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Role of Circulating Osteogenic Progenitor Cells in Calcific Aortic Stenosis

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Objectives The purpose of this study was to determine the role of circulating endothelial progenitor cells with osteoblastic phenotype (EPC-OCN) in human aortic valve calcification (AVC).

Background Recent evidence suggests that rather than passive mineralization, AVC is an active atherosclerotic process with an osteoblastic component resembling coronary calcification. We have recently identified circulating EPCs with osteogenic properties carrying both endothelial progenitor (CD34, KDR) and osteoblastic (osteocalcin [OCN]) cell surface markers.

Methods Blood samples from controls (n = 22) and patients with mild to moderate calcific aortic stenosis (mi-moAS, n = 17), severe calcific AS (sAS, n = 26), and both sAS and severe coronary artery disease (sCAD) (n = 33) were collected during diagnostic coronary angiography. By using flow cytometry, peripheral blood mononuclear cells were analyzed for CD34, KDR, and OCN. Resected normal and calcified aortic valves were analyzed histologically.

Results Patients with mi-moAS and patients with sAS/sCAD had significantly less EPCs (CD34+/KDR+/OCN−) than controls. Patients with sAS showed significantly higher numbers of EPC-OCN (CD34+/KDR+/OCN+) than controls. In addition, the percentage of EPC costaining for OCN was higher in all disease groups compared with controls. A subgroup analysis of younger patients with bicuspid sAS showed a similar pattern of significantly lower EPCs but a high percentage of coexpression of OCN. Immunofluorescence showed colocalization of nuclear factor kappa-B and OCN in diseased and normal valves. CD34+/OCN+ cells were abundant in the endothelial and deeper cell layers of calcific aortic valve tissue but not in normal aortic valve tissue.

Conclusions Circulating EPC-OCN may play a significant role in the pathogenesis and as markers of prognostication of calcific AS. (J Am Coll Cardiol 2012;60:1945–53) © 2012 by the American College of Cardiology Foundation

Calcific aortic stenosis (AS) is a common valve disease affecting up to 26% of adults older than 65 years of age (1), with 3% to 5% showing moderate to severe disease (2), making it the most common indication for valve replacement in the United States (3). In contrast to previous paradigms depicting aortic valve calcification (AVC) as a passive degenerative mineralization process (4), recent evidence suggests that AVC is an active process closely related to coronary artery atherosclerosis and calcification (5). In addition to features of inflammation and increased oxidative stress (6,7), animal models have demonstrated that AVC is characterized by an osteoblastic component (8,9). Indeed, we have recently shown that patients with early and severe coronary atherosclerosis have high levels of circulating endothelial progenitor cells with osteoblastic (osteocalcin [OCN]) phenotype (EPC-OCN) (10). Moreover, we have found that these EPC-OCN are retained within the coronary circulation of patients with endothelial dysfunction (11).

See page 1954

The current study was designed to test the hypothesis that patients with varying degrees of calcific aortic stenosis (AS) show high numbers of circulating EPC-OCN, and furthermore that these cells are present in the aortic valve tissue. By comparing patients with varying degrees of calcific AS and coronary artery disease (CAD) with controls, we sought to establish differences in distribution patterns of circulating endothelial progenitor cells (EPCs) (defined in this study as CD34+/KDR+/OCN−) and

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JACC Vol. 60, No. 19, 2012
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Abbreviations and Acronyms

AS = aortic stenosis

AVC = aortic valve calcification

CAD = coronary artery disease

EPC = endothelial progenitor cell

EPC-OCN = endothelial progenitor cells with osteoblastic phenotype

mi-moAS = mild to moderate calcific aortic stenosis

NFkB = nuclear factor kappa-B

OCN = osteocalcin

PBS = phosphate-buffered saline

sAS = severe calcific aortic stenosis

sCAD = severe coronary artery disease

VIC = valve interstitial cell

EPC-OCN (defined as CD34+/KDR+/OCN+). In addition, by using histological techniques, we sought to investigate whether EPC-OCN can be detected in resected valve tissue of patients undergoing aortic valve replacement for symptomatic severe calcific aortic stenosis (sAS).

Methods

The study was approved by the institutional review board of the Mayo Foundation, and all study subjects provided written, informed consent. A total of 59 consecutive patients with clinical and echocardiographic sAS were referred for preoperative coronary angiography. A total of 26 of these 59 patients were found to have sAS but normal coronary arteries; the remaining 33 patients were found to have both sAS and severe coronary artery disease (sCAD). The severity of AS was determined using standard echocardiographic methods (defined as a mean aortic valve gradient of ≥40 mm Hg and an aortic valve area of ≤0.9 cm^2). In addition, we analyzed blood samples from 17 patients undergoing diagnostic angiography for assessment of CAD with only mild to moderate AS. A total of 22 patients with normal coronary angiograms and echocardiographically normal aortic valves served as the control group. Patients with acute coronary syndromes (unstable angina or acute myocardial infarction), heart failure (ejection fraction <50%), or severe renal or liver disease were excluded. None of the subjects had a clinical diagnosis of Paget's disease, a bone fracture within the past 5 years, or end-stage renal disease.

