocytes were significantly higher in BAV patients than in TAV patients (macrophages, P=0.013; T lymphocytes, P=0.011; neopterin, P<0.001; 4-HNE, P=0.008). Double immunostaining for neopterin and macrophages demonstrated that most neopterin-positive cells were macrophages in BAV patients.

Conclusions: Neopterin accumulation in macrophages may increase oxidative stress and contribute to the early onset of AS in BAV.

Keywords: Aortic valve stenosis (AS); bicuspid aortic valve (BAV); neopterin

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Introduction

Congenital bicuspid aortic valve (BAV) is one of the most common congenital cardiac abnormalities, with an estimated prevalence of 0.5% to 2% (1,2). Aortic valve stenosis (AS) is the most common complication in patients with BAV. Only 15% of patients with BAV will function normally in the fifth decade, and frequently, patients with BAV develop AS at a relatively younger age compared to patients with tricuspid aortic valve (TAV) (3). Progression of AS general population is related to many traditional risk factors for the progression of atherosclerotic diseases, such as systemic hypertension, dyslipidemia, and smoking (3,4). However, traditional risk factors alone cannot explain the high prevalence and

incidence of cardiovascular disease in this population. Moreno *et al.* reported that the pathogenesis of AS in BAV is associated with a more aggressive inflammatory process, with increased macrophage infiltration and neovascularization than that in TAV (5).

Neopterin, a by-product of the guanosine triphosphate (GTP) pathway, is produced by activated macrophages after stimulation by interferon-γ released by T cells, and is an activation marker for monocytes/macrophages (6,7). In this study, to elucidate the role of neopterin in the aortic valve tissues, we immunohistochemically studied the presence of neopterin in aortic valve specimens from patients with AS, with either BAV or TAV. We present this article in accordance with the STROBE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1360/rc).

Methods

Patients and study design

Between April 2012 and March 2015, there were 160 patients with severe AS who underwent successful aortic valve replacement (AVR) at Osaka City General Hospital. Patients with moderate or higher grade aortic regurgitation, infectious endocarditis, rheumatic valvular heart disease, chronic renal failure requiring hemodialysis, and chronic inflammatory diseases such as collagen disease and malignancy were excluded from the 160 cases. All BAVs (n=34) and randomly selected TAVs (n=34) were selected for further analyses. In total, 68 frozen aortic valve specimens were obtained from 68 patients who underwent AVR for AS. In addition, frozen aortic valve specimens were obtained at autopsy from nine individuals (mean age, 68 years) who died from non-cardiovascular causes, which were regarded as controls (n=9). Immediately after AVR, the tissue specimens were snap-frozen in all cases, and stored at −80 ℃. The snap-frozen samples obtained during AVR were serially cut to produce sections of 5 μm thickness, that were fixed in acetone. For each patient, the first section was stained with hematoxylin and eosin, whereas the other sections were immunohistochemically stained.

We collected clinical data from the medical charts. The criteria for hypertension, hyperlipidemia, and diabetes mellitus were in accordance with the guidelines of the American Heart Association and American Diabetes Association. All patients underwent preoperative echocardiography, which were performed by experienced echocardiographists. We evaluated AS severity and cardiac function using transthoracic echocardiography, and calculated the aortic valve area using continuity equation. Left ventricular end-diastolic volume, end-systolic volume, and ejection fraction were also calculated.

Among the summarized patient characteristics, only the age differed significantly (*Table 1*).

Highlight box

Key findings

- Neopterin may increase oxidative stress and contribute to the early onset of aortic valve stenosis (AS) in bicuspid aortic valve (BAV).

What is known and what is new?

- It is well-known empirically that AS in BAV is associated with a faster rate of AS progression, but the cause is still unknown.
- The present study is the first immunohistochemically evaluation of neopterin expression in aortic valve tissues of patients with AS. We found that neopterin is expressed significantly more in valve tissue of AS patients with BAV than in AS patients with tricuspid aortic valve (TAV).

What is the implication, and what should change now?

- Immunohistochemistry proved a difference between BAV and TAV in AS patients. The fact that neopterin is involved in the rate of calcification of the aortic valve may lead to a future in which the rate of progression of AS can be controlled if we can understand why neopterin is highly expressed.

