Glycosaminoglycan Profiles of Myxomatous Mitral Leaflets and Chordae Parallel the Severity of Mechanical Alterations

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OBJECTIVES
This biochemical study compared the extracellular matrix of normal mitral valves and myxomatous mitral valves with either unileaflet prolapse (ULP) or bileaflet prolapse (BLP).

BACKGROUND
Myxomatous mitral valves are weaker and more extensible than normal valves, and myxomatous chordae are more mechanically compromised than leaflets. Despite histological evidence that glycosaminoglycans (GAGs) accumulate in myxomatous valves, previous biochemical analyses have not adequately examined the different GAG classes.

METHODS
Leaflets and chordae from myxomatous valves (n = 41 ULP, 31 BLP) and normal valves (n = 27) were dried, dissolved, and assayed for deoxyribonucleic acid, collagen, and total GAGs. Specific GAG classes were analyzed with selective enzyme digestions and fluorophore-assisted carbohydrate electrophoresis.

RESULTS
Biochemical changes were more pronounced in chordae than in leaflets. Myxomatous leaflets and chordae had 3% to 9% more water content and 30% to 150% higher GAG concentrations than normal. Collagen concentration was slightly elevated in the myxomatous valves. Chordae from ULP had 62% more GAGs than those from BLP, primarily from elevated levels of hyaluronan and chondroitin-6-sulfate.

CONCLUSIONS
The GAG classes elevated in the myxomatous chordae are associated with matrix microstructure and elastic fiber deficiencies and may influence the hydration-related “floppy” nature of these tissues. These abnormalities may be related to the reported mechanical weakness of myxomatous chordae. The biochemical differences between ULP and BLP confirm previous mechanical and echocardiographic distinctions.

Myxomatous changes in mitral valves affect 2.4% to 5% of men and women in the U.S. and other industrialized nations (1,2), but the pathogenesis of this disease remains unknown. The characteristic gross changes in myxomatous mitral valves (thickening, enlargement, leaflet hooding, annular dilation, elongated and/or ruptured chordae) (3) are accompanied at the microscopic level by fragmented, irregular elastin and collagen (4), abundant matrix metalloproteases (5), and an accumulation of glycosaminoglycans (GAGs) and proteoglycans (4,6,7), particularly in the spongiosa layer of the leaflets (4).

Despite visible changes in the leaflet spongiosa, previous biochemical analyses of myxomatous valves have focused less on GAGs and more on collagen. Two studies suggested that myxomatous leaflets have amplified collagen synthesis (8,9). Another study reported that myxomatous chordae but not leaflets have elevated collagen concentrations relative to normals (10). This same study found total GAG concentrations almost double those of controls.

Because proteoglycans and GAGs serve several purposes in connective tissues (11), including hydration, compliance, viscosity, and regulation of collagen fibrillogenesis, their accumulation in myxomatous tissues likely influences the accompanying extracellular matrix and the mechanical behavior of the mitral valve. Mitral valves normally contain several classes of GAGs (12), usually within proteoglycans, such as versican or decorin. Three studies have suggested that the greater GAG concentration of myxomatous valves results mainly from an elevation in the GAG hyaluronan (HA) (10,13,14), but only one of these studies actually investigated specific GAGs in human myxomatous valve tissues (via enzymatic histology) (14).

It is also unclear whether myxomatous changes affect the different components of the mitral valve apparatus to the same extent. We have previously shown that the mechanical abnormalities of myxomatous valves are far more severe in the chordae than in the leaflets (15,16). Indeed, the greater extensibility and lower strength of myxomatous chordae is consistent with reports that most cases of myxomatous mitral regurgitation that require surgical correction are due to chordal elongation or rupture, as opposed to isolated leaflet prolapse due to annular dilation (3). We therefore
hypothesized that myxomatous disease imparts different biochemical effects on the extracellular matrix of the mitral leaflets and chordae, which then result in their varying functional severities. Furthermore, we suspected that there may be additional biochemical differences between the two most common presentations of myxomatous mitral valves—unileaflet prolapse (ULP) and bileaflet prolapse (BLP). We have reported previously that myxomatous valves with ULP are weaker than valves with BLP (17). To test these hypotheses, we compared concentrations of water, deoxyribonucleic acid (DNA), collagen, total GAGs, and specific GAG classes in normal and myxomatous mitral leaflets and chordae and performed a subgroup comparison of ULP versus BLP.