Flow cytometry. Peripheral blood mononuclear cells were isolated from fresh blood samples (collected in ethylenediaminetetraacetic acid tubes) using a Ficoll density gradient, and immunofluorescent cell staining was performed using the following fluorescent conjugated antibodies: CD34-PerCP Cy 5.5 (Becton Dickinson, Franklin Lakes, New Jersey), kinase insert domain receptor allophycocyanin (vascular endothelial growth factor receptor 2, R&D Systems, Minneapolis, Minnesota), and the appropriate isotype controls as previously described (10,11). In addition, osteocalcin (OCN+) cells were identified using an anti-human OCN antibody (Santa Cruz Biotechnology, Santa Cruz, California) with a fluorescein isothiocyanate secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) (10). Live cells were detected using propidium iodide exclusion (Becton Dickinson). Cell fluorescence was measured immediately after staining (Becton Dickinson, FACS Calibur), and data were analyzed using CellQuest software (Becton Dickinson). A total of 150,000 events were counted, and final data were obtained within the lymphocyte gate (determined using light scatter [forward and side scatter]). Representative examples of data plots have been published (10). The investigator performing the cell analysis was unaware of the results of the patient classification.

Immunofluorescence. Severely calcified aortic valves were resected at the time of clinically indicated aortic valve replacement for severe AS. Normal aortic valve specimens were acquired during autopsies.

To confirm the correlation of CD34 with OCN and nuclear factor kappa-B (NFkB) with OCN, double-staining immunofluorescence was performed in frozen cross sections (nondecalcified tissue). Polyclonal (OCN, 1:100, Santa Cruz Biotechnology) and monoclonal (NFkB, 1:50, Cell Signaling Technology Inc., Danvers, Massachusetts; CD34, 1:500, Invitrogen, Grand Island, New York) primary antibodies were used. The secondary antibodies were used according to the vendor's instruction.

Frozen sections were fixed in ice-cold methanol for 15 min and washed 3 times in phosphate-buffered saline (PBS) for 5 minutes. Antigen retrieval was done by steaming the tissue for 25 min followed by 5 min PBS washing. Endogenous peroxidase activity was blocked by a 50/50 solution of methanol and 3% H_2O_2 followed by 5 min of PBS washing. The sections were blocked in 5% bovine serum albumin in PBS for 25 min and the first antibody diluted in blocking buffer was added overnight. Sections were then washed 3 times with PBS for 5 min and the secondary antibody was added in diluted blocking buffer for 30 min at room temperature. Sections were finally washed 3 times with PBS for 5 min and then mounted with DAPI.

Images were acquired by using a computer-aided image-analysis program (AxioCam digital camera, AxioObserver. Z1 and AxioVision software v4.7.2.0, Carl Zeiss MicroImaging, Inc., Thornwood, New York). Under Apo Tome mode, pictures were first taken separately for each individual color channel (red for CD34 or NFkB, green for OCN, and blue for DAPI) at the same area of the tissue for part inspection, and then an additional multichannel merged image was automatically generated through which the correlations between the positive stainings and the stainings with nucleus are presented.

Statistics. We used the Shapiro-Wilk test to determine normal distribution. Data that are not normally distributed are presented as median (interquartile [25th to 75th percentile] range). Normally distributed data are shown as mean ± SD. Continuous variables were tested using 1-way analysis of variance. Dichotomous variables were compared using the Fisher exact test. Analysis of variance was used to test for group differences in EPC results. Skewed data were transformed using a log-transformation. Pairwise comparisons of the 3 diseased groups with the normal group were adjusted using Dunnett's method. Analysis of covariance

was used as a secondary analysis to assess how the associations changed when adjusting for cardiovascular risk factors. A p value <0.05 was considered statistically significant. The Statistical Package for the Social Sciences version 18.0 (SPSS Inc., Chicago, Illinois) and SAS version 9.2 (SAS Institute Inc., Cary, North Carolina) were used to conduct analyses.

Results

Patient demographics. Patient characteristics are summarized in Table 1. Patients with normal endothelial function and normal aortic valves were younger, less likely to be male, and less likely to present with history of hypertension and diabetes mellitus than patients in the disease groups.

Circulating osteoblastic progenitor cells. Patients with sAS and normal coronary arteries showed significantly higher numbers of circulating EPC-OCN than controls (Fig. 1). The percentage of EPCs costaining for OCN was higher in all disease groups compared with controls, reaching statistical significance in all but the sAS group (Fig. 2).

