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Regional structure-function relationships in mouse aortic valve tissue

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Abstract

Site-specific biomechanical properties of the aortic valve play an important role in native valve function, and alterations in these properties may reflect mechanisms of degeneration and disease. Small animals such as targeted mutagenesis mice provide a powerful approach to model human valve disease pathogenesis; however, physical mechanical testing in small animals is limited by valve tissue size. Aortic valves are comprised of highly organized extracellular matrix compartmentalized in cusp and annulus regions, which have different functions. The objective of this study was to measure regional mechanical properties of mouse aortic valve tissue using a modified micropipette aspiration technique. Aortic valves were isolated from juvenile, adult and aged adult C57BL/6 wild type mice. Tissue tensile stiffness was determined for annulus and cusp regions using a half-space punch model. Stiffness for the annulus region was significantly higher compared to the cusp region at all stages. Further, aged adult valve tissue had decreased stiffness in both the cusp and annulus. Quantitative histochemical analysis revealed a collagen-rich annulus and a proteoglycan-rich cusp at all stages. In aged adult valves, there was proteoglycan infiltration of the annulus hinge, consistent with observed mechanical differences over time. These findings indicate that valve tissue biomechanical properties vary in wild type mice in a region-specific and age-related manner. The micropipette aspiration technique provides a promising approach for studies of valve structure and function in small animal models, such as transgenic mouse models of valve disease.

Keywords

valves; mice; tissue mechanical properties; histochemistry; micropipette aspiration

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Conflict of Interest Statement

The authors have nothing to disclose.

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1. Introduction

Aortic valve disease (AVD) is a significant cause of cardiovascular morbidity in humans and occurs in greater than 2% of people in the US (Nkomo et al., 2006; Otto, 2006). There is increasing interest in the development of durable aortic valve bioprostheses for the treatment of AVD (Vesely, 2005; Gallegos, 2006; Sacks et al., 2009b). Aortic valve malformation (AVM) is a heritable condition that underlies the majority of AVD cases, suggesting a developmental etiology (Hoffman et al., 2002; Cripe et al., 2004; Roberts et al., 2005). AVD is associated with valve cell-matrix abnormalities (Otto et al., 1994; Fedak et al., 2003; Hinton et al., 2006) and consequently, extracellular matrix (ECM) disorganization may alter the mechanical microenvironment of valves (Matsumoto et al., 2002; Yip et al., 2009). Aging is a significant risk-factor for AVD (Otto, 2006; Tzemos et al., 2008), and recent studies have demonstrated that aging induces alterations in ECM material properties of porcine valves (Stephens et al., 2007; Stephens et al., 2009). Small animal models, including targeted mutagenesis mouse models of valve disease, provide an effective tool to study genetic factors underlying valve disease pathogenesis (Yutzey et al., 2007). However, the mechanical properties of normal mouse valve tissue remain largely unknown.

Positioned between the left ventricle and aorta, the aortic valve functions to maintain forward blood flow throughout the cardiac cycle. Late embryonic valvulogenesis is characterized by ECM remodeling and organization that continues postnatally (Armstrong et al., 2004; Aikawa et al., 2006; Hinton et al., 2006). The aortic valve is situated within the aortic root, and is composed of discrete cusp and annulus regions (Figure 1). Cusps consist of highly organized connective tissue hinged to a crown-shaped fibrous annulus (Yacoub et al., 1999; Anderson, 2000). The valve annulus functions as a supporting structure for valve cusps; however, regional valve structure-function relationships have not been clearly defined. Importantly, changes in valve anatomy and underlying matrix composition may lead to alterations in valve mechanical properties and therefore affect its function.

To date, mechanical studies of aortic valves as a viscoelastic tissue have focused predominantly on cusps in valves from large animals or humans (Christie et al., 1995a; Billiar et al., 2000; Sacks et al., 2009a). Previous studies have identified differences between different heart valves such as aortic and pulmonary valves (Christie et al., 1995b; Merryman et al., 2007; Merryman, 2010), and reported the mechanical properties of the aortic root in large animal models (Grande et al., 1998; Nicosia et al., 2002; Yap et al., 2010). However, regional differences in valve biomechanics and the mechanical properties of the annulus region in particular, remain largely unexplored.

