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Relationship between Collagen Fibrils, Glycosaminoglycans, and Stress Relaxation in Mitral Valve Chordae Tendineae

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Abstract—The tensile properties of mitral valve chordae tendineae derive from their structural make-up. The objectives of this study were to compare the stress relaxation properties of different types of chordae and relate their variation to structural features. Fifty chordae from eight hearts were subjected to stress relaxation tests. The percent stress relaxation and the relaxation rates were found to increase in the order of marginal, basal, and strut chordae. The water content of the three types of chordae was the same (marginal 77.1 ± 5.9%, basal 77.0 ± 3.4%, strut 78.0 ± 2.3% wet weight). The collagen, elastin, and glycosaminoglycan (GAG) content in chordae were quantified using hydroxyproline assay, fastin elastin assay, and fluorophore-assisted carbohydrate electrophoresis, respectively. Collagen content of marginal chordae was only slightly less than that of basal and strut chordae (marginal 56.6 ± 8.2%, basal 61.4 ± 5.6%, strut 63.8 ± 3.9% dry weight). There was also no significant difference in elastin content between the chordae (marginal 5.3 ± 3.2%, basal 5.4 ± 2.7%, strut 4.6 ± 1.7% dry weight). However, the concentrations of unsulfated chondroitin/dermatan sulfate, 6-sulfated chondroitin sulfate, and 4-sulfate chondroitin sulfate significantly decreased in the order of marginal, basal, and strut. The total GAG-content also decreased in the order of marginal, basal, and strut ($p = 0.06$). The greater amount of GAGs in marginal versus strut chordae is consistent with our previous observations that marginal chordae have a greater collagen fibril density and thus more GAG-mediated, fibril-to-fibril linkages. The greater number of proteoglycan linkages may prevent the slippage of fibrils with respect to each other, and thus reduce stress relaxation. The different viscoelastic properties of mitral valve chordae can thus be explained morphologically.

Keywords—Fibrillar collagen, Viscoelastic properties, Proteoglycan, Connective tissue.

INTRODUCTION

The mitral valve helps maintain the unidirectional flow of blood through the heart by opening and closing in response to ventricular contraction.[31] The mitral valve is composed of four elements: annulus, leaflet, papillary muscles, and chordae tendineae.[18] Chordae tendineae prevent prolapse of the closed valve into the left atrium and are thus essential for proper valve function and ventricular geometry.[26] When the chordae tendineae become diseased and rupture, the resulting valvular prolapse and regurgitation need to be surgically corrected.[27] The growing interest in mitral valve repair requires a better understanding of chordal mechanics.[6,7,24,38]

Chordae tendineae are tendinous tissues, consisting of collagen fibrils, proteoglycans, elastin, fibroblasts, and water.[23,37] The function of the chordae is thus similar to that of tendons and ligaments, except that chordae undergo continuous repetitive loading, with a relatively high frequency (70 cycle/min).

Collagen fibrils are the main building blocks of chordae tendineae.[2] The diameter of collagen fibrils in chordae tendineae is in the range of 40–70 nm,[21] falling into the lower range of the typical 20–180 nm fibril diameter of tendinous tissue.[36] Chordal collagen fibrils, interacting with the glycosaminoglycans (GAGs) of the ground substance, assemble into parallel fiber bundles with a sinusoidal crimp pattern, having a crimp period in the range of 10–20 μm.[21] The crimp period of chordae is much smaller than that usually reported for tendinous tissue, e.g., human achilles (20–50 μm) and rat tail tendon (100 μm).[9] The crimp angle of chordae is in the range of 20–40°,[21] much greater than that found in human achilles (6–9°) and rat tail tendon (12°).[9]

Although mitral valve chordae have size-related and type-related variations of extensibility and tensile modulus,[16,22] the intrinsic structural reasons for these variations have been elucidated only recently.[21] We have determined that the thicker strut chordae are more extensible because they have more highly crimped collagen, and are less stiff because their collagen fibrils have fewer proteoglycan interactions.[21] It is likely that these different interactions between fibrils in chordae would affect more than just their static elastic properties. We hypothesized that structural differences in the different types of chordae would manifest as different viscoelastic properties. This study was therefore designed to measure the viscoelastic properties of the

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different types of chordae and relate any variation in viscoelasticity to their intrinsic structural features.

