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Cellular and Extracellular Matrix Basis for Heterogeneity in Mitral Annular Contraction

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Abstract

Purpose—Regional heterogeneity in mitral annular contraction, which is generally ascribed to the fibrous vs. muscular annular composition, ensures proper leaflet motion and timing of coaptation. It is unknown whether the fibroblast-like cells in the annulus modulate this heterogeneity, even though valvular interstitial cells (VICs) can be mechanically “activated.”

Methods—Fourteen sheep underwent implantation of radiopaque markers around the mitral annulus defining four segments: septal (SEPT), lateral (LAT), and anterior (ANT-C) and posterior (POST-C) commissures. Segmental annular contraction was calculated using biplane videofluoroscopy. Immunohistochemistry of annular cross sections assessed regional matrix content, matrix turnover, and cell phenotype. Micropipette aspiration measured the Young's modulus of the leaflets adjacent to the myocardial border.

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Results—Whereas SEPT contained more collagen I and III, LAT demonstrated more collagen and elastin turnover as shown by greater decorin, lysyl oxidase, and matrix metalloprotease (MMP)-13 and smooth muscle alpha-actin (SMaA). This greater matrix turnover paralleled greater annular contraction in LAT vs. SEPT (22.5% vs. 4.1%). Similarly, POST-C had more SMaA and MMP13 than ANT-C, consistent with greater annular contraction in POST-C (18.8% vs. 11.1%). Interestingly, POST-C had the greatest effective modulus, significantly higher than LAT.

Conclusions—These data suggest that matrix turnover by activated VICs relates to annular motion heterogeneity, maintains steady-state mechanical properties in the annulus, and could be a therapeutic target when annular motion is impaired. Conversely, alterations in this heterogeneous annular contraction, whether through disease or secondary to ring annuloplasty, could disrupt this normal pattern of cell-mediated matrix remodeling and further adversely impact mitral valve function.

Keywords

mitral annulus; extracellular matrix; matrix metalloproteases; collagen; annular contraction

Introduction

The mitral valve (MV) annulus demonstrates heterogeneity in both structure and motion. Structurally, the MV annulus is an asymmetric, hyperbolic paraboloid or saddle-shaped oval ring [1]. Annular deformation during the cardiac cycle is complex and diverse, involving tilting around multiple axes [2-3], translation [2], and contraction [4-6], each occurring to varying degrees throughout the different regions of the annulus. In general, lateral annular contraction is substantially greater than that of the septal annulus during the cardiac cycle [4-6]. Given that elimination of this dynamic movement of the MV annulus *in vivo* has been shown to result in depressed cardiac function [7-9], this characteristic is clearly necessary for proper valve function. This heterogeneity has been primarily attributed to the fibrous vs. muscular composition of the septal and lateral annulus, respectively [4-6,10], but the specific nature of this compositional diversity, particularly the valve leaflet insertion, have not been previously investigated.

In addition, cells from a wide range of tissues [11], including valves [12-13], both respond to and influence their mechanical environment via phenotypic modulation and synthesis of extracellular matrix. The relationship between the highly dynamic *in vivo* mechanics and composition of the valve annulus, however, remains unknown. Establishing such a link between annular motion and composition would have important implications regarding the ultrastructural mechanisms of annular dilation in valve diseases, the potential for preventative treatments, and the design of annular repair strategies and annuloplasty rings.

Therefore, the aim of this study was to correlate the heterogeneity of normal MV annular contraction with regional measurements of matrix turnover and valve cell activation using immunohistochemistry (IHC). IHC was performed for a number of proteins related to cell activation as well as the abundance, synthesis, and degradation of extracellular matrix, and micropipette aspiration was performed to establish a link between extracellular matrix

characteristics and mechanical properties of the mitral leaflets at the border of their insertion into the annulus.

Methods

All animals received humane care in accordance with the guidelines of the US Department of Health and Human Services (NIH Publ. 85-23, Revised 1985). The use of animals in this study was approved by the Stanford Medical Center Laboratory Research Animal Review Committee.