Physical interrogation strategies to determine biomechanical properties of valve tissue have been developed for large animals. However, conventional uniaxial or biaxial mechanical testing of valves in smaller animals such as mice is complicated by tissue size. Micropipette aspiration systems have been used previously for measuring mechanical properties of chondrocytes and their pericellular matrix (Guilak et al., 1999; Alexopoulos et al., 2003), valve interstitial cells (Merryman et al., 2006b; Zhao et al., 2009), and embryonic chicken atrioventricular endocardial cushions (Butcher et al., 2007). The objective of this study was to use a modified micropipette aspiration technique coupled with quantitative histology to measure structure-function relationships of regional mouse aortic valve tissue, and to determine how these properties change with aging. Based on previous findings in humans (Roberts, 1970; Otto et al., 1994), the hypothesis was that the aortic valve annulus region would demonstrate significantly higher tissue stiffness compared to the cusp region, and the entire valve would stiffen with age.

In summary, tensile stiffness in the annulus region is significantly greater than the cusp region at all stages, and interestingly, tissue stiffness in both valve regions decreases with advanced age in mice. Histochemical analyses were consistent with the biomechanical findings both spatially and temporally. These results establish the feasibility of micropipette aspiration in mouse valve tissue and provide a basis to evaluate the mechanical and structural properties of targeted mutagenesis mouse models of valve disease. This approach may improve our fundamental understanding of age-related phenomena in valve tissue and ultimately contribute to the development of new therapies for AVD.

2. Materials and methods

Aortic valves were isolated in situ from C57BL/6 wild type mice at three postnatal developmental stages: juvenile (1 month old (mo)), adult (4 mo), and aged adult (12 mo). Ten mice per stage were used for mechanical studies and three mice per stage were used for histochemistry. All protocols were approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Medical Center.

Valve tissue isolation and preservation

The heart was excised after the mice were euthanized. The dissection extended superiorly from the left ventricular apex to the interface between the anteriorly oriented pulmonary artery and posteriorly oriented aorta. Further dissection around the proximal aspect of the circumference of the aortic valve was performed. The tubular aortic valve structure within the aortic root from the ventriculo-arterial junction to the sinotubular junction was removed intact (Figure 1A). The tissue was washed and frozen for less than 72 hours in Dulbecco's Phosphate Buffered Saline (PBS, Hyclone) and supplemented with protease inhibitor (1:100, Sigma) for biomechanical testing. Previous studies demonstrate that short-term freezing does not adversely affect tissue biomechanical properties (Woo et al., 1986). Tissue was processed for histochemical analyses as previously described (Hinton et al., 2006).

Valve tissue preparation for mechanical testing

Glass slides coated with the silicone polymer polydimethylsiloxane (PDMS) were mounted on the microscope chamber, and a notch exposing the right aspect of the chamber was created (Figure 2A). The isolated aortic valve wheel was dissected open to facilitate accessibility (Figure 2B) such that the ventricular surface of the valve cusp and annulus regions were oriented perpendicular to the plane of the micropipette. Tissue hydration was maintained for the duration of testing.

Mechanical testing of mouse valve tissue using micropipette aspiration

Micropipettes with an inner radius of 25–30 μm were used to ensure that the geometric influences on the measured mechanical properties were negligible (Aoki et al., 1997; Ohashi et al., 2005). Upon contact, aspiration pressure (ΔP) was applied using small incremental steps of less than 0.5 kPa to the tissue surface through the micropipette via a custom-built syringe water reservoir, resulting in a small portion of the tissue surface aspirating into the pipette. Seal formation was observed when resistance to aspiration increased sharply and tissue was appreciated within the micropipette, with the corresponding tare pressure levels at about 0.7 kPa. Detailed analysis of the data in the linear region of the aspiration length vs. pressure curves showed no noticeable creep over periods up to 60 seconds, indicating that the viscoelastic effects have dissipated instantaneously and the measured response reflected elastic material properties. The pressure values were measured using an in-line pressure transducer having a resolution of 1 Pa (models DP15-28/40, Validyne Engineering, Northridge CA), as described previously (Alexopoulos et al., 2003; Merryman et al., 2006b). Videos of the tissue aspiration process were recorded to a DVD-R with a CCD camera

through a bright-field microscope (Diaphot 300, Nikon, Melville NY) with a 20X objective and a 10X wide-field eyepiece. Applied pressure and time were displayed on a video monitor using a digital multiplexer (Vista Electronics, Ramona CA). Two videos were analyzed such that each experiment included two measurements from one valve region.

