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Glycosaminoglycans and proteoglycans in normal mitral valve leaflets and chordae: association with regions of tensile and compressive loading

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This study was designed to identify the specific proteoglycans and glycosaminoglycans (GAGs) in the leaflets and chordae of the mitral valve and to interpret their presence in relation to the tensile and compressive loads borne by these tissues. Leaflets and chordae from normal human mitral valves ($n = 31$, obtained at autopsy) were weighed and selected portions digested using proteinase K, hyaluronidase, and chondroitinases. After fluorescent derivatization, fluorophore-assisted carbohydrate electrophoresis was used to separate and quantify the derivatized saccharides specific for each GAG type. In addition, the lengths of the chondroitin/dermatan sulfate chains were determined. Proteoglycans were identified by western blotting. The regions of the valve that experience tension, such as the chordae and the central portion of the anterior leaflet, contained less water, less hyaluronan, and mainly iduronate and 4-sulfated N-acetylgalactosamine with chain lengths of 50–70 disaccharides. These GAGs are likely associated with the small proteoglycans decorin and biglycan, which were found in abundance in the tensile regions. The valve regions that experience compression, such as the posterior leaflet and the free edge of the anterior leaflet, contained significantly more water, hyaluronan, and glucuronate and 6-sulfated N-acetylgalactosamine with chain lengths of 80–90 disaccharides. These GAGs are likely components of water-binding versican aggregates, which were abundant in the compressive loading regions. The relative amounts and distributions of these GAGs are therefore consistent with the tensile and compressive loads that these tissues bear. Finally, the concentrations of total GAGs and many different chondroitin/dermatan sulfate subclasses were significantly decreased with advancing age.

Key words: compression/decorin/proteoglycans/tension/versican

Introduction

The mitral valve is one of the most complex connective tissue structures in the entire body. It consists of two leaflets and numerous chordae tendineae. These chordae have a highly aligned collagenous core and a thin outer sheath of elastic fibers and endothelial cells. Both leaflets are laminated tissues containing a heavily collagenous layer on the ventricular side; a predominantly elastic layer on the atrial side; and an inner spongiosa layer containing abundant proteoglycans (PGs) and hyaluronan (HA). The relative thicknesses of these layers vary between the two leaflets and also within each leaflet from its attachment edge to its free edge (Kunzelman *et al*., 1993).

The variability of the different leaflet layers, and hence the structural constituents within the mitral valve, are determined by the specific functional roles of the leaflets and chordae. The closed valve, in particular, maintains a balance of tensile and compressive loads, in which the chordae and the flat central region of the anterior leaflet are in tension, whereas the free edge of the anterior leaflet and most of the posterior leaflet are in appositional compression. Accordingly, the most collagenous components of the mitral apparatus are the chordae and the portion of the anterior leaflet between the annulus and the upper appositional border (Kunzelman *et al*., 1993). In the posterior leaflet and in the free edge of the anterior leaflet, the collagenenous layer is relatively thinner, whereas the PG-rich spongiosa is substantially thicker.

The wide diversity of glycosaminoglycans (GAGs) and their parent PGs exert considerable yet variable control over the physical properties of the extracellular matrix (Hardingham and Fosang, 1992; Junqueira and Montes, 1983). Over the past 50 years, exhaustive biochemical analyses have been conducted on the GAGs of heart valves of pigs (Castagnaro *et al*., 1997), cows (Bostrom *et al*., 1963; Deiss and Leon, 1955; Honda *et al*., 1976; Lowther *et al*., 1970; Meyer *et al*., 1969; Moretti and Whitehouse, 1963; von Figura *et al*., 1973), rodents (Colvee and Hurle, 1981; Hallgrimsson *et al*., 1970), and humans (Baig *et al*., 1978; Masuda, 1984,; Murata, 1981; Torii and Bashey, 1966; Torii *et al*., 1965). These studies have examined the influence of subject age on the synthesis and content of the classes of GAGs in all the heart valves. However, these previous studies agree only moderately about the general proportions of HA and the chondroitin/dermatan sulfates (CS/DS) in valve tissues. Although there is a consensus regarding the decrease of total GAG concentration with age, reports disagree on age-related changes in specific GAG classes. Despite the many studies of mitral valve leaflets, only one report describes the GAG classes in

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chordae (von Figura *et al*., 1973). Furthermore, the majority of previous studies have considered the mitral valve leaflets to be homogeneous, which clearly is not the case. One study did examine the regional biosynthesis and content of GAGs in mitral leaflets, but did not differentiate between GAG classes (Bostrom *et al*., 1963). There has also been only one group that attempted to link these GAG profiles with the presence of a particular PG (Toole and Lowther, 1968). In general, there is scant information regarding the influence of GAGs and PGs on the mechanical properties and function of the valve tissues.

