ASSOCIATION OF MYOCARDIAL ANGIogenEsis WITH STRUCTURAL AND FUNCTIONAL VENTRICULAR REMODELING IN AORTIC STENOSIS PATIENTS WITH NORMAL EJECTION FRACTION

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BACKGROUND: Although rarefaction of myocardial angiogenesis has been shown to be associated with left ventricular (LV) systolic dysfunction in animal models of ventricular hypertrophy, this relationship has not been investigated in depth nor validated in humans. We aimed to analyze the relationship between myocardial angiogenesis with various functional and structural ventricular remodeling parameters and asymptotic aortic stenosis (AS) patients with normal LV ejection fraction (LVEF).

METHODS: A total of 38 moderate or severe AS patients with LVEF > 50% were enrolled for the current study and all patients underwent LV endomyocardial biopsy at the septum during aortic valve replacement. The biopsy specimens were stained for platelet endothelial cell adhesion molecule-1 (CD31) to analyze the density of blood vessels in the myocardium.

RESULTS: The degree of myocardial angiogenesis tended to increase with worse myocardial systolic function, LV filling pressure and progressed ventricular hypertrophy (Spearman’s $\rho = -0.388$, $p = 0.016$ for LVEF; Spearman’s $\rho = 0.442$, $p = 0.007$ for $E/e'$; Spearman’s $\rho = 0.424$, $p = 0.008$ for LV mass index). The degree of myocardial angiogenesis was also significantly associated with the degree of aortic valve stenosis (Spearman’s $\rho = -0.368$, $p = 0.023$). There was significant difference in the degree of myocardial angiogenesis according to the LV geometry ($p = 0.016$ for mean difference between different LV geometry groups by analysis of variance). Significant predictors of myocardial blood vessel density were LV mass index ($\beta = 0.398$, $p = 0.010$) and LVEF ($\beta = -0.313$, $p = 0.028$).

CONCLUSION: There is a close relationship between myocardial angiogenesis and LV remodeling in moderate to severe AS patients with normal LVEF, with angiogenesis increasing with LV hypertrophy. Further studies to demonstrate the mechanism underlying this phenomenon is warranted.

KEY WORDS: Angiogenesis · Aortic stenosis · Echocardiography · Ventricular remodeling.

INTRODUCTION

Aortic stenosis (AS) is a prototypical disease of pressure overload to the left ventricle, which affects 2–4% of the elderly population. It is a gradual but constantly progressive disease with a prolonged asymptomatic period. However, the prognosis is poor when symptoms develop and the only curative measure is replacement of the diseased valve at the right time. Therefore, understanding the mechanism of left ventricular (LV) response to pressure overload in these patients is important not only for the optimal timing of surgery but also, for predicting the outcome and possibly, excavating new therapeutic targets.

With a prolonged period of pressure overload, hypertrophy of the myocardium develops as a compensatory mechanism. Microscopically, this is associated with increase of myofiber size and interstitial fibrosis in various human and animal models of ventricular hypertrophy. More importantly, the degree of hypertrophy in asymptomatic AS patients has been associated with clinical outcome in numerous previous reports and although commonly regarded as ‘normal’ systolic func-
tion, some patients do have subclinical LV systolic dysfunction. These results suggest that investigating the mechanism of ventricular hypertrophy, even in patients with ’normal’ systolic function, may be important for understanding the process of ventricular remodeling in AS patients. However the microscopic changes associated with the process of ventricular hypertrophy, especially before the LV systolic dysfunction starts, is largely unknown in humans.

Angiogenesis is a dynamic process that goes side-by-side with the growth and regression of an organ. Specifically, angiogenesis has been shown to be associated with ventricular hypertrophy in various animal models and several investigators have tried to harness angiogenesis for treating cardiac hypertrophy. Although the disruption of coordinated ventricular hypertrophy and myocardial angiogenesis has been shown to contribute to overt LV systolic dysfunction in animal models and also in humans, this relationship has not been investigated in depth nor validated in humans with normal LV systolic function. Also, the phenomenon that has been demonstrated in animals has to be correlated with various parameters of ventricular function in humans, in order to be translated into clinical research in the future.