MATERIALS AND METHODS

Mitral valve chordae were subjected to uniaxial stress relaxation tests, and variations were related to their collagen, elastin, and GAG content. Stress relaxation test and biochemical analysis were carried out on a different batch of chordae tendineae. Hydroxyproline assay, Fastin elastin assay, and Fluorophore-assisted carbohydrate electrophoresis (FACE) were used to quantify chordal collagen, elastin, and GAG content, respectively. All chordae were harvested from freshly slaughtered pigs (Hampshire/Yorkshire, average 6-month old, majority were male). Chordae from a total of 32 hearts were used in this project.

Relaxation Testing

Mitral valve chordae tendineae ($n = 50$) were harvested from eight hearts and stored in physiological saline (Hanks' solution with 0.002% germicide-algicide) before testing with an Instron servohydraulic machine (Model 8511, Canton, MA, USA). During testing, chordae were immersed in a bath of Hanks' solution at 37°C to mimic the physiological environment. Chordae were gripped in #280 emery cloth-lined Delrin clamps and loaded to 150 g at a rate of 4 mm/s. The application of 150 g elongated the chordae into the lower part of the linear region of the load–displacement curve. The decay of the induced load was recorded at 200 Hz for 100 s. We used engineering stress in this study, the force being normalized to chordal cross-sectional area measured at zero load. Since the selected chordae were very nearly round and longitudinally uniform, we measured their diameter with a calibrated optical microscope in the load-free condition prior to testing, and then calculated their cross-sectional area from the formula of $A = \pi \cdot r^2$.

To validate the relaxation tests, nine chordae were marked with hematoxylin dye dots and imaged with a fast imaging system (Dalsa CCD image capture, Canada) during the relaxation tests. During the relaxation process, the distances between dots in the tissue and the two grips did not change, indicating that there was no slippage from the grips.

In the analysis of stress relaxation, stress was normalized to the initial stress. The percent stress relaxation and rate of stress relaxation were then used to characterize the viscoelastic properties of the chordae. The normalized stress relaxation curve was plotted versus logarithmic time, and was found to be linear. The best-fit slope of this linearized curve was used to characterize the rate of stress relaxation.

Hydroxyproline Assay

Collagen content of chordae from 12 hearts was estimated using the hydroxyproline assay. Collagen contains 10–15% of 3- and 4-hydroxyproline, and thus its content in tissues can be estimated biochemically.[23,40]

Each sample assayed for hydroxyproline-contained tissue from 2 strut, 4–6 basal, or 4–6 marginal chordae. Twelve samples of each chordal type were assayed (24 strut chordae, 44 basal, and 44 marginal). Chordae were freeze dried and subjected to HCl (6 N) hydrolysis for 16 h at 115°C. The HCl was then boiled off and the hydrolyzate dissolved in dH_2O. Duplicate samples in 20 μl aliquots and 20 μl aliquots of standards (0, 10, 25, 50, 100, 150, and 300 μg/ml) were prepared and 250 μl chloramine-T reagent added. This material was kept at room temperature for 20 min, then 250 μl aldehyde/perchloric acid solution was added. All samples were heated to 60°C for 15 min. Then 250 μl aliquots were pipetted into 96-well plates, and absorbance was read at 558 nm. The collagen content calculated from the measured OH-proline was normalized to both chordal dry weight and wet weight. Water content of chordae was calculated from the above samples from the difference between their wet weight and their dry weight after 16 h of lyophilizing.

Fastin Elastin Assay

The commercially available Fastin Elastin Assay is a quantitative dye-binding method designed for *in vitro* analysis of elastins from biological tissue (Biocolor Ltd., Ireland).[3] The dye reagent binds to a unique "basic" and "nonpolar" amino acid sequence in mammalian elastin.

Each sample subjected to assay contained tissue from 2 strut, 4–6 basal, or 4–6 marginal chordae. Four samples (chordae from four hearts) of each chordal type were assayed (8 strut chordae, 18 basal, and 20 marginal). Chordae were digested in 4 ml NaOH (0.1 M) in a 98°C water bath for 70 min to isolate the insoluble elastin, which was then subjected to oxalic acid (0.25 M) at 95°C for 60 min to extract elastin. The oxalic acid extracts were centrifuged at 10,000*g* for 10 min in microcentrifuge tubes with membrane filters (MW ~15,000 cutoff). The existing liquid is the oxalic acid-free α-elastin since it molecular weight is approximately 80,000. The elastin was precipitated out overnight in the refrigerator after adding 1 ml cold Fastin Precipitating Reagent. The vial was then centrifuged at 10,000–12,000*g* for 10 min to get an elastin pellet. All liquid was removed from the elastin pellet, and the Fastin dye reagent was added. This step was followed by ammonium sulfate to make the elastin–dye complex precipitate out of solution. Samples were then centrifuged at 10,000*g* for 10 min to isolate the elastin–dye complex, and a Fastin Destain Reagent was added to bring the dye into solution. Sterile 1 mg/ml α-elastin from bovine neck ligament was used as a standard. Samples and standards (100 μl aliquots) were pipetted into a 96-well plate and read using a micro-well plate reader (SOFT MAX PRO) at 513 nm.

Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

Chordae from eight hearts were subjected to FACE analysis for quantification of their GAG types.[4,5,12] Each valve yielded three samples of 2 strut chordae, 4–6 basal, and 4–6 marginal chordae. Eight samples of each chordal type were assayed (16 strut chordae, 32 basal, and 40 marginal). Chordae were minced with fine scissors and digested with a 100 μl aliquot of proteinase-K solution (10 mg/ml) in 1 ml of 100 mM ammonium acetate buffer (pH 7) for 16 h at 60°C. The aliquots containing approximately 5 μg hexuronic acid were diluted to 100 μl in buffer and then subjected to digestion in hyaluronidase SD and chondroitinase ABC, and in chondroitin ACII (Seikagaku, Tokyo, Japan) for 3 h at 37°C, to enable identification of GAG classes. The digested samples were then dried with a vacuum concentrator, resuspended and fluorotagged with 2-aminoacridone (AMAC) and sodium cyanoborohydride ($NaCNBH_4$). Samples were then electrophoresed for 75 min at a constant 500 V on a monosaccharide gel (Glyko, Inc., Novato, CA, USA). Known quantities of maltotriose were added to samples to obtain a fluorescence intensity calibration curve. Disaccharide standards were run with the samples to identify the specific bands (Fig. 1). The gels were imaged with a Quantix CCD camera (Photometrix, Tucson, AZ, USA) and images analyzed with the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA). The quantities of the GAG types were determined according to the integrated optical density of the bands, the calibration curve, and the aliquot volume used for the enzyme digest. Computed quantities were normalized to the sample dry weight.

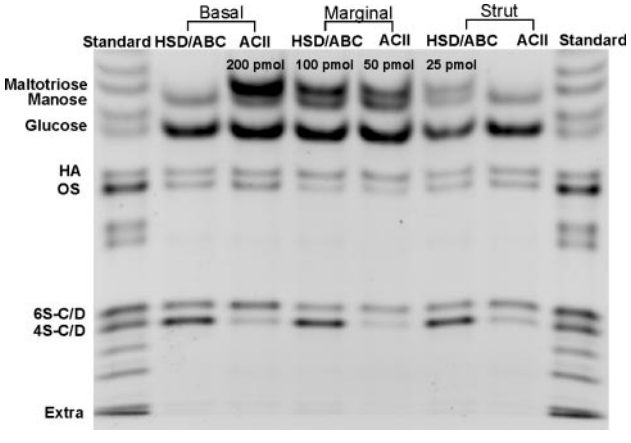

FIGURE 1. Fluorophore-assisted carbohydrate electrophoresis (FACE) gel of three types of chordae tendineae (basal, marginal, and strut). The lanes contain different enzymatic treatments of the chordae. HSD/ABC: hyaluronidase SD and chondroitin ABC; ACII: chondroitinase ACII. The maltotriose standards are for quantification of fluorescence intensity. HA: hyaluronan; OS: unsulfated chondroitin/dermatan sulfate; 6S-C/D: 6-sulfated chondroitin/dermatan sulfate; 4S-C/D: 4-sulfated chondroitin/dermatan sulfate.

Statistical Methods

Statistical comparisons of stress relaxation data and biochemical assays were calculated using One-way Analysis of Variance (ANOVA), with significance accepted at the $p < 0.05$ level. The Tukey test was used for the multiple comparisons. SigmaStat (Sigma Inc, Chicago, IL, USA) was used for statistical analyses.

