Mouse heart valve structure and function: echocardiographic and morphometric analyses from the fetus through the aged adult

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The genetically modified mouse has become an animal model of choice for the study of human cardiovascular disease pathogenesis (7, 8, 49). In vivo analytical methods of developmental and pathological information are important to complement in vitro analyses; however, these modalities are limited in number. Studies of the mouse cardiovascular system have employed noninvasive transthoracic echocardiography, and normal reference standards have been established for ventricular mass and function (9, 14, 30, 41, 50). Several mouse models of valve disease have been described recently (12, 31, 37, 42, 45, 46, 48); however, heart valve structure and function have not been systematically evaluated in a population of normal mice over time to provide standards for the evaluation of mouse models of viable valve disease. The purpose of this study is to present comprehensive echocardiographic data for normal mouse heart valve structure and function from late fetal to aged adult stages and to correlate these findings with histological and morphometric data.

In human valve disease, the majority of patients have a congenital malformation that can manifest as disease in the newborn or more commonly in the aged adult (11, 32). Echocardiography offers the advantage of longitudinal analysis, which provides important data regarding changes in functional status within a single organism and the development of latent disease over time (6, 30). Genetically modified mice used to evaluate the effects of gain or loss of function of genes known to be involved in valvulogenesis often die during embryogenesis, limiting their applicability to human disease (17). However, increasingly sophisticated transgenic models using spatial and temporal regulation of gene expression (8, 49) and the appreciation of the importance of late fetal and early postnatal valve development (1, 18) have led to the search for viable models of valve disease that more closely recapitulate the human phenotype. In vitro analyses of valve morphology during fetal and juvenile mouse development have been described, and various outcome measures have been developed (5, 20, 26); however, these measures have not been systematically studied in a normal population or compared with standard echocardiographic data.

This study provides the first comprehensive assessment of normal mouse valve structure and function from late fetal development through senescence. We have established normal in vivo echocardiographic indexes of mouse valve structure and function and correlated these findings with in vivo histological and morphometric analyses. Echocardiographic indexes of valve structure demonstrate linear growth through the young adult stage, whereas indexes of valve function demonstrate decreased flow velocities during fetal stages and constant flow velocities subsequently. Histological and morphometric analyses demonstrate progressive valve elongation and thinning from the fetus to the young adult. Interestingly, the mouse aortic valve hinge thickens with advancing age in a manner similar to aortic valve sclerosis (27, 28), a marker for human aortic valve disease; however, age-related calcification was not appreciated in the mice we studied. These findings provide a basis for future studies using genetically modified mouse models of valve disease and should become an important reference standard for the study of valve disease natural history and pathogenesis.
MATERIALS AND METHODS

Study design. Wild-type mixed-sex C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were studied at embryonic day (E) 18.5 (late fetal), 10 days (newborn), 1 mo (juvenile), 2 mo (young adult), 9 mo (old adult), and 16 mo (aged adult). Fourteen mice were examined at each time point. Staged litters of embryos were analyzed and collected at E18.5, with evidence of a copulation plug counting as E0.5. Since annulus dimension can vary by age or size, we measured body weight at each time point for normalization. The Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center approved these protocols.

Echocardiographic evaluation. Cross-sectional, two-dimensional and Doppler transthoracic echocardiography was performed by experienced sonographers using a Visual Sonics Vevo 770 Imaging System (Toronto, Canada). A 30-MHz transducer was used for postnatal studies, and a 40-MHz transducer was used for embryonic studies. The chests of the mice or abdomens of pregnant females were shaved and treated with a chemical hair remover to reduce ultrasound attenuation. Heart rate and core temperature were continuously monitored. Normothermic mouse core temperatures were maintained using a heated platform. Mice were anesthetized with 1% to 2% isoflurane. Images were stored in the ultrasound system hard drive and transferred to an image server for off-line analysis.