Recent developments in carbohydrate analysis using fluorophore-assisted carbohydrate electrophoresis (FACE) (Calabro *et al*., 2000a,b) have enabled the study of GAGs in heart valves using novel and efficient methods. The resolution of the bands in FACE enables differentiation between 4- and 6-sulfation of N-acetylgalactosamine (galNAc) on iduronate (idoA)-based disaccharides. The quantification of terminal saccharides (primarily sulfated galNAc) on the same gels simplifies the calculation of the CS/DS chain lengths. Therefore this new technology can quickly provide characteristics about the GAG profile, chain lengths, and sulfation patterns that can provide clues to the identity of particular PGs within mitral valve tissues and might be used to postulate their mechanical contributions to mitral valve function.

This study was designed with several objectives. The first was to determine if the fine structure and chain length of GAGs present in mitral valve tissues are dependent on the predominant type of tissue loading. For this purpose, mitral valves from normal human autopsy subjects were cut into regions of compressive and tensile loading, and their GAG characteristics were measured using FACE. The second objective was to determine if the resulting load-related GAG profiles are dependent on subject demographics (age, body surface area, gender, and race). The third objective was to compare the measured GAG profiles, chain lengths, and sulfation patterns with published data characterizing different PGs to identify the main types of PGs contained within the different loading regions of the valve. Last, western blotting was used to confirm the identity of these PGs in valve tissues.

Results

Mitral valve anatomy

The mitral valve contains two leaflets that attach to the junction between the left atrium and left ventricle (Figure 1). The free edges and ventricular surfaces of the leaflets are connected to the papillary muscles of the left ventricle by numerous chordae tendineae. Mitral valve closure is