Immunohistochemistry

The cellular components were single-stained and analyzed using monoclonal antibodies against smooth muscle cells (1A4; DAKO, Glostrup, Denmark), macrophages (EBM11; DAKO), T lymphocytes (CD3; DAKO), neopterin (Biogenesis Inc., Paterson, NJ, USA), and 4-hydroxy-2-nonenal (4-HNE; NOF Corporation, Tokyo, Japan), which is a product of oxidative stress. Sections were incubated at 4 ℃ overnight or for 1 hour at room temperature and then subjected to a three-step staining procedure using the streptavidin-biotin complex (SABC) method for detection. Peroxidase activity was visualized using 3-amino-9-ethylcarbazole (10 minutes, room temperature), and the sections were counterstained with hematoxylin. Non-immune mouse immunoglobulin G (IgG) serum (DAKO) served as a negative control.

To identify the type of cells stained positive for neopterin, we also performed double immunostaining

Table 1 Patients' characteristics

Variables	Bicuspid patients (n=34)	Tricuspid patients (n=34)	P value
Age (years)	66±9	76±8	<0.001
Male	18 [53]	12 [35]	0.18
Hypertension	21 [62]	29 [85]	0.13
Dyslipidemia	17 [50]	19 [56]	0.75
Diabetes mellitus	7 [21]	14 [41]	0.16
Smoking	8 [24]	6 [18]	0.67
Coronary artery disease	4 [12]	8 [24]	0.44
ACEI/ARB	16 [47]	14 [41]	0.67
Statin treatment	14 [41]	11 [32]	0.53
Creatinine (mg/dL)	0.89±0.43	0.85±0.34	0.4
Aortic valve area (Doppler) (cm^2)	0.65±0.19	0.72±0.16	0.056
LVEF (%)	60.3±13.6	62.1±11.7	0.53
Peak AVPG (mmHg)	94.7±34.8	77.9±24.3	0.052
Mean AVPG (mmHg)	0.65±0.19	0.72±0.16	0.056

Data are presented as mean ± SD or n [%]. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; LVEF, left ventricular ejection fraction; AVPG, aortic valve pressure gradient; SD, standard deviation.

with smooth muscle cells or macrophages and neopterin, based on a previously reported method with minor procedural modifications, in which alkaline phosphatase was visualized with fast blue BB (blue, smooth muscle cells, or macrophages), and neopterin was visualized with 3-amino-9-ethyl-carbazole development (red, neopterin).

Quantitative methods

The tissue area occupied by the immunostained macrophages, neopterin, and 4-HNE was quantified using computer-aided planimetry and expressed as a percentage of the total surface area of the tissue section. Furthermore, based on these quantifications, a "neopterin-positive macrophage score" was defined as follows: neopterin-positive macrophage score = neopterin-positive area/macrophage-positive area. Immunohistochemical staining would depict neopterin-positive cells as if they were attached to each other, making it impossible to count cells one by one. Therefore, the stained area was measured. This stained area was defined as the neopterin-positive area. The same definition was used for macrophage-positive cells. The number of CD3-positive T-cells in the entire tissue section was counted and expressed as the number of cells per mm^2 of tissue. Since macrophages are activated by interferon-γ secreted by T cells, we can presume that the macrophages are activated if there are more T cells. Morphometric analysis was performed by a single investigator who was blinded to the patients' characteristics and histological classifications.

Statistical analyses

All data are shown as mean ± standard deviation (SD) for continuous variables, and percentages for categorical variables. The two groups were compared using an unpaired Student's *t*-test or Mann-Whitney *U* test when the variance was heterogeneous. Categorical variables were compared using the χ^2 test or Fisher's exact test. Statistical significance was set at P<0.05. When comparing the three groups, the Bonferroni correction was used after the nonparametric Kruskal-Wallis test.