Circulating EPCs. Patients with mild to moderate calcific aortic stenosis (mi-moAS) and those with both sAS and sCAD showed significantly fewer circulating EPCs (CD34+/KDR+/OCN−) than controls (Fig. 3).

Analysis of covariance after adjustment for confounding risk factors. Analysis of covariance showed that after adjustment for diabetes, hypertension, and smoking, the statistically significant differences held except for the comparison between normal subjects and subjects with sAS/sCAD for percent OCN of CD34+/KDR+ cells (Table 2).

Subgroup analysis: Severe calcific bicuspid aortic valve stenosis. To address the effect of age on the number of EPCs and EPC-OCN, we compared 12 patients with severe calcific bicuspid aortic valve stenosis with 12 controls of similar age (53 ± 2 years vs. 59 ± 9 years, p = NS). In this subset of study subjects, there was a significantly higher percentage of OCN costaining of EPCs 32 ± 33%) versus controls (8 ± 12%, p = 0.026). The numbers of circulating EPCs (CD34+/KDR+/OCN−) were significantly lower in the calcific bicuspid AS group compared with controls (median [interquartile range]: 9 [27] vs. 27 [55], p = 0.028). Patients with early sAS due to bicuspid aortic valve disease showed higher numbers of circulating EPC-OCN than controls, without reaching statistical significance (CD34+/KDR+/OCN+; median [interquartile range]: 5 [6] vs. 1 [6], p = 0.101).

Immunofluorescence. INFLAMMATION AND OSTEOBLASTIC ACTIVITY. Immunofluorescence staining showed colocalization of the marker of inflammation NFkB and osteoblastic activity (OCN) in severely calcified valves (endothelial and deeper valve tissue layers) (Figs. 4C and 4D). Of note, NFkB/OCN colocalization also was found in noncalcified valves, but only in the deeper valve tissue layers (Figs. 4A and 4B).

IDENTIFICATION OF CD34+/OCN+ CELLS. Immunofluorescence staining showed an abundance of CD34+/

Table 1 Patient Demographics and Clinical Data

	Control (n = 22)	Mi-MoAS (n = 17)	sAS (n = 26)	sAS/sCAD (n = 33)	p Value
Age (yrs)	47 ± 9	68 ± 11	66 ± 11	73 ± 10	0.000
Male (%)	27	76	73	88	0.000
RR_{syst} BP (mm Hg)	130 ± 20	126 ± 23	122 ± 25	129 ± 21	0.600
RR_{diast}	74 ± 10	68 ± 12	66 ± 10	63 ± 11	0.004
Total cholesterol (mg/dl)	181 ± 32	165 ± 36	162 ± 38	165 ± 36	0.262
LDL (mg/dl)	106 ± 28	89 ± 23	88 ± 27	94 ± 28	0.103
HDL (mg/dl)	53 ± 11	44 ± 16	53 ± 18	43 ± 10	0.015
eGFR (MDRD)	81 ± 12	71 ± 19	72 ± 16	66 ± 19	0.017
hsCRP*	2.5±2	1.9 ± 1.8	1.9 ± 1.3	3.7 ± 5.2	0.583
BMI (kg/m^2)	29±6	30 ± 5	28 ± 5	30 ± 5	0.275
AV area/TVI (cm^2)	—	1.7 ± 0.4	0.96 ± 0.29	0.87 ± 0.16	0.000
Mean AV gradient (mm Hg)	—	19 ± 9	56 ± 15	48 ± 11	0.000
Ejection fraction (%)	—	54 ± 14	64 ± 12	59 ± 12	0.043
HTN (%)	36	82	54	79	0.002
HLP	59	65	69	88	0.088
DM	0	18	8	33	0.004
FH (%)	73	29	35	39	0.017
Smoking†	32	41	38	52	0.523

Values are mean ± SD, %, or n. p values are overall tests for a difference between any 2 groups analysis of variance. Data are presented as mean ± standard deviation. *High-sensitivity C-reactive protein data available in n = 22, 4, 8, and 10 patients, respectively. †Includes ex-smokers.

AV = aortic valve; BMI = body mass index; BP = blood pressure; DM = diabetes mellitus; eGFR = estimated glomerular filtration rate (Modification of Diet in Renal Disease [MDRD] method); FH = family history; HDL = high-density lipoprotein; HLP = hyperlipidemia; hsCRP = high-sensitivity C-reactive protein; HTN = hypertension; LDL = low-density lipoprotein; mi-moAS = mild to moderate calcific aortic stenosis; RRsyst/diast = systolic and diastolic blood pressure; sAS = severe calcific aortic stenosis; sCAD = severe coronary artery disease; TVI = tissue velocity index.