METHODS

Myxomatous mitral valve specimens were obtained from 72 patients after surgery to correct severe mitral regurgitation due to a prolapsing valve (Table 1). The use of these tissues was authorized by the Institutional Review Board. Two patients underwent mitral valve replacement with a bioprosthetic valve; the remainder underwent quadrilateral resection of the posterior leaflet and implantation of an annuloplasty ring. Eleven patients had 12 concomitant procedures (7 coronary artery bypass grafts, 4 tricuspid valve repairs, and 1 aortic valve replacement). Myxomatous classification of the tissues was based on gross pathology, and the presence of ULP or BLP was confirmed by surgical and echocardiographic findings. Normal mitral valves were obtained at autopsy (<24 h post mortem) from people who died of non-cardiac-related causes. All tissues were stored at −20°C before analysis. Our laboratory has determined that valve tissues kept at 4°C retain their matrix-based mechanical properties for up to five days. We therefore believe that the extracellular matrix in these cadaveric valves was not measurably degraded during the postmortem period.

Only posterior leaflets and associated chordae were studied, because these tissues are most commonly resected during surgical repair of myxomatous valves, even in BLP (3). The chordae from each specimen were cut free and pooled for analysis. After the wet weights, lyophilized dry weights, and water concentrations were determined, each leaflet and pooled chordal sample was dissolved in 100 mM ammonium acetate buffer (pH 7) containing proteinase-K (1 mg/ml) at 60°C for 16 h (18). Aliquots were then taken to measure the concentrations of hydroxyproline (for collagen) (19), DNA (for cells) (20), and hexuronic acid (for GAGs) (21). The total amount of GAGs was estimated by both the hexuronic acid assay and by adding together the amounts of the different GAG classes found by fluorophore-assisted carbohydrate electrophoresis (FACE).

To quantify the different GAG classes, the samples were analyzed by an enzymatic digestion followed by FACE (18). For each sample, two identical aliquots containing at most 5 μg of hexuronic acid were incubated with either: 1) 2 μl of hyaluronidase SD (Streptococcus dysgalactiae) plus 3 μl of chondroitinase ABC (Seikagaku America, Falmouth, Massachusetts; each 10 mU/μl, termed HABC); or 2) 3 μl of chondroitinase ACII (10 mU/μl, termed ACII) for 3 h at 37°C. The HABC treatment cleaves the HA, chondroitin sulfate (CS), and dermatan sulfate (DS) chains into disaccharides, but the ACII treatment will not cleave bonds adjacent to iduronic acid (a component of DS). Therefore, ACII provides a measure of the minimum CS concentration, allowing the maximum DS concentrations to be estimated by subtraction from HABC. After digestion, the samples were dried, fluorotagged, mixed with glycerol and an internal standard (see below), and electrophoresed on a monosaccharide gel as previously described (18). The gel bands were imaged and analyzed using Gel-Pro (Media Cybernetics, Silver Spring, Maryland).