Ethics

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Osaka City General

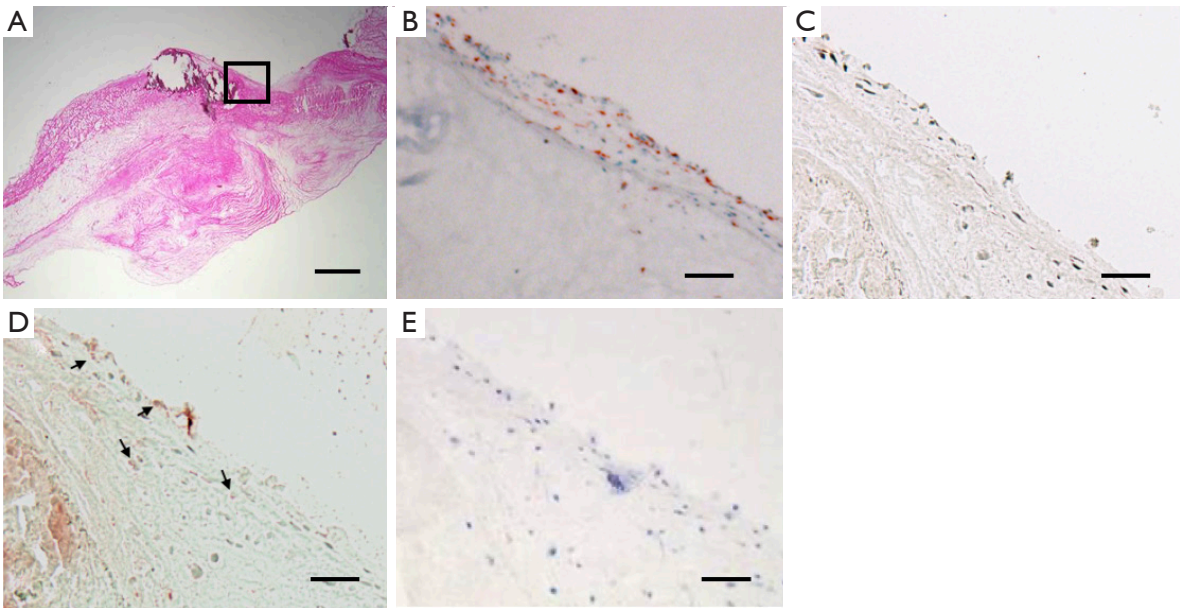

Figure 1 Micrographs of an aortic valve specimen obtained from a patient with TAV (A-E). (A) Hematoxylin-eosin stain. The cross section shows distinct calcifications (the boxed area). The boxed area is enlarged in (B-E). (B) The section stained with anti-CD68 antibody shows scattered macrophages in the lesion. (C) The adjacent section is stained with anti-CD3 antibody, no CD3-positive T lymphocytes are found in this section. (D) The adjacent section stained with anti-neopterin antibody reveals that macrophages are scant with positivity for neopterin. The allows indicate neopterin-positive cells. (E) The adjacent section stained with anti-4-HNE antibody shows that there are not 4-HNE-positive macrophages. Bar: (A) 500 μm; (B-E) 100 μm. TAV, tricuspid aortic valve; 4-HNE, 4-hydroxy-2-nonenal.

Hospital (No. 1504010) and informed consent was taken from all the patients.

Results

Clinical presentation and echocardiographic findings

The clinical and demographic characteristics of the patients at admission are shown in *Table 1*. There were no differences in sex, prevalence of coronary artery disease, or presence of risk factors between patients with BAV and those with TAV: factors associated with calcific valve disease mirror those associated with coronary atherosclerosis (8). However, the age prevalence was significantly lower in patients with BAV than in those with TAV. Among the echocardiographic data, there were no differences in the left ventricular ejection fraction, peak aortic valve pressure gradient, mean aortic valve pressure gradient, or aortic valve area. In summary, there was no significant difference in the severity of AS between the two groups.

Histological findings

In the lesions of TAV patients, the boxed area is calcification (*Figure 1A*), and scattered macrophages were identified around the calcified lesions (*Figure 1B*). Only a few CD3-positive T lymphocytes were observed (*Figure 1C*). We found a small number of neopterin-positive macrophages (*Figure 1D*) and no 4-HNE-positive cells in the lesions of TAV patients (*Figure 1E*). Calcified areas were also observed in patients with BAV (*Figure 2A*). In contrast to TAV, macrophages were abundant in all the lesions of patients with BAV (*Figure 2B*). In addition, large numbers of CD3-positive T lymphocytes and neopterins were observed (*Figure 2C,2D*). 4-HNE-positive cells were partially found where macrophages were identified (*Figure 2E*). The large number of purple cells in neopterin (red) and macrophage (blue) double immunostaining indicated that the majority of neopterin-positive cells were macrophages in aortic valve specimens from BAV patients (*Figure 3*). In the aortic valve specimens obtained from reference cases, there were a few