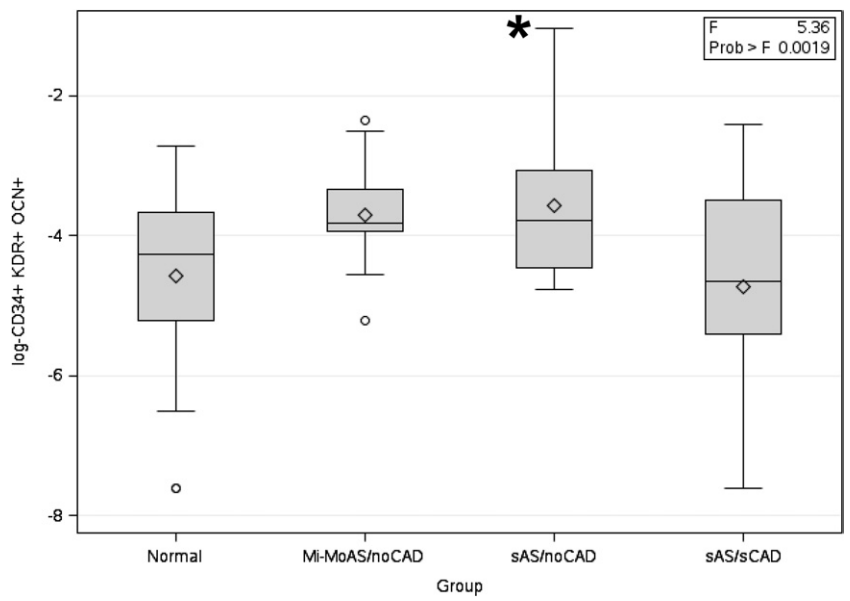

Figure 1 **Circulating EPC-OCN in All 4 Study Groups**

Patients with mi-moAS and sAS showed higher numbers of circulating endothelial progenitor cells with osteoblastic phenotype (EPC-OCN) than normal subjects, whereas patients with both sAS and sCAD had numbers of circulating EPC-OCN similar to those of normal subjects. *p = 0.024 versus normal subjects. Median values and interquartile ranges are shown; **diamonds** represent mean values. CAD = coronary artery disease; mi-moAS = mild to moderate calcific aortic stenosis; sAS = severe calcific aortic stenosis; sCAD = severe coronary artery disease.

OCN+ cells within the endothelial cell layer and the deeper cell layers of resected calcific aortic valve specimen but not in normal valves (Fig. 5).

Discussion

The present study shows for the first time that patients with varying degrees of calcific aortic valve disease have a high fraction of circulating EPC-OCN (CD34+/KDR+/OCN+) with osteogenic activity. The current study supports a potential role of osteogenic EPCs as biomarkers for prognostication of disease progression and in the mechanism of AVC. However, the absolute numbers of EPCs and EPC-OCN show interesting variations between the disease subgroups.

Patients with both sAS and sCAD have significantly reduced numbers of circulating EPCs (CD34+/KDR+/OCN−) compared with controls, but a high percentage of these circulating EPCs are EPC-OCN. Of note, patients with only mi-moAS and normal coronaries have an EPC/EPC-OCN profile similar to that of patients with both sAS/sCAD.

In contrast, patients with sAS and normal coronaries show normal EPCs and high EPC-OCN numbers with a high percentage of EPC costaining for OCN. One could hypothesize that patients with sAS but normal coronaries may represent a subgroup of patients whose bone marrow is capable of a surge of EPC release in response to injury, leading to a more favorable EPC/EPC-OCN profile. In contrast, patients with both sAS and sCAD may not be capable of such a surge and, thus, show progression of calcific atherosclerosis in multiple vascular territories because of an unfavorable imbalance between injury and repair (i.e., low EPC numbers with a high percent of EPC-OCN).

Thus, patients with mi-moAS are an interesting group in our current analysis, the majority of whom have what seems to be an unfavorable EPC profile (i.e., low EPC numbers but high EPC-OCN percentages). Because predicting the progression of calcific AS remains difficult and medical intervention trials have been disappointing, long-term follow-up data of this mi-moAS group may provide insights into the role of EPC-OCN for prognostication of disease progression and suitability for decision-making on aggressive medical management.

Data are limited on the potential role of circulating EPCs and osteogenic precursors in calcific aortic valve disease. Matsumoto et al. (12) compared circulating EPC numbers and function in peripheral blood samples from 15 patients with sAS (CAD was excluded) with 18 age-matched controls (sAS and CAD excluded). In addition, they analyzed harvested aortic valve tissue from patients with sAS for endothelial cell senescence. The authors found that endothelial cells located on the aortic side of valves from patients with sAS showed early senescence. Moreover, circulating EPCs in patients with sAS were reduced and showed functional impairment as assessed by a migration assay. The authors attributed the observed changes to "biological aging."