Serial dilutions of 2-sulfated disaccharide (not found in valves) were added to individual samples to provide an internal calibration curve for fluorescence intensity (Fig. 1). Enzyme digestion products were identified by correspondence to bands in a disaccharide standard lane. The quantity of each GAG was determined from the integrated optical

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<th>Table 1. Subject Group Characteristics</th>
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Data are given as mean ± standard deviation. *p < 0.01 normal vs. ULP; †p < 0.05 ULP vs. BLP. BLP = bileaflet prolapse; CHF = congestive heart failure; LA = left atrial; LVEDD = left ventricular end-diastolic diameter; LVEDS = left ventricular end-systolic diameter; MR = mitral regurgitation; PL = posterior mitral leaflet; ULP = unileaflet prolapse.
The water content in all leaflets was greater than that in the chordae (p < 0.001) (Table 2). Water content was higher than normal in chordae from ULP and highest in the leaflets and chordae from BLP (all p < 0.001). Deoxyribonucleic acid concentration relative to dry weight was equivalent between leaflets and chordae in all groups, significantly higher than normal in myxomatous tissues (p < 0.001), and slightly higher for BLP than for ULP (p < 0.05). Normalizing DNA to wet weight, however, eliminated the significant differences in DNA concentration between groups (data not shown), indicating that the volumetric cell density of the more hydrated myxoid tissues was equivalent to that of the less hydrated normals.

In general, the collagen concentration in chordae was greater than in the leaflets in both normal and myxomatous valves (Table 2). Collagen concentration in myxomatous leaflets was slightly elevated when normalized to tissue dry weight (p = 0.077). Because of high water contents, normalizing collagen to wet weight resulted in lower collagen concentrations in the myxomatous leaflets as compared with normal (p < 0.002), suggesting that the mechanical load-bearing capabilities of collagen are distributed across a larger loading region.

Leaflets had a greater GAG concentration than chordae when expressed in terms of dry weight (p < 0.001). Myxomatous valve chordae had significantly higher GAG concentrations than normals (p < 0.005), whereas the GAG concentrations in leaflets from ULP were slightly elevated (p < 0.05). Chordae from ULP valves had greater GAG concentrations than chordae from BLP valves (p < 0.001), although the leaflet differences were not significant. The differences among the ULP and normal leaflets and chordae retained approximately the same levels of significance even after normalizing data to wet weights.

**Individual GAG class concentrations.** In normal leaflets, the most common GAGs were HA, dermatan-4-sulfate measured by FACE. Statistical comparisons among water concentration, DNA, cells, GAG class concentrations, proportions, or chain lengths in the two myxomatous groups and the normal group were obtained using an analysis of variance, followed by Tukey tests as needed. Because of the large number of statistical tests performed, two-tailed significance was accepted at p < 0.01.

**RESULTS**

Water, DNA, collagen, and total GAG concentrations. The water content in all leaflets was greater than that in the chordae (p < 0.001) (Table 2). Water content was higher than normal in chordae from ULP and highest in the leaflets and chordae from BLP (all p < 0.001). Deoxyribonucleic acid concentration relative to dry weight was equivalent between leaflets and chordae in all groups, significantly higher than normal in myxomatous tissues (p < 0.001), and slightly higher for BLP than for ULP (p < 0.05). Normalizing DNA to wet weight, however, eliminated the significant differences in DNA concentration between groups (data not shown), indicating that the volumetric cell density of the more hydrated myxoid tissues was equivalent to that of the less hydrated normals.

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| Table 2. Concentrations of Water, DNA, Collagen, and Total GAGs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Normal Leaflets | ULP Leaflets    | BLP Leaflets    | Normal Chordae  | ULP Chordae     | BLP Chordae     |
| Water (% of wet weight) | 87.0 ± 3.5      | 88.3 ± 1.9      | 89.4 ± 1.6      | 75.7 ± 6.5†‡  | 79.0 ± 7.3†‡    | 82.6 ± 5.7†‡    |
| Collagen (% of dry weight) | 50.8 ± 13.5     | 57.8 ± 14.2     | 54.5 ± 13.7     | 63.2 ± 11.6*  | 65.6 ± 13.8*    | 61.3 ± 15.1     |
| DNA (mg/g dry weight)       | 3.1 ± 1.7       | 4.3 ± 2.2†‡     | 5.1 ± 2.0†‡     | 2.9 ± 1.5     | 4.2 ± 2.1†‡     | 4.6 ± 1.7†‡     |
| Hexuronic acid (mg/g dry weight) | 4.5 ± 2.1      | 6.5 ± 3.1       | 5.9 ± 3.4       | 2.4 ± 1.2*    | 6.0 ± 2.7†‡     | 3.7 ± 2.1†‡     |

Data are given as mean ± standard deviation. *p < 0.05 chordae vs. leaflets; †p < 0.05 ULP or BLP vs. normal; ‡p < 0.05, ULP vs. BLP.