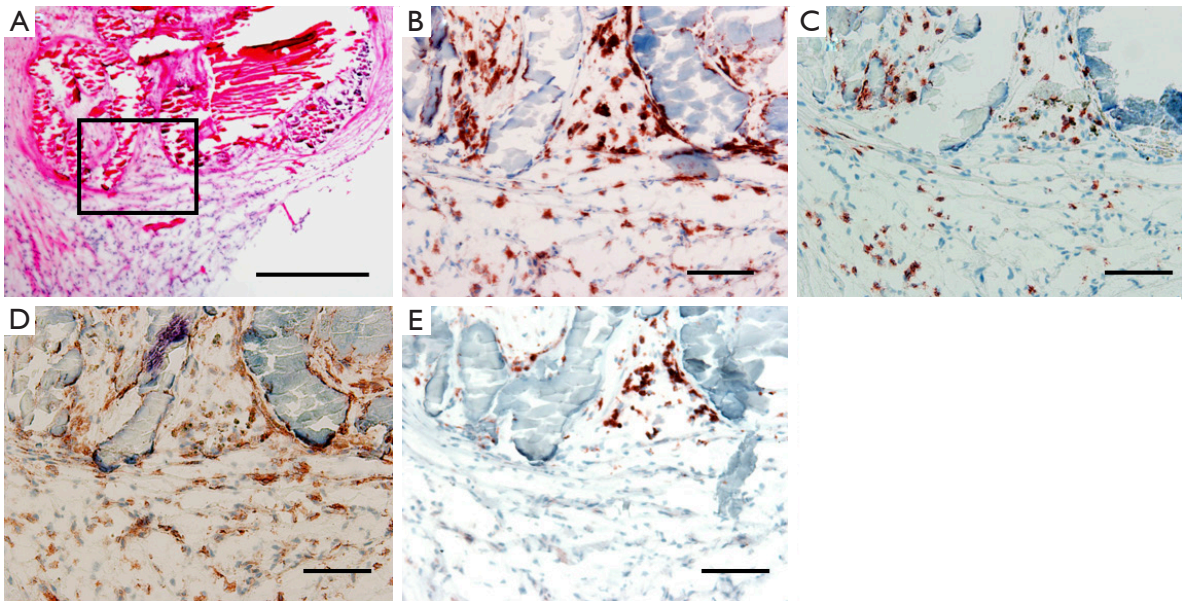

Figure 2 Micrographs of an aortic valve specimen obtained from a patient with BAV (A-E). (A) Hematoxylin-eosin stain. The cross section shows distinct calcification (the boxed area). The boxed area is enlarged in (B-E). (B) The section stained with anti-CD68 antibody reveals abundant macrophages. (C) The adjacent section stained with anti-CD3 antibody reveals the presence of abundant CD3-positive T lymphocytes. (D) The adjacent section stained with anti-neopterin antibody reveals that the presence of abundant neopterin-positive cells. (E) The adjacent section stained with anti-4-HNE antibody shows the presence of 4-HNE-positive cells. Bar: (A) 500 μm; (B-E) 100 μm. BAV, bicuspid aortic valve; 4-HNE, 4-hydroxy-2-nonenal.

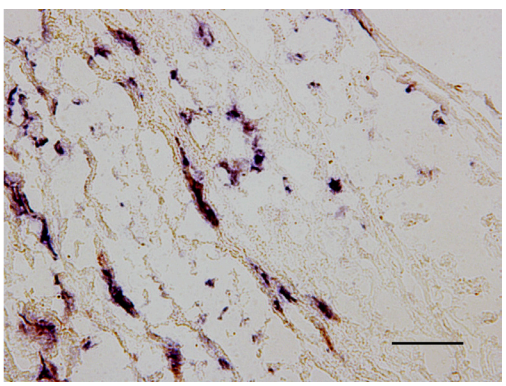

Figure 3 An aortic valve specimen obtained from patients with BAV. Double immunostaining for neopterin (red) and macrophages (blue) demonstrated that the cells exhibiting enhanced staining appeared purple, indicating that the neopterin-positive cells were macrophages. Bar: 50 μm. BAV, bicuspid aortic valve.

macrophages, but no T lymphocytes, Neopterin-positive cells, and 4-HNE-positive cells were found (*Figure 4*).