AS is an excellent human model for studying the change of ventricular function and morphology following chronic pressure overload. In this report, we analyzed the degree of myocardial angiogenesis with various ventricular remodeling parameters, in both function and structure, in moderate to severe AS patients with normal LV ejection fraction (LVEF).

METHODS

PATIENT POPULATION

A total of 38 patients with moderate to severe AS as per current guidelines, e.g., aortic valve area (AVA) < 1.5 cm² and transaortic mean pressure gradient > 30 mmHg or transaortic peak velocity > 3 m/second, were enrolled to this prospective study from September 2009 to September 2012 at Seoul National University Hospital. Patients with significant concomitant valvular disease of more than mild degree, i.e., moderate aortic regurgitation or moderate mitral valve disease, a previous history of cardiac surgery or myocardial infarction and also, patients with significant LV systolic dysfunction (LVEF < 50%) were excluded. All patients gave informed consent to the study, the protocol of which was approved by the Institutional Review Board of Seoul National University Hospital. Baseline laboratory tests, anthropometric measures and medical history were taken at the time of echocardiographic examination. Body surface area (BSA) was calculated using the Mosteller formula.

TWO-DIMENSIONAL ECHOCARDIOGRAPHIC EXAMINATION

We performed a comprehensive echocardiographic examination of each patient with an adequate commercialized equip-

ment (Vivid 7, GE Medical System, Horten, Norway) according to the current recommendations and guidelines. In brief, end-diastolic/systolic LV diameter was measured at the standard parasternal view. The aortic root, i.e., aortic annulus, sinotubular junction and ascending thoracic aorta diameter were measured at the standard parasternal long-axis view.

After securing an adequate standard four-chamber view, we measured peak early and late diastolic velocity (E, A velocity, respectively) at the tip of mitral valve using a standard pulsed-wave Doppler and also, mitral annular velocity (‘e’, ‘a’ velocity, respectively) at the septal annulus using tissue Doppler imaging. We also measured transaortic mean pressure gradient (PG) and maximal velocity at all possible views, for example apical 5 or 3 chamber, subcostal, right parasternal and suprasternal notch view. The AVA was calculated using the continuity equation after acquiring time-velocity integral (TVI) at the aortic valve level and also, LV outflow tract (LVOT) level. Stroke volume was calculated by multiplying TVI at the LVOT level with the cross-sectional area of LVOT and indexed by dividing it with BSA. Valvuloarterial impedance, a measure of the global LV afterload, was calculated using the following equation; (systolic blood pressure + mean transaortic PG) / indexed stroke volume. The LV mass was calculated using the equation of Devereux and Reichek.

All patients had baseline heart rate < 100 bpm. For patients in sinus rhythm, all measurements were an average of 3 consecutive beats. For patients in atrial fibrillation, all measurements were an average of 5 beats according to the current recommendations. The pattern of ventricular remodeling was classified according to the previous literatures, using LV mass index and relative wall thickness (RWT), into normal geometry, eccentric hypertrophy or concentric hypertrophy. Specifically, the cut-off value of LV mass index were 134 g/m² for men, 109 g/m² for women and the cut-off value of RWT was 0.45 for both sex.

TWO-DIMENSIONAL SPECKLE TRACKING IMAGING ANALYSIS

We obtained standard two-dimensional speckle tracking images at a frame rate of 50~100 frame/second from the three standard apical views after securing a steady breath hold. The LV endocardium was tracked at the end-systolic phase with special caution not to include the pericardium. The region-of-interest was defined semi-automatically by an adequate offline analysis program (EchoPac 5.0.1 for PC, GE Medical Systems, Milwaukee, WI, USA) between the endocardial and epicardial borders. For an adequate measurement of global longitudinal strain (GLS), we traced at least five segments of each windows. An independent observer blinded to the objective of the study obtained the whole strain curves. Peak GLS was defined as the peak negative value of the strain curve in a single cardiac cycle and calculated for the entire U-shaped LV myocardium as follows; global strain = [L (end-systole) - L (end-diastole)] / L (end-diastole) × 100 (%) (L: whole LV myo-
cardium as one big segment). Global strain is the myocardial deformation of the myocardium as a whole and not an average of each segmental strain as in previous literatures concerning average strain in severe AS patients. Peak GLS was averaged from GLS values analyzed at apical two, four and three chamber views.