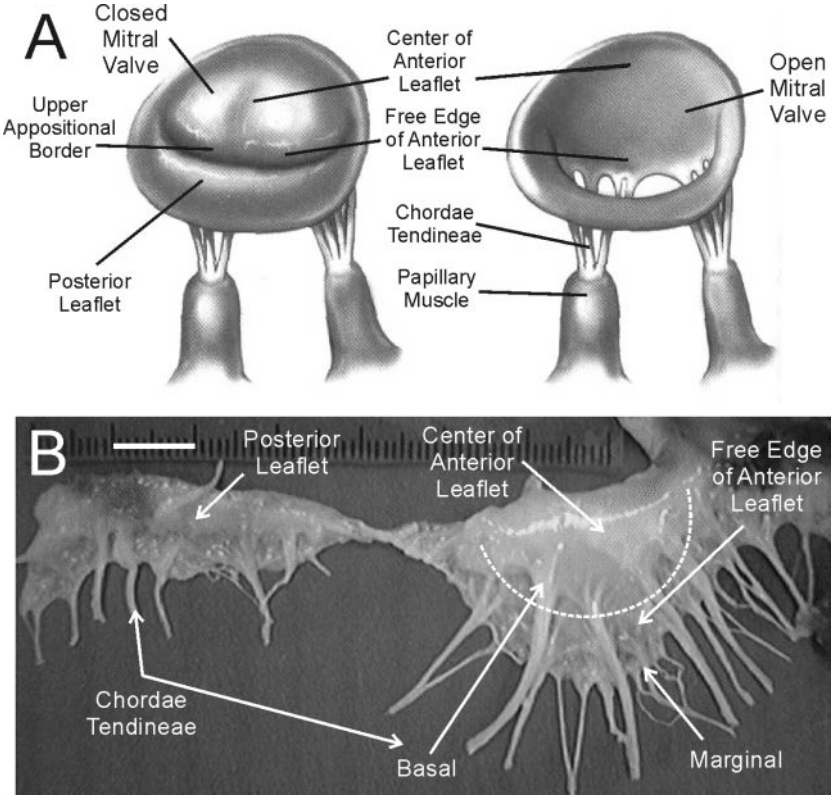

Fig. 1. (**A**) Atrial aspect of the mitral valve, in the closed (left) and open (right) position. (**B**) Ventricular aspect of the mitral valve cut open between the leaflets and laid flat. The dotted line indicates the upper appositional border of the anterior leaflet, which was used to divide the center from the free edge. Chordae tendineae may have basal or marginal insertions into the leaflet, as indicated. The thick white bar in (**B**) is 1 cm long. Illustration in part (**A**) adapted with permission from *Cleveland Clinic Heart Book*, by Cleveland Clinic. Copyright © 2000 Cleveland Clinic. Reprinted by permission of Hyperion.

maintained by apposition of large portions of the anterior and posterior leaflets, the tension on the chordae following contraction of the papillary muscles and the strength of the pressurized anterior leaflet. Mitral valves from 35 normal autopsy subjects were dissected into anterior leaflets (free edge and center), posterior leaflets, and chordae tendineae. Thirty of these valves were used for the entire study; five were used to measure water concentration only.

Water and total GAGs

The hydration and GAG composition of mitral valve tissues were straightforwardly examined by measuring the concentrations of water and total GAGs. Not surprisingly, the water content followed GAG content between different regions of the valve (Figure 2). The concentrations of both water and total GAGs were greatest in the two compressive regions of the valve (posterior leaflet and free edge of the anterior leaflet), significantly less in the center of the anterior leaflet ($p < 0.001$ for water, $p = 0.002$ for total GAGs), and lowest in the chordae ($p < 0.001$ for both variables). Chordal type was also a significant factor, in that the basal chordae that insert directly underneath the leaflets contained less water content than the *marginal* chordae that insert into the leaflet free edges (73% versus 77%, $p = 0.003$).

GAG compositional analysis

GAG composition was measured from the solubilized tissue samples by enzymatic cleavage of the GAG chains into disaccharides, followed by fluorescent derivatization and FACE. This procedure resulted in the quantitation of ΔDiHA, glucuronate (glcA)-based disaccharides containing either 4-, 6-, and unsulfated galNAc, idoA-based disaccharides containing either 4- or 6-sulfated galNAc, di- and trisulfated CS/DS, and the terminal N-acetylhexosamines (Figure 3). (In this

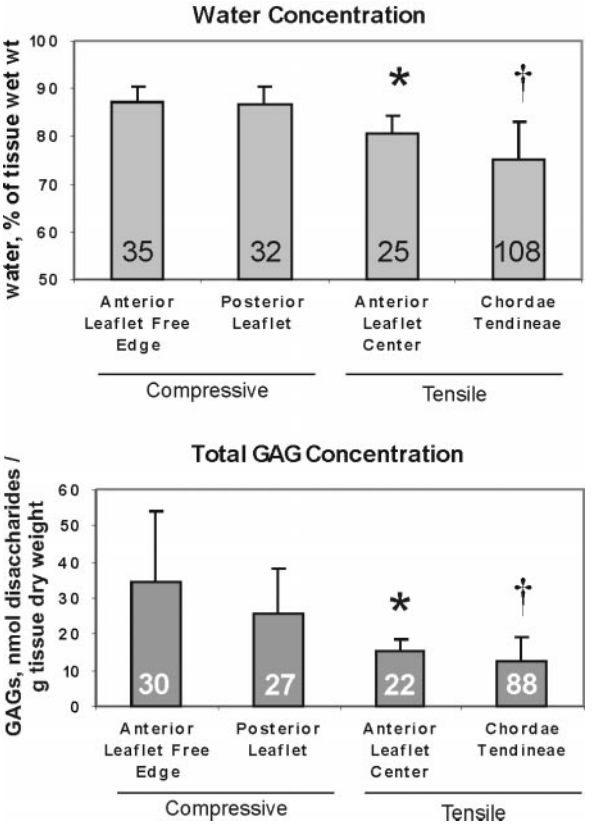

Fig. 2. Water and total GAG concentrations in compressive (first two columns) and tensile (second two columns) regions of the mitral valve. Mean ± SD. Numbers within the columns indicate sample size. The number of chordal samples is high because they were divided into subgroups to indicate basal or marginal insertion into the anterior or posterior leaflet. *ANOVA $p < 0.01$ versus posterior leaflet and anterior leaflet free edge. †ANOVA $p < 0.01$ versus leaflet groups.