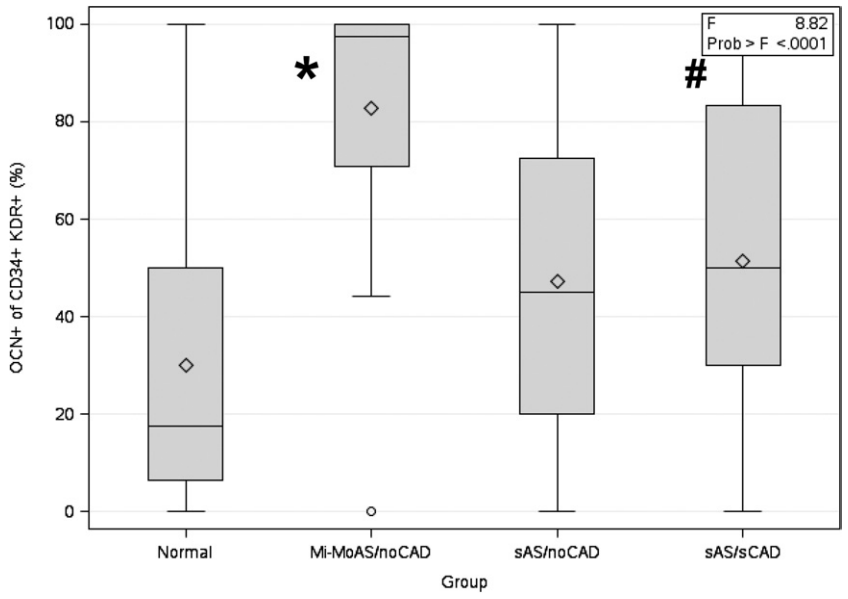

Figure 2 **Percentage of OCN Costaining of EPCs in All 4 Study Groups**

All disease groups showed a higher percentage of costaining of circulating EPCs for osteocalcin (OCN) than normal subjects, reaching statistical significance in all but the sAS group. *p < 0.001, #p = 0.043 versus normal subjects. Median values and interquartile ranges are shown; **diamonds** represent mean values. Abbreviations as in Figure 1.

In contrast to the study by Matsumoto et al. (12), in the current study patients with sAS and no CAD showed numbers of circulating EPCs similar to those of controls. This discrepancy can be explained by the fact that the control group in the study by Matsumoto et al. (12) was recruited from patients undergoing coronary angiography for angina symptoms. Some of these controls may have had coronary endothelial dysfunction, which has been shown to

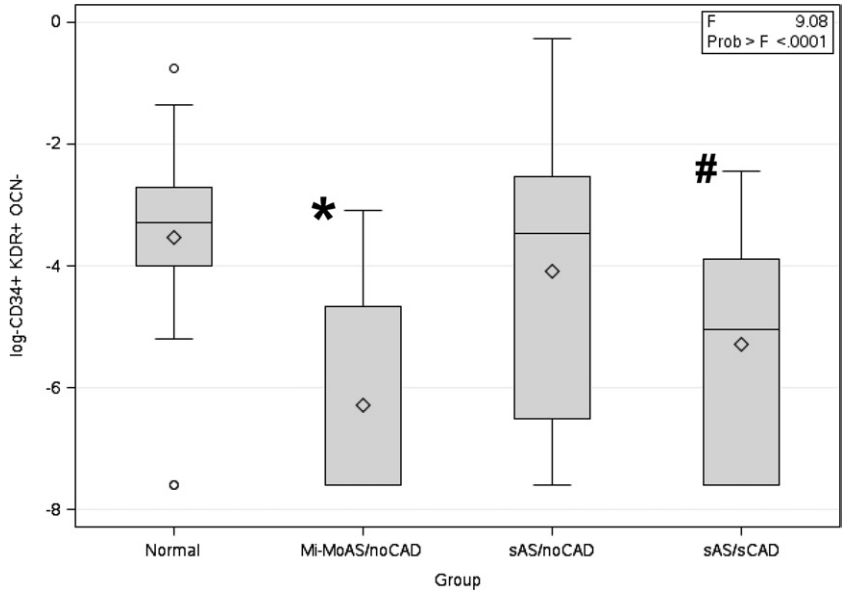

Figure 3 **Circulating EPCs in All 4 Study Groups**

Patients with mi-moAS and patients with both sAS and sCAD showed significantly less circulating EPCs than normal subjects. *p < 0.001, #p = 0.003 versus normal subjects. Median values and interquartile ranges are shown; **diamonds** represent mean values. Abbreviations as in Figure 1.