Annulus dimensions were obtained in the parasternal long-axis view during systole for semilunar valves and the apical four-chamber view during diastole for atrioventricular valves (6, 21). The aortic root measurements were obtained in a modified parasternal long-axis view during diastole. Doppler interrogation was performed on the atrioventricular valve inflow in the apical four-chamber view and semilunar valve outflow in the parasternal long-axis view to assess for stenosis and regurgitation using a sample volume toggle to optimize the angle of interrogation. A modified parasternal long-axis view was required in some cases to ensure ascertainment of the maximum velocity. All measurements were obtained using an angle of interrogation <30° (6, 39). Cardiac chamber dimensions were measured and ventricular function assessed from two-dimensional-directed M-mode echocardiographic images obtained from the parasternal short-axis view, and Doppler images were obtained from the apical four chamber view in accordance with consensus guidelines (6, 21). All measurements were obtained in triplicate and averaged. Embryonic studies proceeded from left to right following the sequential order of fetuses in the uterine horns. Mice with left ventricular systolic dysfunction, as previously determined, were excluded (14, 33, 34).

Histochemical analysis. After echocardiographic imaging, mice were euthanized by carbon dioxide asphyxiation, and hearts were harvested for histological and morphometric analyses. Hearts were stored in the ultrasound system hard drive and transferred to an image server for off-line analysis.

Valve thickness was measured at the proximal (hinge), middle, and distal aspects of the valve cusps and leaflets. The proximal thickness was measured at the widest aspect of the hinge of the valve, the middle thickness was measured at the thinnest aspect of the middle third of the valve, and the distal thickness was measured at the widest aspect of the distal third or tip of the valve. Valve length was measured from the annulus to the tip excluding the chordae tendinae in the mitral valve, and valve area was measured from the annulus to the tip. Sequential sections were reviewed and measurements were obtained from representative sections. Three sections were measured in triplicate and averaged from three mice.

Statistical analysis. Analyses were performed using SAS and Stat View (SAS Institute, Cary, NC). All measures are presented as means ± 2SD to approximate normal distribution. Logarithmic transformation and regression analyses were performed, and nomograms were produced based on the following relationship: \( z = \ln(\text{mean annulus dimension}) - \ln(\text{mean weight})/\sqrt{\text{mean square error}} \). Morphometric measurements were indexed to body weight (each mouse’s morphometric measure was divided by its weight, e.g., valve length/body weight) for purposes of comparison between ages. One-way ANOVA was used to compare continuous variables by age group, and Scheffe’s test was used to adjust for multiple comparisons. A P value <0.01 was considered significant.

RESULTS

We sought to systematically evaluate heart valve structure and function by echocardiography and histological morphology in a population of normal mice from late fetal to aged adult stages. Average heart rate and body weight for mice of different ages are shown in Table 1. An age-appropriate heart rate of 250 beats/min in the fetus and 450 beats/min in postnatal mice was maintained in all mice, consistent with previous studies (13, 41, 43). The average body weight demonstrated linear growth during the fetal, embryonic, juvenile, and young adult stages and was constant during the adult stages.

Echocardiographic analysis of heart valve structure and function. Since annulus dimension is used as a direct measure of the severity of human valve disease (4, 38, 40, 44), we sought to determine normal murine valve annulus dimensions in all four heart valves as a function of age at serial time points. Representative measurements of the aortic and mitral valves from a 2-mo-old young adult mouse by two-dimensional echocardiography are shown in Fig. 1, A and B, double-headed arrows. The annulus dimensions show linear growth over the late embryonic, neonatal, juvenile, and young adult stages and plateau over the young adult, old adult, and aged adult stages (Fig. 1, C–F), consistent with findings in humans (10, 16). For example, normal aortic valve annulus dimension in a young adult mouse (2 mo old) ranges from 1.1 to 1.3 mm. Of note, an eccentric opening, impaired closure, abnormal morphology, or qualitative thickening of cardiac valves was not appreciated in any mice at any stage.

Since valve size is related to body size (10, 16, 44) and different mouse strains may have different body sizes, the annulus dimensions were independently analyzed as a function of body weight. With the use of regression analysis and logarithmic transformation, valve annulus dimension \( z \)-scores were calculated based on mouse body weight. All four valve annulus dimensions are positively correlated with body weight (data not shown). \( R^2 \) values for annulus dimensions as a function of body weight were 0.92, 0.87, 0.93, and 0.86 for the aortic, mitral, pulmonary and tricuspid valve annulus dimensions, respectively. Nomograms were generated for each heart valve annulus dimension, providing a method to determine normal valve size range based on mouse body weight (Fig. 2). For example, normal aortic valve annulus dimension in a 25-g mouse ranges from ~1.0 to 1.5 mm. Analyzing reference data as a function of two different factors (age and weight) provides...
regurgitation and since root dilation may accompany semilunar valve disease, we evaluated left atrial size, left ventricular end-diastolic dimension, aortic root, and ascending aorta dimensions in normal mice. During the embryonic, neonatal, and juvenile stages, linear growth was appreciated and, during the adult stages, the dimensions plateaued (Fig. 4), similar to valve growth. Furthermore, we evaluated indexes of systolic and diastolic left ventricular function over time. Left ventricular fractional shortening and fractional area change were calculated as markers of systolic function, whereas mitral inflow E-wave-to-A-wave ratio and isovolumic relaxation time were measured as markers of diastolic function. Although standard data have been reported for both fetal and young adult stages, the findings of this study provide the first report of normal ventricular function from fetal to aged adult stages (Fig. 5).