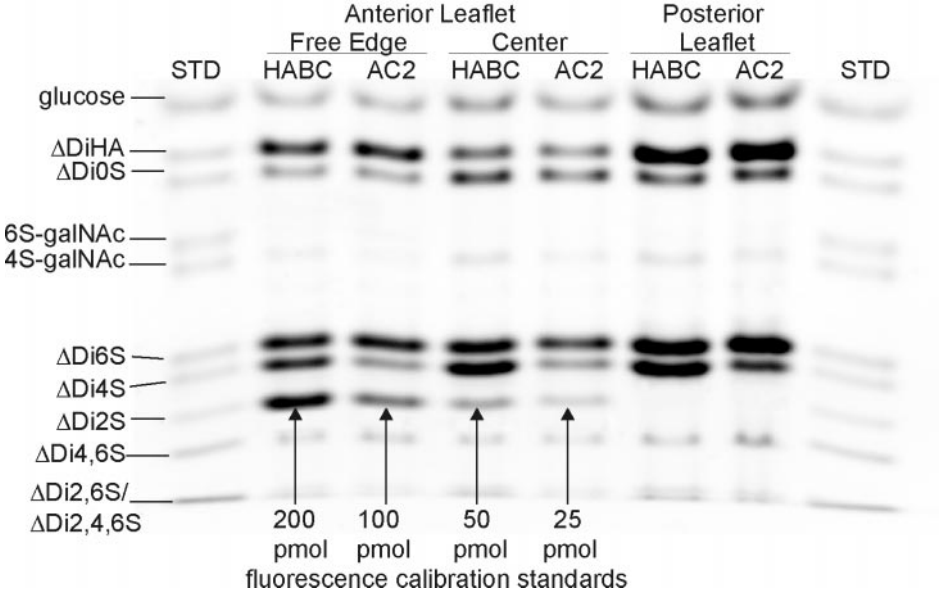

Fig. 3. FACE gel with HABC and ACII digests of different regions of the mitral valve in lanes 2–7, and monosaccharide and disaccharide standards in lanes 1 and 8. Serial dilutions of the chondroitin 2-sulfate were added to the samples in lanes 2–5 before running the FACE gel to provide a calibration curve between concentration and fluorescence intensity.

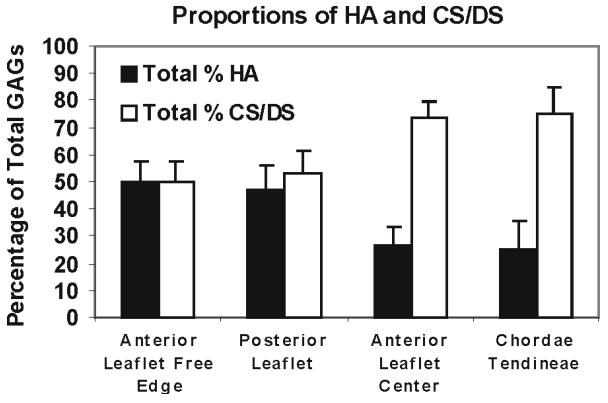

Fig. 4. Proportions (mean ± SD) of HA and CS/DS in compressive and tensile regions of the mitral valve. Sample sizes are given in Table I.