JACC Vol. 60, No. 19, 2012
November 6, 2012:1945–53

Table 2 Analysis of Covariance After Adjustment for Confounding Risk factors

Overall ANOVA p Values and Dunnett-Adjusted Pairwise Comparisons on Transformed Data After Adjustment for Diabetes, Hypertension, and Smoking

Comparison	CD34+ KDR+ OCN+ (Log-Transform)	OCN+ of CD34+ KDR+ (No Transform)	CD34+ KDR+ OCN− (Log-Transform)
Overall (ANOVA)	0.001	<0.001	<0.001
Normal vs. mi-moAS/no CAD	0.13	<0.001	<0.001
Normal vs. sAS/no CAD	0.029	0.23	0.66
Normal vs. sAS/sCAD	0.84	0.26	0.012

ANOVA = analysis of variance; CAD = coronary artery disease; mi-moAS = mild to moderate calcific aortic stenosis; sAS = severe calcific aortic stenosis; sCAD = severe coronary artery disease.

be associated with reduced numbers of circulating EPCs (10,13). In contrast, our controls have normal coronary arteries and normal endothelial function (invasively assessed) (10). Nevertheless, both studies implicate endothelial injury with an impaired repair process in calcific AS. The current study further elucidates the mechanism of valvular calcification that may occur during the attempted repair. Patients with sAS have not only have functionally impaired EPCs but also a high number of circulating EPC-OCN. Thus, rather than repair, engraftment of EPC-OCN may lead to progressive calcification of the aortic valve.

Further evidence for the active role of circulating osteogenic cells in valvular calcification comes from studies of CD45+/OCN+ cells in patients with end-stage aortic valve disease and heterotopic ossification. Egan et al. (14) showed that CD45+/OCN+ cells represented 1.1% of circulating mononuclear cells in their study population and were localized to regions of heterotopic ossification.

The fact that we rarely found CD34+/OCN+ cells in normal valves but an abundance of CD34+/OCN+ cells in the endothelial cell layer and the deeper valvular tissue of severely calcified aortic valves further supports the concept of attraction of osteogenic precursors to sites of valvular injury and their active role in AVC.

Evidence of colocalization of inflammation (expression of activated NFkB) and osteoblastic activity (OCN) within deeper layers but not the endothelium of normal (noncalcified) valves in our study may suggest a role of resident osteogenic cells that are activated by an early inflammatory process. One may speculate that resident osteogenic cells are the first responders to early, modest valvular injury/inflammation, and after more significant injury, particularly to the valvular endothelial cell layer, circulating osteogenic precursors are attracted, as suggested by the abundant colocalization of inflammation and osteoblastic activity,

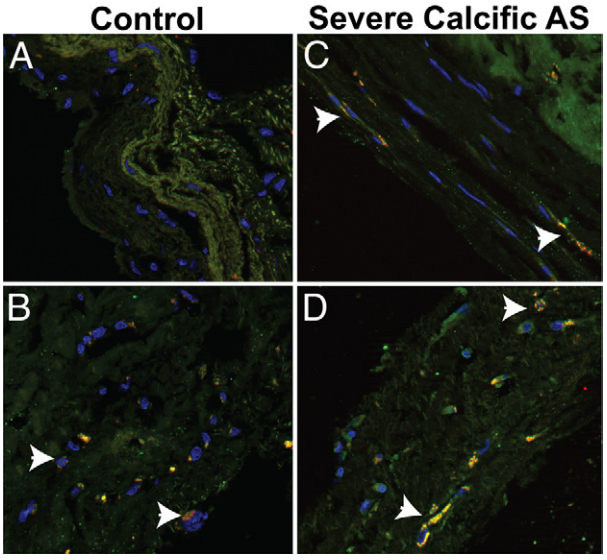

Figure 4 Colocalization of Inflammation and Osteoblastic Activity

Immunofluorescence staining for nuclear factor kappa-B (NFkB) **(red)** and osteocalcin (OCN) **(green)** of control **(A, B)** and severely calcified aortic valves (**C, D; blue** represents DAPI stain). NFkB and OCN demonstrate colocalization (**yellow,** examples indicated by **white arrowheads**), indicating that the osteoblastic process is associated with inflammation. Whereas calcified aortic valves show NFkB/OCN costaining within the endothelial cell layer **(C)** and the deeper valve tissue layers **(D)**, control valves show costaining only within the valve **(B)** and not within the endothelial cell layer **(A)**. Magnification ×40. For individual color channels, please see Online Figures 1 and 2. AS = aortic stenosis.