Animal Protocol

Eight (8) radiopaque markers were implanted to delineate four annular segments (Fig. 1): septal (SEPT, the distance from marker 2 to 4) and lateral (LAT, marker 6-8) annulus, and anterior (ANT-C, marker 4-6) and posterior (POST-C, marker 2-8) commissures. Procedures for marker implantation and biplane video fluoroscopy have been described previously [14]. Annular segment contraction was calculated as the percentage change from maximum to minimum segment lengths both at baseline and during calcium infusion [5] throughout the cardiac cycle. The hearts were then harvested and stored in formalin.

Histology and Immunohistochemistry

For each segment of the MV annulus, a 3-5 mm wide strip was cut from the myocardium to the leaflet free edge (Fig. 1). Cross sections were embedded in paraffin and sectioned to a thickness of five microns. Each sample was stained with Movat pentachrome as well as with standard immunohistochemistry procedures to demonstrate specific extracellular matrix components, enzymes involved in matrix turnover, and cell phenotype (Table 1). The staining intensity in each layer (fibrosa, atrialis, and muscle, Fig. 2) of the MV annulus of blinded samples was quantified using ImageJ software (NIH, Bethesda, MD).

Micropipette Aspiration

Sheep hearts for micropipette aspiration were obtained from a local abattoir. A total of 12 mitral valves were obtained. A 5 mm square sample was obtained from each segment of the annulus, corresponding to marker positions 3 (SEPT), 7 (LAT), 5 (ANT-C) and 1 (POST-C). Fresh tissues were then tested by micropipette aspiration as previously described [15] on the atrial side of the leaflet adjacent to the myocardium at the annular border. Experimental images captured during the testing were analyzed using ImageJ software (NIH) to measure the aspiration length and pipette diameter, and pressure data was acquired from a pressure transducer via a National Instruments DAQ system. Data analysis was performed using a finite-strain hyperelastic exponential constitutive model as previously described, and the effective modulus (M) describing the local stiffness of the tissue was derived from the product of the two material constants in the model [16].

Statistical Analysis

Data are presented as mean and standard deviation, unless otherwise noted. Multifactorial analysis of variance (ANOVA) was performed using SigmaStat (SPSS, Chicago, IL) with the level of significance set at 0.05. Data that was not normally distributed was rank

transformed before analysis. Paired t-tests were used to compare annular contraction with and without Ca^{++} . Correlations between staining intensities of different proteins within individual annular layers and segments were used to assess protein co-localization related to matrix turnover and cell phenotype. Correlations between protein intensities and annular contraction were analyzed to investigate the mechanobiology of compositional differences. These correlations were calculated using a Pearson rank order test for normally distributed data and Spearman rank order test for non-normally distributed data. For correlations between intensities of different proteins $p = 0.02$ was considered a trend and $p = 0.00625$ considered significant (since 8 proteins were being compared); for correlations between intensities of proteins and annular contraction each protein was considered individually, therefore $p = 0.05$ was considered significant, but only the most significant of these correlations with $p < 0.03$ are listed.

Results

Heterogeneity of Annular Segment Contraction

Without calcium infusion, annular contraction (percent reduction from max to min segment length) was greatest in LAT, followed by POST-C, ANT-C, and lastly SEPT (Table 2, $p < 0.001$). With calcium infusion, annular contraction was greatest in LAT and POST-C, followed by ANT-C, and lastly SEPT (Table 2, $p < 0.001$). The contraction of each annular segment except SEPT increased with calcium infusion compared to baseline (each $p < 0.036$).

Differences in Leaflet Insertion Structure

Histological analysis showed that the fibrosa in the SEPT leaflet continued as a deep insertion into the musculature as a substantial, collagen-rich band, whereas in LAT the fibrosa remained more superficial to the annular musculature. Additionally, in LAT the leaflet appeared to be a continuation of the muscular tissue (Fig. 3), as opposed to an independent structure into which the muscle inserted. In contrast, in both ANT-C and POST-C the leaflet was more structurally distinct from the adjacent musculature, with moderate insertion of the fibrosa into the musculature but not to the same degree as in the SEPT region of the annulus. Regions of the annulus where the fibrosa inserted into the musculature to a substantial degree were the same regions that showed relatively less contraction (Table 2).