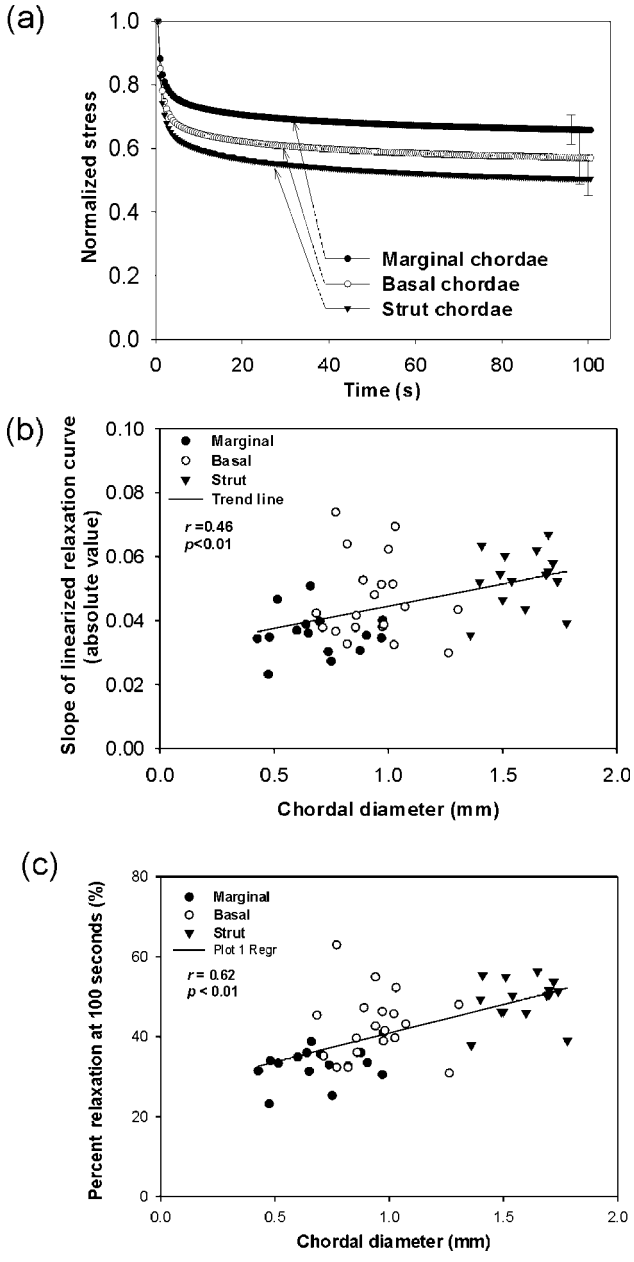

FIGURE 2. Stress relaxation properties of chordae tendineae. Note the different mean stress relaxation curves of the three types of chordae (a), the increase in the relaxation rate with chordal diameter and chordae type (b), and the percent relaxation at 100 s (c).

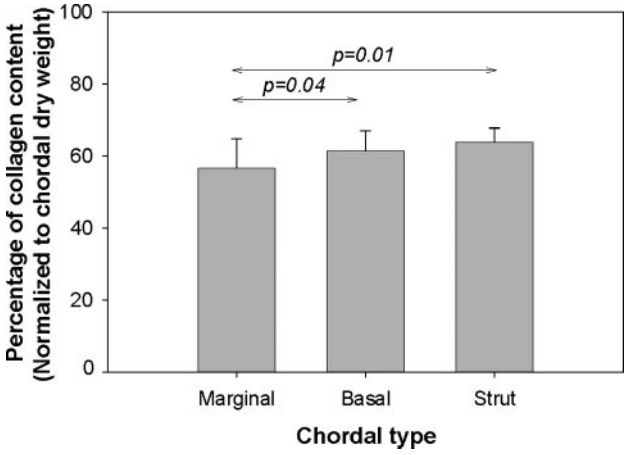

FIGURE 3. Comparison of collagen contents of the three types of chordae, normalized to chordal dry weight. Labeled statistical significances are results of multiple comparisons using the Tukey test.

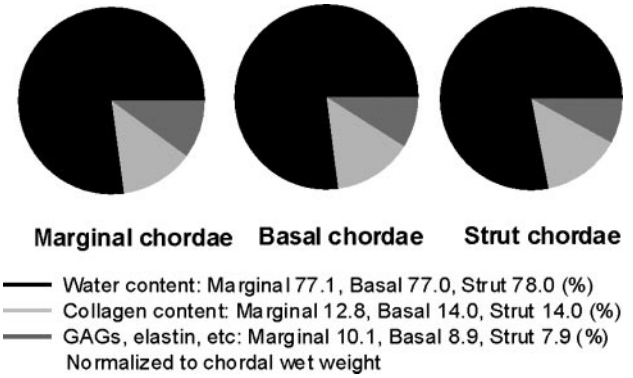

FIGURE 4. Comparison of the main components of chordae tendineae, normalized to chordal wet weight.