Data analysis

Images were captured at 1 frame/sec and analyzed at 50 frames/video (Figure 3A). From each image, the aspiration length (L) and radius (r) of the micropipette were measured using *Image-Pro AMS* software (Media Cybernetics Inc, Bethesda MD). These measurements were conducted by two blinded, independent users. Data were analyzed using a half space punch model (Theret et al., 1988; Alexopoulos et al., 2003). This model assumes that the tissue is elastic, isotropic, incompressible, half-space material with the Young's modulus (E) of the material given by

$$E = \phi(\eta) [(3r)/(2\pi)] (\Delta P/L) \quad (1)$$

where r is the micropipette inner radius, ΔP (in kPa) is the equilibrium aspiration pressure and L is the corresponding aspiration length. ϕ , the wall function was determined by pipette geometry η (ratio of micropipette inner radius to wall thickness). The relationship between $\phi(\eta)$ and has been established previously (Theret et al., 1988). The above equation can be rearranged as,

$$L/r = [3 \phi(\eta)/(2\pi)E] \Delta P \quad (2)$$

For each sample, a plot of (L/r) vs. aspiration pressure (ΔP) was obtained using least-squares fitting algorithm (Figure 3B). A value of 2.2 was estimated for the wall function ($\phi(\eta)$) based on tissue and pipette dimensional measurements. L/r vs. ΔP profile was obtained in triplicate for all 30 animals corresponding to 3 stages and 2 regions. Young's Modulus (E) was calculated from the slope of the linear region using equation 2. E was then used to approximate the tensile stiffness of valve tissue. Given the small size of the micropipette relative to the large dimension of the tissue, the tissue under contact could be considered essentially flat, and the slight concavity or convexity present was not likely to adversely impact the analysis. Importantly, previous studies suggest that given the geometries used in this study (pipette radius $\ll$ tissue thickness), both the half-space and layered models would likely yield similar stiffness values (Guilak et al., 2005).

Histochemistry

Aortic valve tissue from 1 mo, 4 mo and 12 mo mice was processed in both long (in cross section) and short (en face) axes (n=3 per stage per axis). Histochemical staining using Movat's modified pentachrome stain was performed to evaluate regional ECM composition and organization over time (Hinton et al., 2006; Hinton et al., 2008). Pentachrome stain identified elastic fibers as black, collagen fibers as yellow, proteoglycans as blue, muscle as red, and cell nuclei as purple. Quantitative analysis of collagen and proteoglycan staining intensity was performed on pentachrome stained sections (3 animals per stage, 2 sections per animal). The sections were visualized using a Nikon Eclipse Ti microscope and image analysis was performed on 10X RGB images using *NIS Systems* software (Nikon Instruments Inc, Melville NY). Images were white balanced, and the cusp and annulus regions were marked using the region of interest (ROI) tool. To identify collagen (yellow) or proteoglycan (blue) staining, a range of values for Red, Green and Blue component was

chosen for each color. These values were then used to threshold all images to identify yellow and blue areas for collagen and proteoglycan staining respectively. Collagen and proteoglycan average area fractions were determined for each image as the ratio of yellow or blue area to the total area, for both cusp and annulus regions at all stages. In addition, the collagen to proteoglycan area ratio was reported.

Statistical Analysis

Two-factor ANOVA and post-hoc test with Bonferroni corrections (SPSS Inc, Chicago IL) were used to determine the effects of region and stage on tensile stiffness, collagen and proteoglycan area fractions, and collagen to proteoglycan ratio, with significance levels of $\alpha = 0.05$. All data were reported as mean $\pm$ one standard deviation.

3. Results

Plots of applied aspiration pressure ΔP vs. effective aspiration displacement L/r demonstrate tensile viscoelastic behavior including characteristic toe region (fiber realignment), linear region (uniform elastic deformation) and yielding phase (plasticity to failure) in both annulus and cusp regions. The slope of the linear region was used to evaluate regional tissue tensile stiffness (Figure 3B). For all tested animals, R^2 of greater than 0.8 was observed in the least square fit, showing good agreement between the analytical prediction and experimental aspiration data.

In aortic valve annulus tissue, tensile stiffness values were 596 ± 82 kPa in juvenile mice, 580 ± 41 kPa in adult mice, and 549 ± 59 kPa in aged adult mice. In aortic valve cusp tissue, the tensile stiffness values were 316 ± 43 kPa in juvenile mice, 311 ± 38 kPa in adult mice, and 271 ± 27 kPa in aged adult mice (Figure 4). Tensile stiffness in the annulus region was significantly greater than the stiffness of the cusp region at all stages ($p < 0.0001$). Interestingly, there was a slight but statistically significant decrease in tissue stiffness in both the annulus and cusp regions in aged adult mice when compared with juvenile mice ($p < 0.05$), but not between other stages, consistent with modest age-related degenerative changes.