Decreased mitral E-wave-to-A-wave ratio and increased isovolumic relaxation time were demonstrated at E18.5, consistent with fetal circulation, and constant normal ranges were defined at subsequent stages, consistent with previous findings (13, 14, 34, 41, 43). There was a trend toward decreased left ventricular systolic function in the aged adult mice; however, this did not reach statistical significance.

**Histological and morphometric analysis of heart valves.** To determine morphological characteristics of normal valve structure, aortic and mitral valves from mice that previously underwent echocardiography were subjected to histological and morphometric analyses as described in the MATERIALS AND METHODS (Fig. 6 and Table 1). Tissue shrinkage due to process-

<table>
<thead>
<tr>
<th>Heart rate, beats/min</th>
<th>E18.5</th>
<th>10 day</th>
<th>1 mo</th>
<th>2 mo</th>
<th>9 mo</th>
<th>16 mo</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>243±52</td>
<td>477±96</td>
<td>467±94</td>
<td>465±60</td>
<td>420±66</td>
<td>428±70</td>
</tr>
<tr>
<td>Weight, g</td>
<td>1.2±0.2</td>
<td>7.2±0.9</td>
<td>11.2±1.0</td>
<td>17.3±1.4</td>
<td>33.0±6.0</td>
<td>26.4±4.0</td>
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**Aortic valve right cusp**
- **Length, μm**: 214±22, 370±72, 395±164, 423±32, 700±82, 685±12
- **Thickness, μm**: Pro 86±7, 79±18, 75±46, 45±14, 82±4, 92±12
- **Mid**: 60±3, 34±5, 28±7, 21±2, 19±6, 17±2
- **Dis**: 116±12, 82±38, 65±44, 66±8, 93±14, 64±2
- **Area, mm²**: 0.017±0.001, 0.02±0.003, 0.017±0.012, 0.016±0.001, 0.03±0.003, 0.02±0.001

**Aortic valve left cusp**
- **Length, μm**: 218±11, 342±96, 362±124, 474±48, 770±108, 676±24
- **Thickness, μm**: Pro 89±2, 73±15, 60±37, 49±32, 113±30, 147±11
- **Mid**: 60±3, 33±3, 23±11, 21±2, 23±4, 19±2
- **Dis**: 114±10, 80±62, 59±60, 53±22, 67±28, 61±6
- **Area, mm²**: 0.017±0.001, 0.019±0.004, 0.015±0.014, 0.019±0.004, 0.031±0.004, 0.021±0.002

**Mitral valve anterior leaflet**
- **Length, μm**: 427±18, 630±154, 753±208, 921±316, 1,076±188, 903±27
- **Thickness, μm**: Pro 94±6, 68±3, 60±18, 69±22, 78±32, 65±6
- **Mid**: 50±3, 39±13, 22±10, 27±6, 15±4, 13±2
- **Dis**: 76±3, 57±13, 35±26, 54±2, 43±5, 36±8
- **Area, mm²**: 0.03±0.001, 0.03±0.008, 0.024±0.01, 0.032±0.006, 0.031±0.003, 0.024±0.001

**Mitral valve posterior leaflet**
- **Length, μm**: 243±16, 332±164, 504±271, 697±320, 701±234, 714±11
- **Thickness, μm**: Pro 88±4, 78±10, 66±26, 62±34, 72±38, 62±5
- **Mid**: 53±3, 41±23, 17±10, 29±18, 15±2, 13±2
- **Dis**: 81±5, 56±42, 44±32, 45±3, 43±26, 27±6
- **Area, mm²**: 0.02±0.001, 0.016±0.011, 0.014±0.012, 0.025±0.014, 0.016±0.008, 0.018±0.003