BLP = bileaflet; DNA = deoxyribonucleic acid; GAGs = glycosaminoglycans; ULP = unileaflet prolapse.
D-4-S), and chondroitin-6-sulfate (C-6-S) (Fig. 2). Myxomatous leaflets, however, had more C-6-S (BLP, p \( < 0.033 \); ULP, p \( < 0.009 \)) and slight elevations in almost every other GAG class (with respect to dry weight). Normal chordae contained predominantly D-4-S, with less HA and C-6-S. The myxomatous chordae had elevated concentrations of HA, unsulfated chondroitin, chondroitin-4-sulfate, and C-6-S (p \( < 0.003 \)). The relative changes in GAG concentrations were far greater in myxomatous chordae than that in the leaflets. Although there were no differences between the two myxomatous leaflet groups, ULP chordae contained slightly more HA and C-6-S than did the BLP chordae (p \( < 0.05 \)).

Myxoid valves also contained altered proportions of the various GAG classes (Fig. 3). Myxomatous leaflets from ULP had slightly greater proportions of C-6-S than normal leaflets (p \( = 0.013 \)), whereas leaflets from BLP had significantly greater proportions of C-6-S (p \( = 0.002 \)) and slightly lower proportions of D-4-S (p \( < 0.035 \)), but overall there were few differences between groups. The myxomatous chordae, however, had slightly higher proportions of HA (p \( < 0.05 \)), significantly greater proportions of C-6-S (p \( < 0.001 \)), and lower proportions of D-4-S (p \( < 0.001 \)) and oversulfated CS/DS (p \( < 0.004 \)) than did normal chordae. The changes in the GAG class proportions were therefore greater in the myxoid chordae than they were in the myxoid leaflets, although they showed the same general trends.

**GAG chain length.** In normal mitral valves, the CS/DS chains were longer in leaflets than in chordae (p \( < 0.001 \)) (Fig. 4). In myxomatous valves, no such difference was found. There was also no difference in chain lengths between leaflets from ULP and normal valves, but leaflets from valves with BLP contained longer GAG chains (p \( < 0.004 \)). Chordae from myxomatous valves contained longer GAG chains than did normal chordae (p \( < 0.001 \)).

**DISCUSSION**

The first major finding of this study was that the biochemically measurable effects of myxomatous mitral valve disease and the differences between ULP and BLP valves appear to be more pronounced in chordae than in leaflets. These greater magnitude changes in the chordae support our previous mechanical findings (15–17), in which both myxomatous leaflets and chordae were more extensible and less stiff than normal tissues, but changes in stiffness and failure strength were more severe in the chordae. Our mechanical
and biochemical findings are therefore consistent with the clinical observations that chordal rupture and elongation are the most frequent mechanisms responsible for myxomatous mitral regurgitation (3).

The second finding was that myxomatous and normal chordae contain distinctly different "profiles" of individual GAG classes. Normal chordae contained primarily D-4-S in chains approximately 50 to 60 disaccharides long, whereas the myxomatous chordae had greater proportions of C-6-S and HA, a reduced proportion of D-4-S, and significantly longer CS/DS chains. The GAG profile of myxomatous chordae was therefore less like normal chordae and more like normal and myxomatous leaflets. The GAG profile of myxomatous leaflets was almost uniformly elevated across all the GAG classes but was otherwise essentially unchanged from that of normal leaflets. Interestingly, although HA had previously been suggested as the GAG mainly responsible for the myxomatous change (10,13,14), we found that the GAG most elevated was C-6-S. We attribute this new finding to the sensitivity and repeatability of the FACE methodology in resolving the different GAG classes. Otherwise, the changes in the collagen, DNA, and hexuronic acid concentrations described here are in almost complete agreement with findings from previously published studies (8–10).