Quantitative analysis

Quantitative analysis showed that the percentage of macrophage-positive areas was significantly higher in BAV patients than in either TAV patients or controls (BAV *vs.* TAV, P=0.013; BAV *vs.* controls, P<0.001). In addition, lesions from TAV patients or controls compared to lesions from BAV patients, had significantly higher numbers of CD3-positive T lymphocytes (BAV *vs.* TAV, P=0.011; BAV *vs.* controls, P<0.001), neopterin-positive macrophage scores (BAV *vs.* TAV, P<0.001; BAV *vs.* controls, P<0.001), and 4-HNE-positive areas (BAV *vs.* TAV, P=0.008; BAV *vs.* controls, P<0.011) (*Figure 5*). The neopterin-

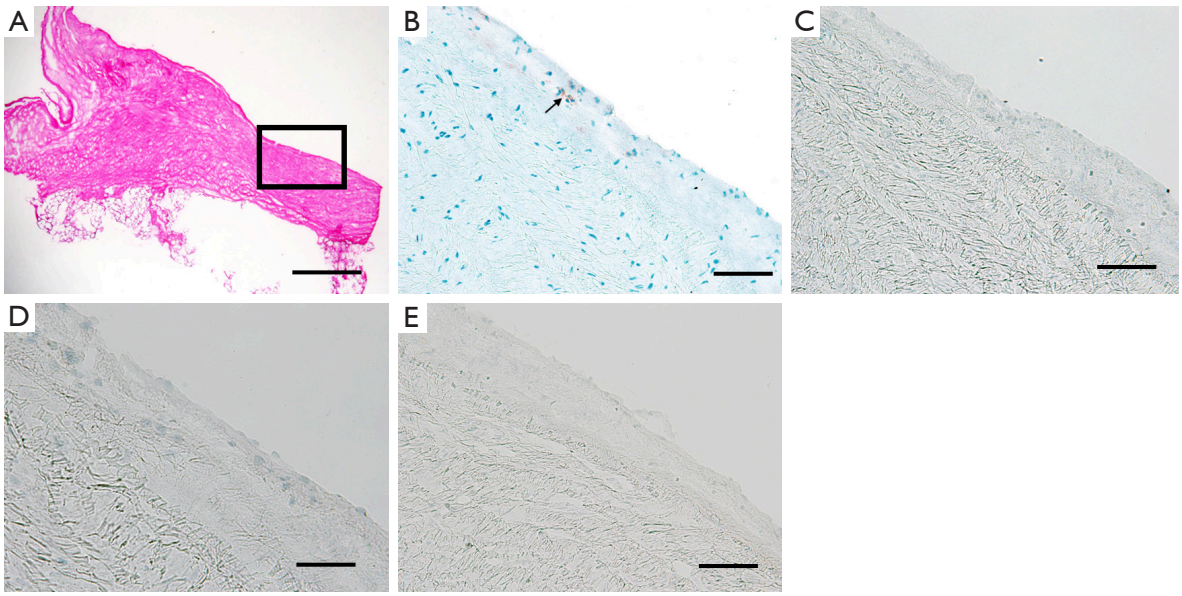

Figure 4 Micrographs of an aortic valve specimen obtained from a control case (A-E). (A) Hematoxylin-eosin stain. The boxed area is enlarged in (B-E). (B) The section stained with anti-CD68 antibody shows a few macrophages in the lesion. The allow indicates macrophages. (C) The adjacent section stained with anti-CD3 antibody reveals that there are no T lymphocytes. (D) The adjacent section stained with anti-neopterin antibody reveals no positivity for neopterin. (E) The adjacent section stained with anti-4-HNE antibody shows that there are not 4-HNE-positive macrophages. Bar: (A) 1,000 μm; (B-E) 100 μm. 4-HNE, 4-hydroxy-2-nonenal.

positive macrophage score correlated positively with 4-HNE-positive area and the number of CD3-positive T lymphocytes (4-HNE, R=0.317, P=0.008; T lymphocytes, R=0.296, P=0.014).

Discussion

In this study, through immunohistochemical and quantitative analyses of aortic valves, neopterin expression significantly increased in patients with AS and that neopterin expression was even more significantly increased in patients with BAV than in those with TAV. These results suggest that increased expression of neopterin in the valve tissue may play an important role in AS progression and may be a cause of AS progression in young patients with BAV.

As a sensitive biomarker for detecting T cell immune activation in humans, neopterin can be a useful biological indicator of inflammatory diseases. Several studies have reported plasma or urinary neopterin levels as biomarkers of inflammatory diseases such as human immunodeficiency virus (HIV), malignant tumors, coronary diseases, heart failure, neurodegenerative diseases, diabetes, and normal aging process diseases (9-19).