RESULTS

Each of the three chordal groups had stress relaxation curves that were statistically different (Fig. 2). The percent relaxation increased in the order of marginal, basal, and strut chordae (marginal 33.2 ± 4.7%, basal 42.4 ± 8.3%, strut 49.1 ± 5.4%, $p < 0.01$) [Fig. 2(a)]. The rate of relaxation, characterized by the slope of linearized relaxation curves, showed an increasing trend with chordal diameter ($r = 0.46$, $p < 0.01$) [Fig. 2(b)]. Compared by chordal groups, the rate of relaxation increased in the order of marginal (0.036 ± 0.007), basal (0.047 ± 0.013), and strut (0.053 ± 0.009, $p < 0.01$).

Marginal chordae had slightly less collagen content normalized to chordal dry weight than basal and strut chordae (marginal 56.6 ± 8.2%, basal 61.4 ± 5.6%, strut 63.8 ± 3.9%, $p = 0.002$) (Fig. 3). When normalized to wet weight, however, no statistically significant differences in collagen content were found (marginal 12.8 ± 2.9%, basal 14.0 ± 1.7%, strut 14.0 ± 1.8%). There was no significant difference in elastin content (normalized to dry weight) between chordal types (marginal 5.3 ± 3.2%, basal 5.4 ± 2.7%, strut 4.6 ± 1.7%).

The water content (normalized to wet weight) of the three types of chordae was also the same (marginal 77.1 ± 5.9%, basal 77.0 ± 3.4%, strut 78.0 ± 2.3%). In all chordae, water was the dominant component (Fig. 4), with collagen being the second most dominant component.

The total concentration of glycosaminoglycans (GAGs) decreased in the order of marginal, basal, and strut ($p = 0.06$). With regard to the specific GAG classes, no trend was found for hyaluronan nor for 6-sulfated dermatan sulfate (Fig. 5). However, the concentration of unsulfated chondroitin/dermatan sulfate, 6-sulfated chondroitin sulfate, and 4-sulfated chondroitin sulfate decreased in the order of marginal, basal, and strut, with a statistic significance $p = 0.001$, $p = 0.022$, and $p = 0.015$, respectively. The 4-sulfated dermatan sulfate also showed the decrease trend in the order of marginal, basal, and strut, but was not statistically significant ($p = 0.303$).

DISCUSSION

The typical stress relaxation test of tendon and ligament uses a step increase in strain followed by a hold phase.[1,17,32,42] Because marginal chordae have much smaller extensibility than basal chordae and strut chordae (marginal 4.3 ± 1.6%, basal 8.5 ± 3.0%, strut 17.5 ± 3.3% strain),[21] it was not possible to select a single strain that would elongate all chordae into the linear region of their load–displacement curve. We therefore loaded the chordae to 150 g, which ensured that all three types of chordae were in the linear region, and held them at the corresponding elongation during stress relaxation. In this situation, however, the marginal chordae were subjected to higher initial stress than strut chordae. Stress relaxation curves

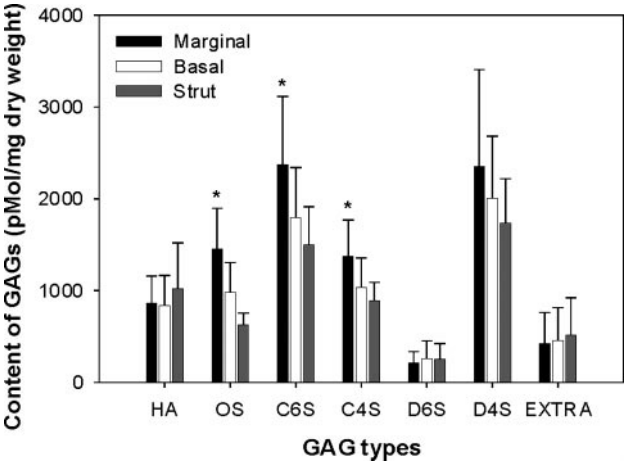

FIGURE 5. Comparison of glycosaminoglycan types between the three types of chordae tendineae. HA: hyaluronan; OS: unsulfated chondroitin/dermatan sulfate; C6S: 6-sulfated chondroitin sulfate; C4S: 4-sulfated chondroitin sulfate; D6S: 6-sulfated dermatan sulfate; D4S: 4-sulfated dermatan sulfate; EXTRA: other GAG types.

were therefore normalized with respect to maximum stress prior to analysis.