Values are means ± 2SD. Measurements were indexed to body weight for purposes of comparison between ages. Pro, proximal; Mid, middle; Dis, distal. *P < 0.01, embryonic day (E) 18.5 vs. 10 day; †P < 0.01, 10 day vs. 1 mo; ‡P < 0.01, 1 mo vs. 2 mo; §P < 0.01, 2 mo vs. 9 mo; ¶P < 0.01, 2 mo vs. 16 mo. No significant differences were observed in any measurements between 9- and 16-mo-old mice.
ing occurred, such that the aortic and mitral valve annulus dimensions were 34–54% smaller at all time points by morphometric analysis (data not shown), consistent with previous reports (15, 24). Movat’s pentachrome staining showed that the E18.5 valves consisted of bilayered cusps and leaflets, whereas neonatal, juvenile, and adult valves showed normal ECM trilaminar stratification (18). Over time, the aortic valve cusps and mitral valve leaflets progressively elongated and thinned (Fig. 6). Morphometric analyses of the aortic and mitral valves were performed and absolute measurements are reported in Table 1. These analyses demonstrated significantly increased valve length of all cusps and leaflets through the juvenile stages; interestingly, atrioventricular valve leaflets continued to lengthen into adulthood, after semilunar valve cusps ceased lengthening. The valve thickness was significantly decreased in the proximal, middle, and distal aspects of both the aortic and mitral valves from the fetal through the juvenile stages. In general, valve thickness was constant during adult stages. However, there was significantly increased thickness of the proximal aspect (hinge) of aortic valve cusps in aged adult mice without gross histochemical abnormalities. The aortic and mitral valve areas remained relatively constant over time; however, when indexed to body weight, they decreased significantly, consistent with a dramatic decrease in valve thickness and a modest increase in valve length. Taken together, these findings suggest that postnatal valve growth is due to the lengthening and thinning of the primitive valve cusps and leaflets, consistent with continued ECM remodeling and stratification after birth.

To determine the potential for age-related valve calcification, we examined two markers of calcification at serial time points using alizarin red and vonKossa staining (19, 23). Alizarin red staining was negative at all time points including the aged adult stage, suggesting that age-related valve calcification is not present in C57BL6 mice (Fig. 7A). With vonKossa staining, black particulate deposits were observed at all time points in aortic valve cusps and mitral valve leaflets but not in the
aorta or aortic valve annulus (Fig. 7B). In light of recent findings describing melanocytes in heart valves (25), we used hematoxylin and eosin staining to evaluate for the presence of pigmented cells, which also appear as dark particulate deposits. In these analyses, the same pattern of dark particulate staining was observed for both aortic and mitral valves, demonstrating a lack of calcification, consistent with the alizarin red staining. Pigmented cells were demonstrated in the valve interstitium (Fig. 7C), consistent with previous findings (25), but were not observed in white mice with a FVBN genetic background of any age (data not shown). Taken together, these findings suggest that age-related calcification is not present in C57BL6 mice with the caveat that vonKossa staining in these mice may be identifying melanocytes, giving a false impression that calcification is present. Therefore, alizarin red should provide a more accurate assessment of valve calcification in mouse models of valve disease.
DISCUSSION

This study reports for the first time a comprehensive evaluation of mouse valve structure and function from the embryonic to aged adult stages and correlates these in vivo echocardiographic findings with in vitro histological and morphometric analyses. Defining standard echocardiographic and morphometric measurements through normal growth and development provides valuable controls for the evaluation of mouse models of viable or latent valve disease. For example, standard data allow the analysis of valve pathology that may manifest as valve malformation in the fetus and progress to valve disease only in the aged adult. The ability to correlate longitudinal in vivo valve structure and function with morphometric findings at various stages will allow a more rigorous evaluation of valve malformation and disease progression, thereby contributing to the elucidation of both the natural history and pathogenesis of this important clinical problem.