**ENDMYOCARDIAL BIOPSY**

All patients gave written consent on the intraoperative biopsy. In brief, after a standard aortotomy and removal of the diseased aortic valve, 3 mm sized endomyocardial biopsy specimen was collected from the basal septum of the LV cavity using a standard bioprobe.

**IMMUNOSTAINING AND MORPHOMETRIC ANALYSIS**

All samples were stored overnight in 10% formaldehyde solution and embedded in paraffin. Four microgram section was cut for immunohistochemistry and treated for antigen activation. Nonspecific binding sites were pre-blocked using 3% hydrogen peroxide for 30 minutes. The primary antibody used for detection of blood vessel was rabbit anti-human platelet endothelial cell adhesion molecule-1 (PECAM-1, 1:250, Millipore, Billericia, MA, USA). The primary antibody was incubated overnight at 4°C and a secondary biotinylated anti-rabbit IgG (1:100, Promega, Sunnyvale, CA, USA) antibody was incubated following the primary incubation. Finally, the staining results were visualized using a standard DAB kit (Vector lab., Burlingame, CA, USA) according to the manufacturer’s recommendation. Morphometric measurements and analysis were done with a semi-automatic dedicated software (ImageJ, http://rsb.info.nih.gov/ij). The result of the morphometry was expressed as the PECAM-1 positive % area of the whole image.

**STATISTICAL ANALYSIS**

Continuous variables are tested for normality with Kolmogorov-Smirnov test and presented as mean ± standard deviation or median (25–75th percentile) as appropriate. The difference between the groups was compared using Student’s t-test or Mann-Whitney U test and analysis of variance (ANOVA) between three groups. Bivariate correlation analysis between the parameters of myocardial structure and function are drawn and the strength of correlation presented as Spearman’s ρ. Dichotomous variables are presented as percentages and compared using χ²-test. All analysis was done with SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) and two-tailed p-value of < 0.05 was considered statistically significant.

**RESULTS**

A total of 38 moderate to severe AS patients were prospectively enrolled for the current study. The baseline clinical characteristics are summarized in Table 1. We included only those with normal LVEF, i.e., LVEF > 50%, to analyze how angiogenesis was specifically related to the process just before the transition to heart failure. In brief, there was no significant difference between the three groups of LV remodeling.

All patients could be divided into 3 groups according to the LV geometry, normal (n = 9), eccentric hypertrophy (n = 11) or concentric hypertrophy (n = 18). There was no patient with concentric remodeling geometry. We analyzed the echocardiography data in these patients (Table 2). The dimension, wall thickness and mass index of LV were significantly different between the three groups as expected. Although there were no significant differences in the E velocity, e' velocity, transaortic peak velocity nor transaortic mean PG between the three groups, the annulus diameter and the AVA was smaller in the concentric hypertrophy group (p = 0.024 for aortic annulus diameter; p = 0.043 for AVA by ANOVA).

Next, we analyzed the blood vessel density in the myocardium that was taken at the time of aortic valve replacement. There was a wide range of vessel density in the given myocardial sample that ranged from 1% to nearly 4% of the whole section. Various echocardiography parameters including parame-