Some previous studies on tendon and ligament have shown none or only slight strain dependence for stress relaxation,[15,29,39] while others have shown considerable strain dependence.[13,19,30] In the recent work by Provenzano,[30] it was found that stress relaxation rate of ligament (medial collateral ligaments of rat) was dependent on initial strain in the low range (0–2% strain), whereas at high strains (2–5% strain), stress relaxation rate was not influenced by strain level.[30] This implies that strain dependence occurs mainly in the nonlinear range of the extensibility curve, not in the high stiffness, linear range. In our case, all chordae were loaded well into their linear range, thus minimizing the effects of initial strain/stress level. Indeed, chordae within any given group were found to have no dependence of relaxation on initial stress (Fig. 6).

The main mechanical differences between chordae identified from this study were in the slopes of the linearized stress relaxation curves, and the percent stress relaxation. The thicker strut chordae relaxed more and faster than the thinner marginal chordae. In view of our previous ultrastructural studies,[21] it is likely that this difference in mechanical behavior resulted from their different composition. The marginal, basal, and strut chordae, however, have nearly the same amount of water and collagen content when normalized to wet weight (Fig. 4). We can therefore conclude that water and collagen content were not the main reasons for the differences in viscoelastic properties. Elastin can also be excluded, since concentrations from different chordae were the same, and occurred in only trivial amounts. The remaining component that varied with chordal type, and could have affected stress relaxation, therefore, is the GAG content.

As reported previously, the ultrastructures of chordal types are significantly different from each other.[21] Marginal chordae have smaller average fibril diameter and higher fibril density than basal and strut chordae. The area percentages occupied by the fibrils, however, are identical for all chordae.[21] Since the collagen content for all chordae is the same, marginal chordae, which have the smaller average fibril diameter and higher fibril density, have more fibril surface area for interaction with the ground substance.[21] The ground substance of chordae is mainly composed of proteoglycans and water. Proteoglycans consist of a protein core to which glycosaminoglycan (GAGs) side chains are attached. The GAGs may interact with collagen fibrils directly[33] or bind to hyaluronic acid and form aggregates.[34] The main proteoglycans in chordae are the small proteoglycans that bind orthogonally with specific sites on the D-period of collagen fibrils to form the fibril-proteoglycan network.[20] The collagen fibril interactions that we found in chordae are similar to those of other tendinous tissue.[11,35] These proteoglycans were proposed to have two functions: (i) as a collagen fibril organizer—a yardstick that limits fibril dimensions during assembly,[33] and (ii) as a mediator of force transmission between adjacent collagen fibrils.[8,28,34] We have shown that proteoglycans linking adjacent fibrils become skewed when the chordae are stretched[20] and have thus supported the concept of proteoglycan-mediated fibrillar interaction. Indeed, it was the greater interaction between fibrils of marginal chordae that we used to explain the greater elastic modulus of marginal chordae in a previous publication on this topic.[21]