Human valve disease that presents early in life is characterized by thick valves with disorganized matrix; however, more commonly valve disease presents later in life and is characterized by disorganized matrix and calcification (18, 19). The findings of this study demonstrate that there is an increase in aortic valve thickness in the proximal aspect (hinge) of the valve with advancing age in normal mice. This finding is consistent with age-related valve sclerosis, a marker for human valve disease risk (27, 28), and suggests the thickening seen in valve sclerosis may originate in the valve annulus. This marker may identify those patients at risk for aortic valve disease, including calcific valve disease. It is unclear whether calcification represents a latent manifestation of an abnormal develop-
opmental program or a secondary epigenetic sign of end-stage disease. The findings of this study suggest that age-related calcification is not present in C57BL6 mice. Interestingly, the presence of melanocytes may confound the analysis of valve calcification in black mice as determined by standard vonKossa staining. Therefore, alternative assays may be necessary to confirm true calcification and potentially distinguish and evaluate different sources of aberrant valve mineralization (e.g., vascular calcification vs. ossification).

Developing standard reference data facilitates the evaluation of mouse models of valve malformation and disease since they provide a baseline for the accurate assessment of potential markers of dysmorphic and dysfunctional valves. In the absence of standard data, it is conventional to compare small groups (e.g., 3 transgenic vs. 3 wild-type mice), resulting in the potential risk of finding statistically significant differences between groups when both groups actually fall within the normal range (12, 42). In light of the current results, standard reference data for C57BL6 mouse valve structure and function can be used for more informative analyses. It is important to acknowledge that different genetic backgrounds may impact mouse valve structure and function; however, the results of the current study control for potential differences in body size by establishing normals derived by valve dimensions indexed to

Fig. 6. Histological analysis of aortic and MV anatomy over time. Working model of semilunar valve anatomy demonstrating the components of a valve: the fibrosa, spongiosa, ventricularis, hinge, and annulus (A). Representative morphometric measurements of valve length, thickness, and area are shown in yellow (B); valve thickness is measured in the distal (D), middle (M), and proximal (P) aspects of the cusp. Pentachrome staining at serial time points, including E18.5 (embryonic; C), 10 days (neonatal; D), 1 mo (juvenile; E), 2 mo (adult; F), 9 mo (older adult; G), and 16 mo (aged adult; H), demonstrating progressive elongation and thinning of the valves. There is AoV annulus thickening in the aged adult (*; H). CT, chordae tendinae; IVS, interventricular septum. All images at ×40 magnification.

Fig. 7. Histological analysis of potential age-related calcification in AoV and MV. Alizarin red staining is negative for calcification in 16-mo-old mice, as evidenced by the lack of red staining, suggesting there is no age-related calcification (A). Dark particulate material is stained black (A, arrows); von-Kossa staining is positive in the AoV (B, arrows); however, hematoxylin and eosin staining shows pigmented cells (melanocytes) in the same distribution (C, arrows), indicating that black particulate vonKossa staining is identifying melanocytes rather than calcification. All images at ×200 magnification.
body weight. This is particularly important in the context of studying the natural history of progressive valve disease over time. Importantly, the normal range of Doppler-derived flow velocities was relatively wide, potentially reflecting an inherent technical limitation of Doppler; this confounding variable should be taken into consideration. Valve defects that occur during early embryonic development and primary cardiac morphogenesis as a result of a homozygous mutation are typically embryonic lethal and therefore do not recapitulate viable and progressive valve disease. The use of mouse echocardiography will be important in the assessment of valve malformation and latent valve disease as more sophisticated models of viable valve disease become available and, consequently, will improve our understanding of human valve disease pathogenesis.

Histological and morphometric analyses complement echocardiographic analysis, and the findings of this study provide baseline data for the morphometric analysis of valve structure from the fetus through the aged adult. Specifically, these data describe growth patterns that may be affected by valve malformation or subsequently altered by valve disease. Importantly, tissue shrinkage should be taken into consideration when comparing absolute valve dimension measurements ascertained in vivo and in vitro. The general observations of valve thinning and elongation in the context of constant valve area suggest that postnatal valve growth may be due to reorganization of existing tissue rather than the addition of new tissue. However, the valve area decreases when indexed to body weight, consistent with ECM compaction and postnatal remodeling (1, 20). Observations demonstrating ongoing remodeling of valve structure during postnatal life suggests that cellular mechanisms controlling mature trilaminar ECM organization are dynamic, underscoring the importance of studying morphometric changes over time.

In summary, the findings of this study provide standard echocardiographic data for normal mouse valve structure and function and correlate these findings with detailed histological and morphometric data. These findings provide an important reference standard and a basis for future studies, using genetically modified mouse models of valve disease for the study of valve disease pathogenesis.

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GRANTS

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