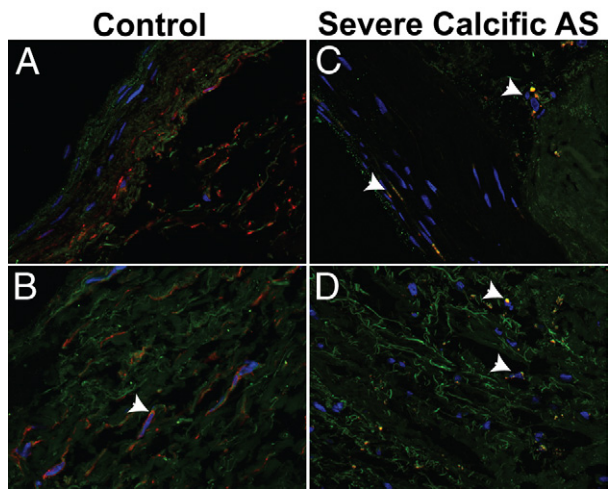

Figure 5 Immunofluorescence Staining for Osteoblastic Progenitor Cells

Immunofluorescence staining for CD34 **(red)** and osteocalcin (OCN) **(green)** of normal **(A, B)** and severely calcified aortic valves (**C, D; blue** represents DAPI stain). CD34/OCN costaining (**yellow,** examples indicated by **white arrowheads**) is rarely observed within deeper valve tissue of control valves **(B)**, but not within the endothelial cell layer **(A)**. In contrast, CD34/OCN cells are abundant within the endothelial cell layer **(C)** and deeper valve tissue **(D)** of severely calcified valves. Magnification ×40. For individual color channels, please see Online Figures 1 and 2. AS = aortic stenosis.

including the endothelial cell layer of severely calcified valves.

Toutouzas et al. (15) demonstrated that aortic valve inflammation is associated with neovascularization in patients with aortic valve stenosis. In the current study, we did not assess aortic valve neovascularization; however, it is conceivable that aortic valve neovascularization serves as an entry port not only for inflammatory cells but also for osteogenic circulating progenitor cells and, thus, promotes valvular calcification.

Our current data are also supported by previous experimental data in osteoprotegerin-deficient mice and a mouse model of accelerated calcification through calcitriol administration. Pal et al. (16) found a strong correlation between the amount of extracted aortic valve calcium and the percentage of circulating OCN(+) EPCs. In addition, in a subgroup of patients with peripheral artery disease, the same authors found higher percentages of OCN(+) EPCs in patients with higher AVC volume assessed by computed tomography (16).

The mechanistic concept of blood-derived procalcific cells is further supported by recent evidence of increased numbers of circulating OCN+/alkaline phosphatase+ myeloid cells in diabetic patients; Fadini et al. (17) demonstrated that type 2 diabetic patients showed elevated numbers of these cells that were also adjacent to sites of calcification in carotid endarterectomy specimens of diabetic patients.

We analyzed a subgroup of our patients who were younger and had severe calcific bicuspid aortic valves scheduled for aortic valve replacement. The comparison with normal subjects of comparable age revealed a similar distribution of EPCs and EPC-OCN seen between normal subjects and patients with both sAS and sCAD. Thus, age does not seem to be a major determinant for the decrease in circulating EPCs, number of EPC-OCN, and percentage of OCN costaining of circulating EPCs in the current study. This is in line with our previous findings in which the absolute number and percentage of EPC-OCN were age-independent predictors of early and severe coronary atherosclerosis (10). Indeed, others have demonstrated that although there is no significant difference in the number of circulating EPCs between old individuals and young, healthy individuals, EPCs from older individuals (61 ± 2 years) show functional impairments (lower survival, migration, and proliferation) (18). EPC senescence induced by telomere shortening may contribute to these functional impairments in older individuals (19).

Not every patient with bicuspid aortic valve disease shows early, severe AVC. One may speculate that genetic factors determine the relative ratio of EPC-OCN and EPCs in an individual and, thus, the onset and rate of progression of vascular calcification. Prospective clinical studies will have to show whether assessing EPC-OCN relative to EPCs in patients with bicuspid aortic valve disease helps to prognosticate the rate of progression of bicuspid AVC and stenosis.

Our subgroup of patients with bicuspid aortic valves also may help to explain the restriction of the severe calcification process to only 1 endothelial territory in some patients. Despite the fact that patients with severe bicuspid calcific aortic valve stenosis show high percentages of OCN costaining and low numbers of circulating EPCs (similar to our sAS/sCAD group), only few show significant CAD. A combination of low total EPCs but high percentage of EPC-OCN may lead to early accelerated progression of tissue calcification in the most injured endothelial territory (e.g., a bicuspid valve exposed to high shear stresses). With aging, this disadvantageous ratio of EPC-OCN/EPC likely leads to multiterritory vascular calcification when more and more endothelial cell layers become significantly injured. In patients with a more favorable ratio of EPC-OCN/EPC, a better functioning repair process may relatively delay the calcification process. In such patients, severe, symptomatic calcification may manifest later in life and only in the endothelial territory that showed aggressive endothelial injury.