We interpret the elevation of HA and C-6-S in myxomatous chordae as indicating a likely overabundance of the proteoglycan versican (23,24). Versican is one of the largest proteoglycans, containing a 450-kD core protein sur-
rounded by 15 to 20 long GAG chains (23), which extend outward from the core protein to create a highly structured, negatively charged macromolecule that frequently binds with HA (24). Thus, it is thought that the versican–HA aggregate provides soft tissues with hydration and compressive resistance (11). An abundance of this proteoglycan aggregate would explain the thickened, spongy appearance of myxomatous valves, particularly in the enlarged, hydrated, translucent spongia. Versican also reportedly has an inverse relationship with elastin: CS chains, such as those found in versican, inhibited the in vitro assembly of tropoelastin into mature insoluble elastic fibers (25), whereas overexpression of a versican variant without any GAG chains produced the opposite effect (26). The overabundance of versican–associated GAGs may lead to the deficient elastic fibers found in myxomatous valves (6).

In myxomatous valves—and chordae in particular—the relative increase in the proportion of HA and long-chain C-6-S was accompanied by a relative decrease in the proportion of 4-sulfated GAGs with shorter chain lengths. These 4-sulfated GAGs are commonly found in the small proteoglycans decorin and biglycan (27). Decorin binds to type I collagen and has been shown to control collagen fibrillogenesis (11). Although the elevated collagen concentration measured in this study did not reach statistical significance, an increase in the ratio of collagen to decorin-associated GAGs suggests an impairment in type I collagen fibrillogenesis and/or the synthesis of alternative types of collagen, such as type III (10), which may not provide the same mechanical strength as type I collagen.

Finally, we provide the first findings that ULP and BLP are two biochemically distinct variants of myxomatous mitral valve disease. The chordae from valves with ULP contained more GAGs, whereas leaflets and chordae from valves with BLP contained more water. Compared with valves from ULP, the leaflets and chordae from valves with BLP had more cells but slightly less collagen. Because differences in biochemical makeup can influence material properties, our findings of biochemical differences among subgroups of myxoid valves supports our previous report of mechanical and echocardiographic diversity between ULP and BLP (17).

Study limitations. One possible limitation of this study is the slightly lower mean age of the control group. Several previous studies, however, have shown that the concentrations of total GAGs, CS (12), and cells (28) in human heart valves decrease with age. Therefore, our younger normal control group underestimates, rather than exaggerates, the findings of our study.

Our proposed explanation for these findings is that the matrix and mechanics of myxomatous chordae are shifting from a normal “chordal phenotype” involving high-tensile loads toward a “leaflet phenotype” involving a combination of compressive and low-tensile loads. It will be important to determine whether the stimulus for this phenotypic shift is genetically based (perhaps related to a heritable connective tissue disorder or a unique aspect of valvular interstitial cells) or is a secondary response to the altered mechanical forces in the regurgitant valve. Although we cannot conclusively establish whether these biochemical changes precipitate the original myxomatous valve dysfunction, these findings nonetheless illuminate the relationships among the altered extracellular matrix, tissue mechanics, and function of myxomatous valves. Our interpretation that these biochemical changes may be responsible for, or perpetuated by, the altered mechanical loads in a vicious cycle of myxomatous degeneration provides additional support for the mechanism of myxomatous pathogenesis recently proposed by Rabkin et al. (5).

Conclusions. These findings show that the mitral valve chordae are more severely affected by myxomatous changes than leaflets and substantiate our hypothesis that there is a biochemical basis for the varying functional severity of the myxomatous mitral valve disease.

Acknowledgments

The authors express appreciation to Jessie Walker, Vincent Hascall, PhD, Anthony Calabro, PhD, and Ronald Midura, PhD.

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