Compositional Heterogeneity Between Septal and Lateral Segments and Between Commissures

Compared to the LAT annulus, the SEPT annulus contained more Col I and III both across the fibrosa, atrialis, and muscle layers (Fig. 4A, each $p < 0.001$) and in the muscle layer individually (each $p < 0.001$). In contrast, LAT showed greater expression of proteins related to collagen and elastic fiber turnover including the collagen-degrading enzyme MMP13 (Fig. 4B, $p < 0.001$); the proteoglycan DCN, which is involved in collagen fibrillogenesis ($p = 0.033$); LOX, an enzyme involved in both elastin and collagen crosslinking ($p = 0.006$); and elastin (Fig. 5, $p < 0.005$). LAT also demonstrated greater expression of SMaA ($p < 0.001$). These results of greater collagen and elastic fiber turnover in LAT compared to SEPT parallel the contractile patterns of these regions.

Compared to ANT-C, the POST-C annulus contained significantly more Col III ($p<0.015$), as well as MMP13 ($p<0.02$) and SMaA ($p=0.006$), which may be consistent with the greater annular contraction in the POST-C annular segment.

Compositional Heterogeneity Among Septal, Lateral, and Commissural Segments

In the comparison of all different annular segments, POST-C showed the greatest staining for Col I and III of all the segments (both overall $p<0.001$). Col I and III in ANT-C were both greater than LAT (both $p<0.001$). Both commissures also had greater expression of MMP13 ($p<0.001$), LOX ($p=0.005$), and elastin ($p<0.001$), compared with the SEPT and LAT segments. Consistent with the greater expression of matrix turnover proteins, ANT-C and POST-C showed the greatest expression of SMaA ($p<0.001$).

When considering all of the annular segments as a data set, several strong correlations were noted between the magnitudes of annular contraction and expression of markers of either cell activation or matrix turnover. In the muscle and fibrosa layers, SMaA expression was strongly correlated with annular contraction, both with and without calcium infusion (muscle: 0.687-0.718, each $p<0.00001$; fibrosa: 0.587-0.611, each $p<0.00035$). MMP13 expression was similarly strongly correlated with annular contraction (muscle: 0.622-0.626, each $p<0.0012$; fibrosa: 0.675-0.732, each $p<0.0002$). The correlation between MMP13 and annular contraction at baseline was particularly strong in the LAT fibrosa and muscle layers ($r=0.780-0.823$, $p<0.023$).

Correlation Between Expressions of Different Proteins

The presence of the myofibroblast cell phenotype was suggested by several correlations between SMaA (the marker for cell activation) and collagen or MMPs. Across all 4 annular segments, there were correlations between MMP13 and SMaA (0.687-0.88 in various layers, $p<0.00001$ for each layer). Further, a correlation was found between MMP9 and elastin (0.590, $p=0.0025$) suggestive of elastic fiber turnover.

Mechanical Properties of MV Regions

Micropipette aspiration revealed a significant difference in the effective modulus of the atrialis side of the posterior commissure (adjacent to the annular border, $M = 4.14$ kPa) as compared to the lateral annular region ($M = 0.71$ kPa). However there were no other differences observed between annular regions (anterior commissure, $M = 1.72$; septal annulus, $M = 1.65$).

Discussion

This study provides evidence documenting a novel relationship between the *in vivo* contraction of various segments of the mitral annulus and the matrix turnover, layered structure, and valvular cell phenotype within that segment. Contraction was the lowest in magnitude in areas showing the greatest amount of fibrous insertion into the muscle, but was directly related to matrix turnover and cell activation. The LAT portion of the annulus demonstrated greater collagen and elastic fiber turnover, as well as cell activation, compared to the SEPT annulus, paralleling greater contraction in LAT compared to SEPT. Similarly,

collagen turnover and cell activation was high in the POST-C annulus, paralleling greater annular contraction in POST-C compared to ANT-C. Overall, the commissural annular segments had the highest collagen content as well as most pronounced collagen and elastic fiber turnover. Throughout all of the segments, there were strong, localized correlations between the expression of myofibroblast phenotypic marker (SMaA) and proteins involved in matrix synthesis and turnover, which suggests the involvement of myofibroblasts in annular compositional heterogeneity.