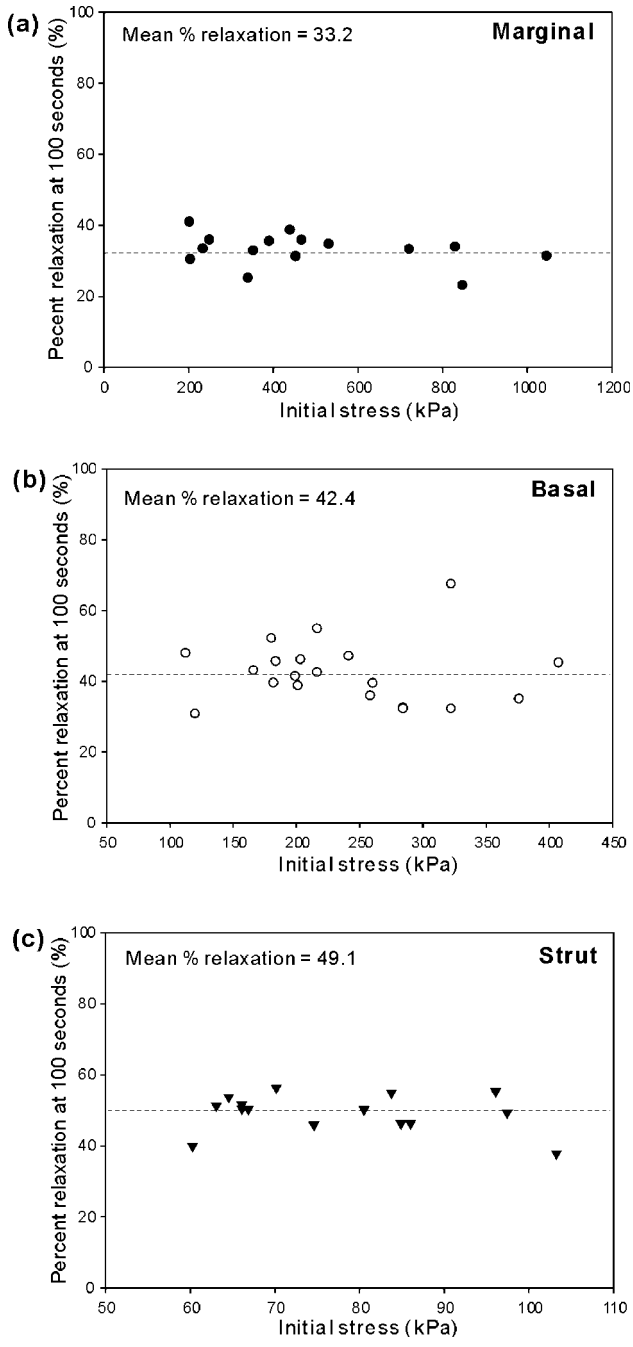

FIGURE 6. The relationship between stress relaxation and initial stress within chordal groups: marginal (a), basal (b), and strut (c). Note the absence of a dependence of stress relaxation on initial stress, and the differences in overall stress relaxation between chordae types.

We now make a similar argument regarding the stress relaxation process of tendinous tissues, mitral valve chordae in particular. Fibrillar interaction will affect the relative slippage between adjacent fibrils, will influence the structural reorganization of the tissue, and will thus affect the relaxation process, and hence the viscoelastic properties of the material. Fibrils with fewer proteoglycan connections will slip more and reorganize more than those with abundant proteoglycan connections. Since marginal chordae have greater collagen fibril density and fibril surface area, they have more GAG-mediated, fibril-to-fibril linkages. Indeed, the greater amount of GAGs anticipated from this argument is supported by the results of the FACE assay (Fig. 5) in two ways: (i) marginal chordae have greater overall GAG concentration; and (ii) the GAG types whose concentrations statistically decrease in the order of marginal, basal, and strut are the GAGs that are believed to be present in the fibril-to-fibril linkages.[33] These are the unsulfated chondroitin/dermatan sulfate, 6-sulfated chondroitin sulfate, and 4-sulfated chondroitin sulfate.

Recently, Elliott *et al*. reported that the tail tendon of decorin knockout mice relaxed faster and to a greater extent than controls.[10] This observation is consistent with our mechanism of proteoglycan-mediated chordal viscoelasticity. It was also reported, however, that chondroitin sulfate markedly increased the viscosity of hyaluronan solutions.[25] The behavior of chondroitin sulfate and hyaluronan in solution, however, is likely different from that which may occur between adjacent collagen fibrils and thus this observation is likely not relevant to chordal viscoelasticity.

The relative importance of chordal viscoelasticity, however, remains in question. Chordae tendineae are loaded for 0.3 s during each heartbeat (70 cycle/min).[14] Quasilinear viscoelastic (QLV) models fit to our data indicate that about 11% of the stress content will relax over this time course. Stress relaxation can thus take place during normal chordal function, although it may appear to be small. It is likely, however, that our *in vitro* loading tests underestimated the potential for chordal relaxation and chordae may in fact relax considerably more during the shock loading of mitral valve closure. Stress relaxation during systole may enable the tissue fibers to partially relax back to their natural state before the subsequent loading cycle, perhaps minimizing total stress.

From this study, we conclude that the greater number of proteoglycan linkages in the marginal chordae is responsible for the reduced capacity for stress relaxation, by way of the fibril–GAG interaction model. The different viscoelastic properties of mitral valve chordae can thus be explained morphologically.

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