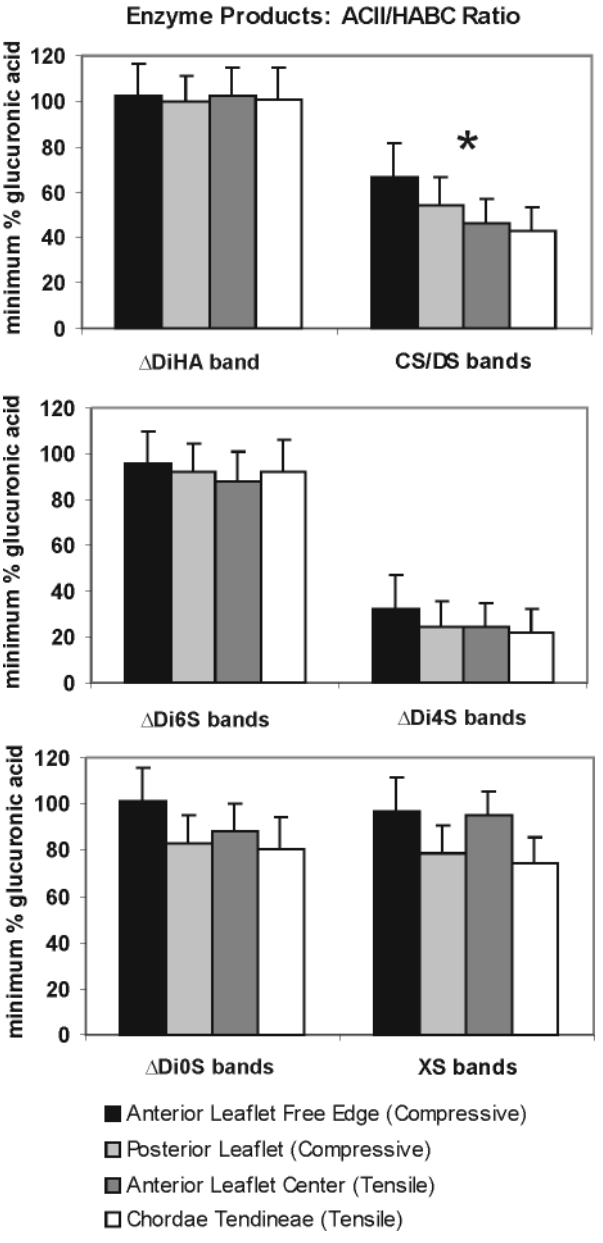

Fig. 5. Ratios (mean ± SD) of ACII/HABC enzyme digest products (indicating the minimum percentage glucuronic acid) found in the GAG compositional analysis of the compressive and tensile regions of the mitral valve. Sample sizes are given in Table I. *ANOVA $p < 0.001$ between groups.

study, the designation of a GAG as containing iduronate or glucuronate was based on the susceptibility to chondroitinase ACII. CS/DS is meant to refer to bands that could contain either iduronate- or glucuronate-based disaccharides. This nomenclature is consistent with the designation of an intact GAG chain as CS if the chain contains exclusively glucuronate and DS if the chain contains any iduronate at all.) In the posterior leaflet and the free edge of the anterior leaflet (compressive regions), the proportions of ΔDiHA and total CS/DS were approximately equivalent (Figure 4). In the chordae and in the center of the anterior leaflet (tensile regions), there was approximately three times as much CS/DS as ΔDiHA.

The minimum percentage of glcA (Figure 5) was determined from the relative band intensities after two different enzyme treatments: (1) chondroitinase ABC combined with hyaluronidase SD (HABC) and (2) chondroitinase ACII (ACII). Because the ACII treatment cleaves bonds adjacent to glcA but not idoA, the ACII treatment measures the minimum glcA content, which was then divided by the HABC product (glcA + idoA) to determine the percentage value. In the first set of columns in Figure 5 (top), the minimum percentage of glcA content in HA was approximately 100%, as expected. These data indicated that the ACII and HABC treatments were capable of liberating equivalent amounts of ΔDiHA. (Because we determined that the chondroitinase ACII enzyme performs equally well as hyaluronidase SD in the digestion of hyaluronan, we have since switched from our HABC mixture to an ACII/ABC mixture (3 μl each) for complete digestion of hyaluronan and CS/DS. This alternative offers substantial economic savings.) The second set of columns (top) shows that the total CS/DS disaccharide bands from valve tissues contained a mixture of glcA and idoA. The minimum glcA present in the posterior leaflet and free edge of the anterior leaflet was higher than in the chordae and the center of the anterior leaflet (analysis of variance [ANOVA] $p < 0.001$ between groups). Likewise, disaccharides appearing in the 6-sulfated or 4-sulfated band positions (Figure 5, middle) could have contained either glcA or idoA. The 6-sulfated position bands contained glcA almost exclusively, whereas the 4-sulfated position bands were largely idoA. Both the unsulfated and the oversulfated CS/DS bands (Figure 5, bottom) contained 75%–100% glcA.