**Table 1. Baseline clinical characteristics of the study participants**

|                               | Total (n = 38) | Normal (n = 9) | Eccentric hypertrophy (n = 11) | Concentric hypertrophy (n = 18) | p-value |
|-------------------------------|---------------|----------------|-------------------------------|---------------------------------|---------|
| Age (years)                   | 67.7 (8.9)    | 70.7 (11.1)    | 63.4 (8.4)                    | 68.9 (7.4)                      | 0.139   |
| Male, n (%)                   | 17 (44.7)     | 5 (55.6)       | 5 (45.5)                      | 7 (38.9)                       | 0.713   |
| SBP (mmHg)                    | 126 (16)      | 134 (17)       | 120 (14)                      | 125 (14)                       | 0.127   |
| DBP (mmHg)                    | 69 (10)       | 74 (4)         | 66 (10)                       | 68 (11)                        | 0.177   |
| BSA (m²)                      | 1.69 (0.14)   | 1.74 (0.16)    | 1.73 (0.13)                   | 1.65 (0.14)                    | 0.569   |
| Hypertension, n (%)           | 17 (44.7)     | 3 (33.3)       | 6 (54.5)                      | 12 (66.7)                      | 0.259   |
| Diabetes, n (%)               | 11 (28.9)     | 2 (22.2)       | 2 (18.2)                      | 7 (38.9)                       | 0.431   |
| Hyperlipidemia, n (%)         | 6 (15.8)      | 2 (22.2)       | 2 (18.2)                      | 2 (11.1)                       | 0.732   |
| Atrial fibrillation, n (%)    | 4 (10.5)      | 1 (11.1)       | 1 (9.1)                       | 2 (11.1)                       | 0.983   |
| Baseline Cr (mg/dL)           | 1.15 (0.97)   | 0.99 (0.27)    | 0.98 (0.25)                   | 1.33 (1.38)                    | 0.188   |

The difference of baseline clinical characteristics between patients with distinct patterns of remodeling, i.e., concentric remodeling, eccentric hypertrophy, concentric hypertrophy was calculated using Student’s t-test, Mann-Whitney U test or χ²-test as appropriate and the results presented as p-value. SBP: systolic blood pressure, DBP: diastolic blood pressure, BSA: body surface area, Cr: creatinine
Fig. 1. Correlation between ventricular function and myocardial blood vessel density. Significant negative correlation between left ventricular (LV) ejection fraction and myocardial blood vessel density (A), in contrast to significant positive correlation between E/e’ and myocardial blood vessel density (B) and also, LV global longitudinal strain (C).
as in the previous literature, there was significant difference of the degree of myocardial angiogenesis between the three groups (p = 0.016) (Fig. 4).

**Discussion**

The main findings of the current study are that 1) within the similar ‘normal’ systolic function, a wide degree of myocardial angiogenesis exists in patients with severe AS, 2) the degree of myocardial angiogenesis correlates with both LV systolic and diastolic function, 3) the degree of myocardial angiogenesis also correlates with the degree of ventricular hypertrophy and finally, 4) there is a significant difference of the degree

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**Table 3.** Predictors of myocardial blood vessel density among aortic stenosis severity parameters

|                          | Univariate |           | p-value |           | p-value |
|--------------------------|------------|-----------|---------|-----------|---------|
| LV ejection fraction     | -0.410     | 0.011     |         |           |         |
| E/e'                     | 0.441      | 0.007     |         | 0.120     | 0.459   |
| LV mass index            | 0.510      | 0.001     |         | 0.398     | 0.010   |
| AVA                      | -0.319     | 0.051     |         | -0.157    | 0.308   |
| Transaortic mean PG      | 0.183      | 0.272     |         |           |         |
| Transaortic Vmax         | 0.141      | 0.397     |         |           |         |
| Valvuloarterial impedance| 0.142      | 0.402     |         |           |         |

The predictors of myocardial blood vessel density were analyzed with linear regression analysis. LV: left ventricle, AVA: aortic valve area, PG: pressure gradient, Vmax: maximal velocity
of myocardial angiogenesis between the different LV geometry. Although there have been various animal and human studies with AS investigating the correlation of angiogenesis with overt heart failure, there has been no studies showing that angiogenesis is associated with subclinical LV dysfunction in ‘normal systolic function’ human hearts.

With chronic pressure overload to the LV in AS, the myofibrils tend to get thicker, which results in ventricular hypertrophy. This also means that LV hypertrophy is a dynamic process involving various complex intracellular signals. Angiogenesis is a dynamic process that is closely related to organ growth and metabolism of the cells needs ingrowth of blood vessels to support this process. Specifically, the disruption of the coordination between ventricular hypertrophy and angiogenesis has shown to be a cause of overt heart failure in murine models, which support the previous hypothesis concerning angiogenesis and ventricular hypertrophy. Furthermore, the decreased expression of myocardial vascular endothelial growth factor has been pointed out as the culprit of systolic dysfunction.