Functional Implication of Heterogeneous Valve Insertion Structure

Differences in the insertion of the heavily collagenous fibrosa within the annular musculature reflect the contractile behavior of the different segments of the mitral annulus. Although this contractile nature of the mitral annulus has been widely investigated [4-6], the microstructural aspects of the mitral leaflets' anchorage within the annulus, and how these vary across annular segments, have been largely overlooked. Across the different segments of the MV annulus, the depth of the fibrosa insertion was generally inversely related to the magnitude of contraction. Regarding the deep insertion of the SEPT leaflet, the substantial bands of collagen demonstrated here are likely related to its continuity with the left ventricular outflow tract (LVOT) and would greatly reduce contraction of the SEPT annular segment relative to remainder of the annulus, as observed in this and other studies [4-5,17-18]. The circumferential anchoring of the SEPT segment of the annulus by the fibrous trigones (between the mitral and aortic valve) would also be a significant factor in the reduction of the contraction relative to the rest of the annulus, which is surrounded by dynamic, flexible muscle tissue. In addition to differences in collagen insertion depth, the segments also showed differences in the overall structure of the proximal leaflet insertion into the annulus, which would likely affect the flexibility of this leaflet region. The thick annular insertion base anchoring the SEPT and commissural leaflets would reduce flexibility compared to the thin, narrow base anchoring the LAT leaflet. Indeed, compared to LAT, the SEPT leaflet displays greater excursion [3] and closure rate [6].

Annular Compositional Heterogeneity and Turnover in Relation to Contraction

The overall abundance of collagen within the various annular segments was consistent with the degree of insertion observed for each segment. For example, the greater collagen content in SEPT vs. LAT reflected the collagen-rich fibrosa inserting into the muscle of SEPT. However, collagen expression was not limited to the fibrosa layer: collagen was also more strongly stained within the muscle layer of SEPT than in the LAT muscle layer. Thus, the frequently described “fibrous” nature of the SEPT annulus is characterized by collagen that is highly organized within the fibrosa layer as well as diffusely abundant throughout the muscular layer.

Within the primary leaflet annular comparison as well as the commissural leaflet annular comparisons, the relative magnitudes of annular contraction paralleled those of expression of markers for matrix turnover and cell activation. This work agrees with a previous *in vivo* study showing that the lateral annulus contracts more than the septal region of the annulus, and we have now correlated this observed behavior with the extracellular matrix structure and cell behavior in these regions [19]. It should be noted that contraction in the

commissures was reported as being limited in the previous study, whereas in this study commissural contraction was not significantly different from contraction of the lateral annulus. This difference between reports is likely due to the definition of the commissural annulus, which, as defined in this and other studies [20,21] encompasses a region from the fibrous trigones to the more muscular lateral annulus, resulting in a transitional region averaging the behavior of both fibrous and muscular regions. In the previous study, the commissures were defined as a small area adjacent to the fibrous trigones, and thus commissural contraction in that work appeared to reflect the stiff, fibrous nature of the tissue region. Further, there were strong correlations between the myofibroblast phenotype and matrix turnover markers within individual annular segments. These results suggest a relationship between annular contraction and matrix turnover/cell activation. It is likely that the highly dynamic mechanical environment in the regions of greatest contraction would necessitate greater matrix turnover to maintain the steady-state matrix composition and structure, leading to increased activation of cells in these regions. Indeed, it was within the muscle and fibrosa layers that the correlations between annular contraction and proteins of matrix turnover and cell activation were predominantly found, which is consistent with the structural and functional roles of these layers.