Clinical data show that 50% of patients with calcific AS have clinically significant CAD (20,21). Moreover, Otto et al. (22) showed that the risk of myocardial infarction is increased in patients with calcific aortic valve disease, even in those without clinically evident CAD. The current data may provide, for the first time, a mechanistic explanation for this intriguing association. Patients with sAS and normal coronary arteries showed high levels of circulating EPCs with an osteoblastic potential, which may promote early initiation and progression of coronary calcification. Coronary calcification, in turn, has been shown to be associated with accelerated plaque progression (23) and higher event rates (24).

Figure 6 summarizes the potential mechanisms by which circulating EPC-OCN may participate in AVC. As summarized by Hermans et al. (25), all 3 typical layers of the aortic valve are populated by valve interstitial cells (VICs) that maintain leaflet integrity. It is thought that in response to injury, endothelial-mesenchymal transition gives rise to quiescent VICs from embryonic progenitor endothelial/mesenchymal cells (26). These quiescent VICs likely maintain valve integrity and inhibit neovascularization. Although activated VICs likely help the repair process, osteoblastic VICs participate in tissue calcification. A fourth type of VICs are progenitor VICs; however, their origin is unknown. One possible origin is the bone marrow via the circulation. On the basis of our data, we hypothesize that circulating EPC-OCN implant into the endothelial cell layer and that the deeper cell layers of aortic valves may represent circulating progenitor VICs. The fact that some patients have high percentages of circulating EPC-OCN may predispose them to earlier and more significant activation of osteoblastic VICs on valve injury.

Alternatively, circulating EPC-OCN could represent markers of ongoing endothelial-mesenchymal transition, in which case they may be useful in the prognostication of progression of aortic and coronary valve calcification. Further experimental studies will have to elucidate whether

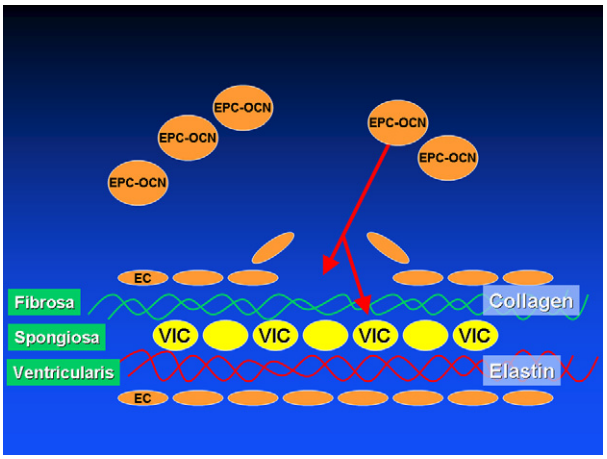

Figure 6 Possible Role of Circulating EPC-OCN in AVC

In the hypothesized interplay among the circulating EPC-OCN, the endothelial cell layers of the aortic valve, and the VICs, EPC-OCN may engraft into the endothelial cell layer or enter deeper valvular tissue layers and become VICs (see "Discussion" for further details). AVC = aortic valve calcification; EC = endothelial cell; EPC-OCN = circulating endothelial progenitor cells with osteoblastic phenotype; VIC = valve interstitial cell.

circulating EPC-OCN actively participate in tissue calcification or are only markers of the disease.

Study limitations. Because our study focused on a different mechanism of calcific aortic valve stenosis, we did not assess valvular endothelial cells or circulating EPCs for senescence, migratory function, or apoptosis. In addition, without using an experimental model, the current study on human surgical specimens does not allow exploring the definite mechanistic role of circulating EPC-OCN in the pathogenesis of AVC.

We used isotype controls instead of fluorescence-minus-one controls in our 4-color flow-cytometry analyses. The use of fluorescence-minus-one controls could have led to a different gating strategy.

Our subgroup analysis of patients with bicuspid aortic valve stenosis is limited to 12 patients, which is mainly due to the low prevalence of the disease in the general population and the fact that not all bicuspid valves calcify to a surgical degree. The current study design does not allow the assessment of prognosis of patients with calcific aortic valve disease and certain levels of EPC-OCN. Prospective long-term follow-up of our patients (especially those with mi-moAS) will demonstrate whether those with higher numbers of EPC-OCN show accelerated progression of calcific aortic valve stenosis.

Conclusions

We show for the first time that the percentage of EPC-OCN in a given EPC population is significantly increased in patients with varying degrees of AVC. Moreover, our data indicate that a combination of low total EPCs but high percentage of EPC-OCN is independent of age and may contribute to early and accelerated valvular and vascular calcification.

Future prospective studies will show whether the number of EPC-OCN relative to EPCs may be a marker of prognostication for the onset and rate of progression of valvular and coronary calcification and may be useful in decision making about the aggressiveness of risk-factor management of patients.

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Key Words: bicuspid aortic valve disease ■ calcific aortic valve stenosis ■ endothelial progenitor cells ■ osteocalcin.

APPENDIX

For supplementary figures, please see the online version of this article.