In this report, we have shown that the degree of myocardial angiogenesis is closely associated with adverse remodeling. Specifically, within patients with normal EF, the systolic/diastolic function, the LV mass and the degree of valve stenosis were all closely associated with the degree of myocardial angiogenesis. Following the natural concept that angiogenesis follows the growth of an organ and that the disruption of angiogenic cytokine in the cardiomyocytes leads to systolic dysfunction, it can be said that if the systolic function are within normal limits, a certain degree of angiogenesis follows the adverse remodeling of the myocardium with chronic pressure overload. This is supported by some old animal data demonstrating evidence of capillary growth in hypertensive rodent models. Furthermore, it is a relatively well-known concept that the coronary vascular resistance/reserve is much reduced with the progression of ventricular hypertrophy, both in animals and in humans. Therefore, it may be logical to say that angiogenesis, which inevitably involves the sprouting of new vessels, may be a compensatory mechanism for the decrease of coronary vascular resistance/reserve.

It was interesting to find that the degree of myocardial angiogenesis is associated with the degree of adverse LV remodeling, for example LV systolic and diastolic function parameters. Specifically, the moderate degree of correlation in nearly all of the echocardiographic data tested shows that the process of LV remodeling, although it may sometimes differ from individual-to-individual, follows a fairly universal process. Following a chronic pressure overload to the LV due to the progression of AS, the ventricle starts to get thicker whilst the systolic and diastolic function deteriorate. Although our data involves only patients with normal LVEF, the results of our analysis demonstrates that the ventricles are undergoing extensive pathological remodeling. Increased angiogenesis may be a compensatory effort of the cardiomyocytes to endure the prolonged stress, which in the long-term may fail. Therefore, as previous reports have demonstrated, employing more sensitive parameters, such as strain, for assessing the ventricular remodeling may be needed as a guide for effective early treatment, which is supported by our data as well. Of course, this should not be mistaken that LVEF is a poor parameter to assess the gross LV systolic dysfunction.

Another interesting finding was that there was a significant difference in the degree of myocardial angiogenesis between the different LV geometry. Previous data have demonstrated that longitudinal strain, a marker of subendocardial fiber contractility, is decreased in patients with concentric hypertrophy compared with other types of geometry. However, neither the circumferential nor the radial strain were affected by the geometry. This finding, in concert with our previous findings and also, the current analysis, demonstrates that the subendocardial layer may be the one that undergoes extensive remodeling in response to chronic pressure overload. It is also notable that all of the biopsy specimens used in our analysis were from endomyocardial biopsy taken at the subendocardium.

Our findings are not without limitations. First, all hemodynamic data, especially LVEF and E/e’, are all load-dependent parameters of LV function. However, all of our patients were in euvolemic status and did not having resting dyspnea on echocardiographic examination. Furthermore, the association of myocardial angiogenesis with systolic function is corroborated by our strain analysis, a relatively load-independent parameter. Second, although we have suggested a possible mechanism for increased angiogenesis following adverse ventricular remodeling in severe AS, we did not prove a definite data for coronary flow reserve nor resistance. Third, although we have
provided a relationship between LV remodeling parameters and myocardial angiogenesis, we have not provided a mechanistic data on how this happens. We plan to present these data in the near future. Fourth, although the degree of myocardial angiogenesis correlated with AVA, there was no significant correlation between the degree of angiogenesis and other AS severity parameters such as transaortic mean PG and peak velocity. Conversely, these data may demonstrate that the degree of LV hypertrophy rather than the hemodynamic stress parameters are important for myocardial angiogenesis, which is partially demonstrated by the results of the multivariate linear regression analysis. It would be interesting to investigate whether this holds true in non-AS left ventricular hypertrophy (LVH) patients, such as patients with LVH by hypertension.

In conclusion, our analysis results demonstrate that there is a close correlation between the degree of myocardial angiogenesis following adverse remodeling of the LV in severe AS patients with normal EF. Specifically, myocardial angiogenesis increases as the degree of adverse LV remodeling increases. Further study is warranted on the mechanism of myocardial angiogenesis following chronic pressure overload in humans and how this is related to outcome in the future.

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