Interestingly, when considering all of the annular segments, the commissures demonstrated not only the greatest collagen content but also the greatest collagen turnover and greatest level of cell activation, even though the commissures as a group had annular contraction that was not significantly different from LAT. Reasons for this inconsistency might include the density of collagen within the fibrosa layer (which was very high in the commissures but less so in the LAT) or the influence of other mechanical stimuli, in addition to contraction, that could affect the annulus in a heterogeneous manner. The expected contraction-turnover relationship, however, was demonstrated in the comparison of POST-C and ANT-C.

Micropipette aspiration enabled the testing of highly localized regions of the annulus, which would not be possible with traditional mechanical testing. As a result, some regional heterogeneity was observed in the mechanical properties of the annulus. The increased collagen content in the septal and commissural areas of the annulus, discussed previously, would suggest that these regions would also be more stiff than the lateral annulus. While only the posterior commissure was found to have higher stiffness than the lateral annulus, these results still demonstrate regional heterogeneity that is likely linked to changes in the regional matrix composition. One limitation to this study was the restriction of mechanical micropipette aspiration testing to the atrial side of the annulus due to practical limitations of finding the border between muscle and annular tissues. It is likely that the ventricular side of the annulus may have more pronounced differences in stiffness, due to the differences in insertion structure and collagen content of the fibrosa side of the valve.

Potential Functional Contributions of Myofibroblasts

Given the known roles of myofibroblasts in matrix remodeling [22,23], the fibrosa layer correlations between the myofibroblast phenotype and markers for matrix degradation imply that myofibroblasts contribute to the annular compositional heterogeneity. Moreover, myofibroblasts are known to respond to their mechanical environment [24]. Therefore, we

speculate that these cells provide an important link between annular cell/matrix composition and function by sensing annular contraction and responding by synthesizing and remodeling extracellular matrix to maintain the native mechanics and function of the tissue. As such, the myofibroblast may be a therapeutic target for diseases in which annular composition and contraction is altered.

Implications for Mitral Disease and Treatment

The link between annular contraction and composition shown in this study implies that altered annular contraction, such as occurs with annuloplasty ring placement [25,26], as well as in disease processes such as ischemic and functional mitral regurgitation [14,27], may lead to alterations in valve cell phenotype and mitral annular composition, which could lead to further deterioration in MV function. Therefore, this study provides further motivation for the ongoing refinement of annuloplasty ring designs to maintain physiologic, heterogeneous annular contraction. Previous studies in ovine models of mitral disease have showed changes in annular contraction [20,21], and correlating these results with potential changes in cellular and extracellular matrix remodeling would provide additional direction in the design of annuloplasty rings. A previous study in human patients has also showed significant changes in annular dynamics between normal, ischemic, and myxomatous mitral valves [28], thus using an ovine model to link such changes in each of these conditions to matrix and cell behavior changes would be valuable. In addition, there was great heterogeneity of leaflet-musculature insertions between the different mitral valve leaflets. Since these insertions are essential mechanical linkages connecting the leaflets to the mitral annular/left ventricular complex, these attachments will need to be carefully replicated in the design of a tissue-engineered mitral valve to ensure proper function. In the future, it will be important to understand and account for the numerous forces acting on the mitral annulus and how those forces interplay with annular composition to provide a more complete picture of the complex structure-function relationship in the mitral annulus.

Study Limitations

As noted above, one study limitation is due to the challenge of performing micropipette aspiration on the ventricular surface of the annulus. An additional limitation is the inherent variability of immunohistochemistry, which was quantified to be 13.7% within batches and 16.5% between batches. In order to limit this variability, duplicate sections were cut from the SEPT and LAT annular segments, and the staining results were averaged. Furthermore, all samples from a given annular segment were stained for a given protein in one batch. A second source of variability is the differences in valve morphology between individual animals, similar to the variability noted in humans [29,30]. Despite these sources of variability, we have found statistically significant results. Additionally, there are some anatomic differences between the annuli of ovine and humans [31], which should influence how these results are applied to the study of human mitral valve disease.