Histochemical analyses were performed to assess regional ECM organization (Figure 5) and composition (Figure 6). At all stages, valve tissue in the annulus region consisted primarily of compact collagens, while tissue in the cusp region consisted predominantly of proteoglycans (Figure 5). Quantitative analysis confirmed the observed regional difference in collagen and proteoglycan area fractions ($p < 0.001$, Figure 6B). The collagen to proteoglycan area ratio was much higher in annulus region compared to the cusp region (Figure 6C). In the aged adult, this pattern persisted, however, there was noticeable proteoglycan infiltration of the valve hinge in the annulus region (Figure 5, Inset). Quantitative analysis showed decreased collagen area fraction and increased proteoglycan area fraction ($p < 0.01$, Figure 6B), and a decreased collagen to proteoglycan area ratio in both regions at the aged adult stage (Figure 6C). Taken together, these histopathologic findings are consistent with the biomechanical results and suggest a potential region-specific mechanism for age-related valve degeneration.

4. Discussion

In this study, we report a novel approach for determining the mechanical properties of mouse aortic valve tissue using a modified micropipette aspiration technique. Importantly, our findings demonstrate the feasibility of this approach in mouse valve tissue. Significant differences were observed in biomechanical properties between annulus and cusp regions of mouse aortic valves. The tensile stiffness was found to be significantly greater in the annulus

region (~550–600 kPa) compared to the cusp region (~270–320 kPa) at all stages of postnatal valve development, consistent with previously reported values of canine and porcine valve and aortic sinus stiffness (Thubrikar et al., 1980; Merryman et al., 2006a; Liao et al., 2008). The higher stiffness values observed in the valve annulus are likely due to the regional abundance of collagen, which is associated with high material stiffness, valve durability and strength (Sacks et al., 1997; Driessen et al., 2003; Balguid et al., 2008), and relative paucity of elastic fibers and proteoglycans (Vesely, 1998). Importantly, the regional differences in valve tissue stiffness observed in our study are in agreement with previously reported modeling-based stress-strain studies that showed differences in stress sharing between valve and aortic root tissue (Grande et al., 1998). Overall, the findings of the current study demonstrate regional variation in valve tensile stiffness, consistent with regional differences in ECM composition and organization.

Age-related changes in ECM organization, and alterations in matrix remodeling in human valve tissue have been reported previously (Otto et al., 1994; Rabkin et al., 2001; Fedak et al., 2003; Hinton et al., 2006). Recent studies examining porcine valves demonstrate specific changes in collagen and proteoglycan composition with advanced age, suggesting that age-associated ECM alterations can play a role in valve degeneration (Stephens et al., 2007; Stephens et al., 2009). Interestingly, proteoglycan content in valves is known to change with age or disease states (Barber et al., 2001; Grande-Allen et al., 2003; Stephens et al., 2008). Our results indicate that collagen decreases while proteoglycans increase with aging in both cusp and annulus regions of mouse valves. Importantly, regional valve tensile stiffness also decreases at this stage suggesting that latent changes observed in valve biomechanical properties are due in part to matrix compositional changes.

Fibrogenesis abnormalities in valve tissue can manifest as fibrosis (too much collagen) or myxomatous change (too much proteoglycan). Based on our findings, we conclude that mice undergo age-associated degeneration that primarily affects the annulus, similar to degeneration in human valves, but involves proteoglycan accumulation instead of collagen accumulation (Roberts, 1970; Otto et al., 1994). We reconcile this important difference by considering the dominant nature of the collagen-rich fibrosa layer in humans and the relatively proteoglycan-rich and collagen-poor mouse aortic valve. While fibrotic disease states may manifest as different changes in ECM composition (Wynn, 2007), this distinguishing feature between aged human and mouse valves has significant biomechanical implications and warrants further study. Taken together, these findings suggest that impaired ECM maintenance and remodeling with aging, i.e., structural degeneration, results in compromised mechanical valve function. Importantly, studies in targeted mutagenesis mouse models of valve disease will help elucidate the mechanisms underlying maladaptive matrix remodeling and fibrogenesis abnormalities, and improve our understanding of the different manifestations of age-associated changes and valve disease.