Neopterins are produced by macrophages activated via the GTP pathway. Activated T-cells produce interferon-gamma, which activates macrophages. In activated macrophages, GTP-cyclohydrolase converts GTP to 7,8-dihydroneopterin by GTP-cyclohydrolase. Neopterin is generated by oxidation of 7,8-dihydroneopterin by hypochlorous acid (HOCl), which is released by neutrophils during inflammation (9,11,13). Neopterin is a product of 7,8-dihydroneopterin oxidation during the antioxidant reactions. In contrast, neopterin acts as a pro-oxidant and enhances oxidative damage in several cell types (16,20).

Naito *et al.* reported that serum neopterin is a useful marker for AS (21). To the best of our knowledge, this is the first study which investigated the localization of neopterin in the aortic valve tissue removed from a patient with severe AS of BAV and TAV, and in normal aortic valve from the deceased. Enhanced expression of neopterin in macrophages is deeply associated with instability of coronary and carotid plaque (9,10). Serum neopterin levels were elevated in patients with unstable coronary artery disease compared to control subjects and patients with stable coronary artery disease (22,23). Furthermore, neopterin levels are associated with the presence of complex coronary lesions, such as those with irregular morphology or ulceration (24,25). Thus, there are some reports mentioning the relationship between

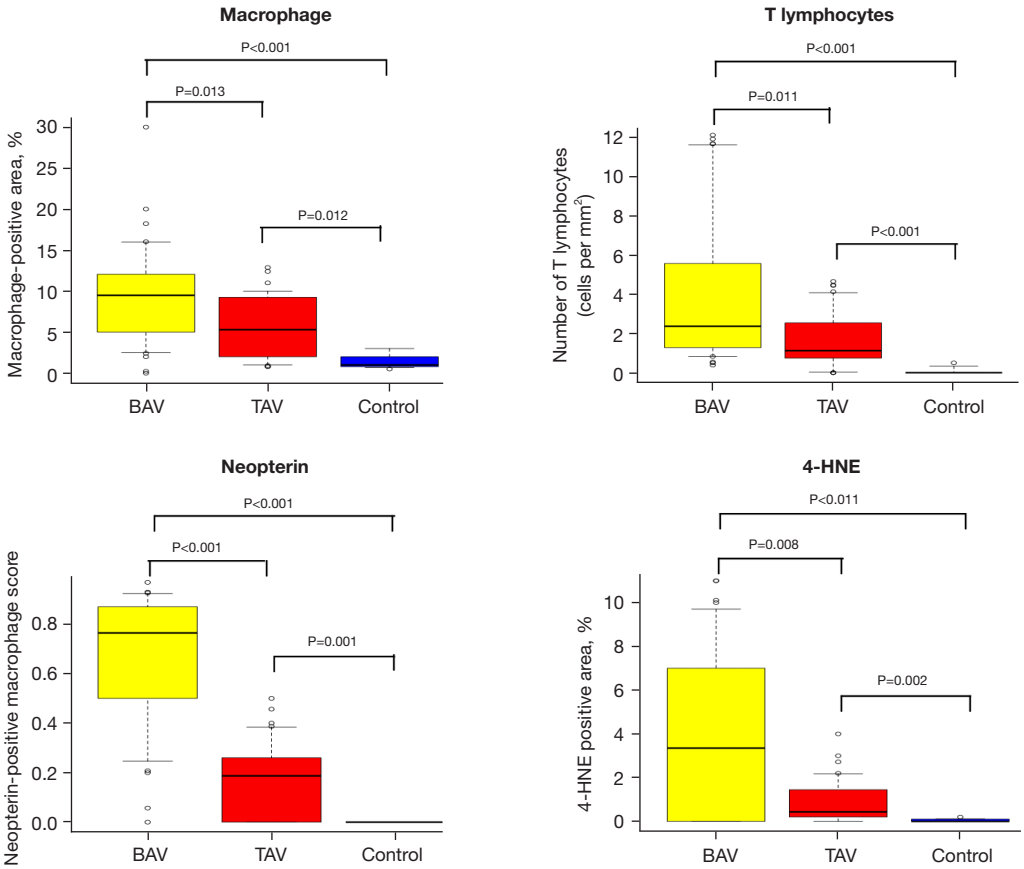

Figure 5 Graphs showing the macrophage-positive area expressed as a percentage of the total surface area, the number of T lymphocytes, the neopterin-positive macrophage score, and the 4-HNE-positive area in the aortic valve specimens obtained from patients with BAV and TAV, and reference cases. BAV, bicuspid aortic valve; TAV, tricuspid aortic valve; 4-HNE, 4-hydroxy-2-nonenal.

neopterin and atherosclerosis.