In conclusion, this study reveals heterogeneity in MV annular composition in terms of individual matrix components, matrix turnover, presence of myofibroblasts, and structure of leaflet insertion. Furthermore, this study provides the first evidence for a connection between the heterogeneity in mitral valve annular matrix composition and heterogeneity of

annular contraction. Such a link could prove instrumental both in clinical strategies to treat valves with altered matrix composition as well as in strategies for designing a heterogeneous tissue engineered heart valve.

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Fig. 1.

Location of the different annular segments from which tissue sections were taken. In the case of septal segment (SEPT) and lateral segment (LAT) 2 tissue sections were analyzed, in the case of the anterior and posterior commissures (ANT-C, POST-C), one tissue section was taken from either side of the tantalum marker based on quality of the leaflet on each side. For calculation of annular contraction, the distance between marker 2 and 4 was defined as SEPT, marker 6-8 as LAT, marker 4-6 as ANT-C, and marker 2-8 as POST-C.

Fig. 2.

Right image shows location of layer of annulus analyzed in tissue section relative to leaflet below and annular fluoroscopy marker seen in left photograph of gross specimen. Arrows point to layers of the MV annulus (atrialis, fibrosa, and muscle). Scale bar represents 2 mm.

Fig. 3. Examples of differences in insertion structure in different MV annular segments. In the left panel is an image representing the typical insertion of the LAT leaflet with the large base, middle panel represents the commissural segments, and right panel represents the SEPT leaflet. Scale bars represent 2 mm.

Fig. 4.

Relative amounts of Col I and III across the different annular segments (A). Overall Col I and Col III each $p < 0.001$. * $p < 0.001$ vs. LAT for a given protein (Col I or Col III). $\wedge p < 0.01$ vs. SEPT and $\dagger p < 0.015$ vs. ANT-C for Col III. Relative amounts of MMP13 and SMaA across the different annular segments (B). Overall MMP13 and SMaA each $p < 0.001$. All comparisons between annular segments were significant for MMP13 ($\dagger p < 0.05$). * $p < 0.001$ vs. SEPT for SMaA. $\wedge p < 0.006$ vs. POST-C for SMaA. For all graphs, error bars indicate standard error of the mean.

Fig. 5.

Relative amounts of elastin across the different annular segments. Overall $p < 0.001$.

* $p < 0.005$ vs. SEPT.

Table 1
Panel of antibodies used in immunohistochemistry

Protein	Function
<u>Collagens and Collagen-Turnover Proteins</u>	
Collagen I (Col I) ^a	Predominant collagen in valve, provides tensile strength
Collagen III (Col III) ^a	Reticular collagen
Matrix Metalloproteinase (MMP)-13 ^b	Collagen degradation
<u>Elastic Fiber-Related Proteins</u>	
Matrix Metalloproteinase (MMP)-9 ^c	Elastin degradation
Lysyl oxidase (LOX) ^d	Involved in collagen and elastin crosslinking
Elastin ^e	Predominant component of elastic fibers
<u>Proteoglycans (PG)</u>	
Decorin (DCN) ^a	PG involved in collagen fibrillogenesis
<u>Valve Cell Activation</u>	
Smooth muscle alpha-actin (SM α A) ^f	Indicates an “activated” myofibroblast-like phenotype

^a gift of Dr. Larry Fisher, NIH (Bethesda, MD);

^b Chemicon (Temecula, CA);

^c Assay Designs (Ann Arbor, MI);

^d Imgenex (San Diego, CA);

^e Abcam (Cambridge, MA);

^f Dakocytomation (Denmark)

Table 2
Percent Contraction of Annular Segments at Baseline and During Calcium Infusion

	Baseline	With Calcium
SEPT	3.9±2.6	4.7±2.7
LAT	18.1±4.4 ^{†‡}	20.9±4.4 ^{*†‡}
ANT-C	9.9±3.5 [†]	12.3±3.5 ^{*†}
POST-C	15.4±5.7 ^{†‡}	20.3±6.0 ^{*‡}

*
p<0.05 v. Baseline;

[†]
p<0.05 v. SEPT;

[‡]
p<0.05 v. ANT-C

Data presented as mean ± standard deviation.

Contraction was calculated as %change from maximum to minimum segment lengths.