One limitation of the current analysis technique is that tensile stiffness determination was based on the assumption that small deformations in the tissue are treated as a half-space layer with infinite lateral dimensions (Theret et al., 1988). Valve tissue in humans and large animals has a complex layered structure and exhibits non-linear stress-strain relationship and regional anisotropy (Lo et al., 1995; Stella et al., 2007; Sacks et al., 2009a). Micropipette aspiration can be extended to include the multi-layered valve structure (Alexopoulos et al., 2003), and will be the subject of future studies. The model used in the present study also requires that all tissue samples be at least 4 times the radius of micropipette, for the effects of geometry to be negligible (Aoki et al., 1997; Ohashi et al., 2005). In these experiments, the validity of this assumption was confirmed by visual microscopic observation.

In summary, these findings demonstrate that micropipette aspiration in combination with quantitative histology can be used to study region-specific age-related degeneration in mouse valve tissue. Importantly, this method provides opportunities for valve biomechanical testing in targeted mutagenesis mouse models of valve disease, which in turn enables a multidisciplinary approach to the study of AVD pathogenesis and natural history. Ultimately, combining engineering and molecular methodologies will elucidate valve structure and function and potentially inform new therapeutic strategies and bioprosthesis development.

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Figure 1.

Anatomy of the mature aortic valve. The aortic valve is made up of three cusps (blue), hinged (red lines) to the supporting annulus (yellow). The aortic root extends from the ventriculo-arterial junction (VAJ, bottom blue ring) proximally to the sinotubular junction (STJ, top blue ring) distally and is composed of fibrous valve annulus tissue and the arterial sinuses of Valsalva (A). When the aortic root is transected, the valve cusp and annulus regions are revealed (B).

Figure 2.

Valve tissue orientation for micropipette aspiration. The microscope stage holds a PDMS-coated glass slide that is designed to accommodate the lateral introduction of a micropipette (A). Valve tissue is secured in the slide with micropins. The micropipette is attached to the pipette holder and accesses the tissue surface in an orthogonal plane; upon contact, a suction pressure is applied (B).

Figure 3.

Regional valve tissue aspiration. The aspirated valve tissue meniscus is clearly seen in both cusp (A, top panel) and annulus (B, bottom panel) regions. Instantaneous aspiration pressure (ΔP), aspiration length (L), and radius (r) are indicated. The dark spots in valve cusp tissue represent melanocytes (A, top panel). The tissue was equilibrated in PBS throughout the entire testing period. A representative plot of L/r vs. ΔP for annulus vs. cusp is shown (B). Higher slope corresponded to higher tensile stiffness of the annulus samples, as compared with cusp, as determined using the half space model equation. Scale bar 50 μm .

Tissue tensile stiffness

Figure 4.

Regional and temporal variation in mouse valve tensile stiffness. Tensile stiffness values in the annulus region were significantly higher than the cusp region at all stages ($n=10$ in each age group, *: $p<0.0001$). The overall decrease in valve tissue (both cusp and annulus) tensile stiffness observed in aged adult valves as compared with juvenile group suggests aging-related degeneration (#: $p<0.001$: 1 mo cusp vs. 12 mo cusp and $p<0.05$: 1 mo annulus vs. 12 mo annulus).

Figure 5.

Qualitative regional histopathology in mouse aortic valve tissue with aging. Histochemistry of aortic valve tissue in long (A, B, C) and short (D, E, F) axes at 1 mo (A, D), 4 mo (B, E) and 12 mo (C, F). Pentachrome staining indicated the presence of proteoglycans in the cusp region (blue staining – arrows) and collagen in the annulus region (yellow staining – arrowheads). In the aged adult, proteoglycan infiltration of the annulus region at the hinge is observed (asterisk, Inset). AoV, aortic valve; LV, left ventricle. Scale bars: A through E, 120 μ m; Inset, 20 μ m.

Figure 6.

Regional and temporal histochemistry quantification in mouse aortic valve tissue. Image analysis of pentachrome-stained valve sections (A, left panel) identified the distribution of collagen (yellow pixels, A middle panel) and proteoglycans (blue pixels, A right panel). Quantitative regional differences in collagen and proteoglycan composition (B; *: $p < 0.001$, annulus vs. cusp) were confirmed at all stages. With advanced age, collagen content decreased and proteoglycan content increased in mice valves in both regions (B). Proteoglycan infiltration in the annulus region is demonstrated by decreased collagen area fraction and increased proteoglycan area fraction (B; #: $p < 0.01$, 1 mo and 4 mo vs. 12 mo) together with decreased collagen to proteoglycan ratio (C). Age-dependent increase in proteoglycan content is consistent with the observed decrease in tissue stiffness.