It is important to note that the aforementioned neopterin as a biomarker, which we mentioned above is also elevated in heart failure and unstable angina, refers to plasma neopterin (12,22-25). We believe that plasma neopterin is significantly unreliable in assessing coronary artery disease and heart failure because it is also elevated in a variety of inflammatory diseases. For example, the current state of knowledge sees the concept of hemodynamic regulation by the heart and kidneys as a complex and dynamic system in which changes in the function of one organ can lead to a spiral of dysfunction in both (26). it is easy to imagine that coronary artery disease and heart failure would cause renal aggravation and thus activate cellular immunity. It is important to emphasize again that in this study, we directly evaluated diseased tissue.

Hoffmann *et al.* reported that neopterin induces nitric oxide-dependent apoptosis in rat vascular smooth muscle cells (27). In previous studies on calcification, Proudfoot *et al.* reported that apoptosis precedes vascular smooth muscle cell calcification and that apoptotic bodies are capable of initiating vascular calcification (28). Freeman *et al.* reported that the histopathologic features and pathologic processes of calcific valve disease are similar to those of atherosclerosis (29). Thus, the previous reports and the results of this study suggest that high neopterin expression may induce smooth muscle apoptosis in valve tissues and contribute to the progression of calcification, which may result in the accelerated progression of AS.

However, neopterin itself is still not fully understood, and a new pathway for neopterin production was only mentioned by Baxter-Parker *et al.* in 2020, who reported that 7,8-dihydroneopterin is converted to neopterin by HOCl and that 7,8-dihydronopterin scavenges superoxide and is subsequently oxidized to neopterin in cellular and cell-free experimental systems (30).

We do not know how neopterin is expressed in the BAV tissue. Neopterin may be congenitally expressed in the BAV. However, it is difficult to confirm this hypothesis because it is not possible to evaluate valve tissue during AS progression. Lorenz *et al.* used four-dimensional (4D) flow magnetic resonance imaging to evaluate the aortic blood flow in patients with BAV. The results showed a strong helicoidal flow across the entire aorta and retrograde blood flow during diastole in patients with BAV, and this phenomenon increases wall shear stress in the aortic wall (31,32). It has also been reported that wall shear stress induces oxidative stress on the vascular endothelium (33). We suspected that this also increases wall shear stress in the valves, thereby causing inflammation and superoxide production. In this study, the neopterin-positive macrophage score was significantly higher in patients with AS with BAV than in those with TAV despite the same degree of AS progression. These findings provide immunohistological evidence that in patients with BAV, not only the effect of degenerative changes but also other factors contribute to this effect. These findings suggest that neopterins in activated macrophages may increase oxidative stress and contribute to AS progression in patients with BAV.

Study limitation

The primary limitation of this study is the small number of enrolled patients. Second, only calcific AS developed because valve samples were surgically obtained. We could not inspect the early AS lesions. Furthermore, to verify that neopterin affects bicuspid valve AS, it is also important to show that non-AS patients with bicuspid valves do not develop neopterin at an older age. However, this could not be verified because it is ethically impossible to harvest normal valve tissue from a living human being. Third, the control group in this study was obtained from autopsy cases and not surgical cases. We did not verify the neopterin measurement result between pre- and post-mortem instances. However, neopterin is a stable molecule (34). Fourth, the harvested aortic valve tissues were mixed during the surgical harvest; thus, it was impossible to distinguish between the left coronary cusp, right coronary cusp, and noncoronary cusp.

Conclusions

The present study is the first immunohistochemically evaluation of neopterin expression in aortic valve tissues of patients with AS. In AS patients with BAV, neopterin-positive macrophage scores positively correlated with 4-HNE-positive areas and T lymphocyte counts. 4-HNE is an oxidative stress marker and a higher number of T lymphocytes is presumably associated with more active macrophages. Double immunostaining of neopterins and macrophages showed that in AS patients with BAV, neopterin-positive cells were macrophages. These findings suggest that neopterin accumulation in macrophages increases oxidative stress and contributes to the early onset of AS in BAV.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1360/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1360/dss

Peer Review File: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1360/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1360/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Osaka City General Hospital (No. 1504010) and informed consent was taken from all the patients.

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