Additional data about the specific GAG disaccharide species were then calculated by subtracting the ACII data from the HABC data to estimate the maximum idoA contents. The ratios between different GAGs and their sulfation patterns were also calculated. In both the posterior leaflet and the free edge of the anterior leaflet, the most common GAG classes were found to be ΔDiHA (47%–50% of total GAG Δdisaccharides) and glucuronate with 6-sulfated galNAc (glcA-6S-galNAc) (Table I, Figures 6 and 7).

Table I. Ratios of GAG epimerization and sulfation characteristics (means ± SD)

Valve region (sample size)[a]	glcA/idoA	galNAc 6S/4S (glcA)	galNAc 4S/6S[b] (idoA)	galNAc 6S/4S (overall)
Anterior leaflet free edge ($n = 30$)	$1.79 \pm 1.05^{\dagger *}$	$2.85 \pm 1.54^{*}$	$11.5 \pm 7.8^{*}$	$0.90 \pm 0.31^{*}$
Posterior leaflet ($n = 26$)	$1.17 \pm 0.53^{\ddagger}$	$2.60 \pm 0.85^{*}$	$14.3 \pm 12.2^{\ddagger}$	$0.72 \pm 0.24^{*}$
Anterior leaflet center ($n = 13$)	0.81 ± 0.48	1.49 ± 0.49	24.6 ± 11.9	0.37 ± 0.08
Chordae tendineae ($n = 82$)	0.62 ± 0.28	1.55 ± 077	35.8 ± 27.9	0.31 ± 0.13

[a]Not all valve regions were available from all valves obtained from autopsy. The number of chordal samples is high because they were divided into subgroups to indicate basal or marginal insertion into the anterior or posterior leaflet.
[b]Because the galNAcs in idoA-containing disaccharides are more frequently 4-sulfated, the 4S:6S ratio is given instead of the 6S:4S ratio.
$^{\dagger}p < 0.01$ versus posterior leaflet.
$^{*}p < 0.01$ vs. anterior leaflet center and versus chordae tendineae.
$^{\ddagger}p < 0.01$ vs. chordae tendineae.

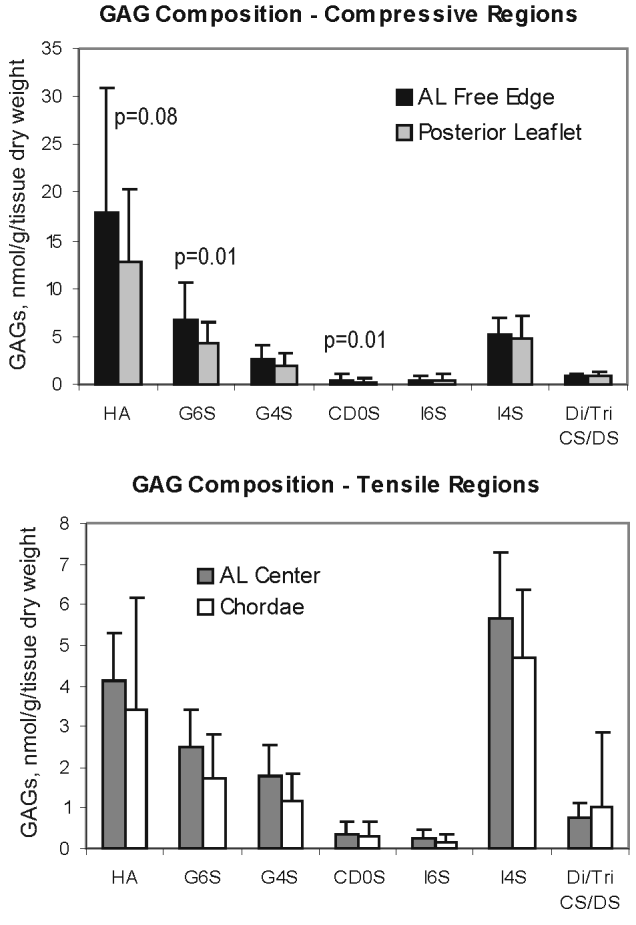

Fig. 6. GAG compositional analysis in the compressive and tensile regions of the mitral valve. Data (mean ± SD) are given in nmol of GAG Δdisaccharide per mg tissue dry weight. G6S, G4S, I6S, and I4S, are defined in the abbreviations. Sample sizes are given in Table I.

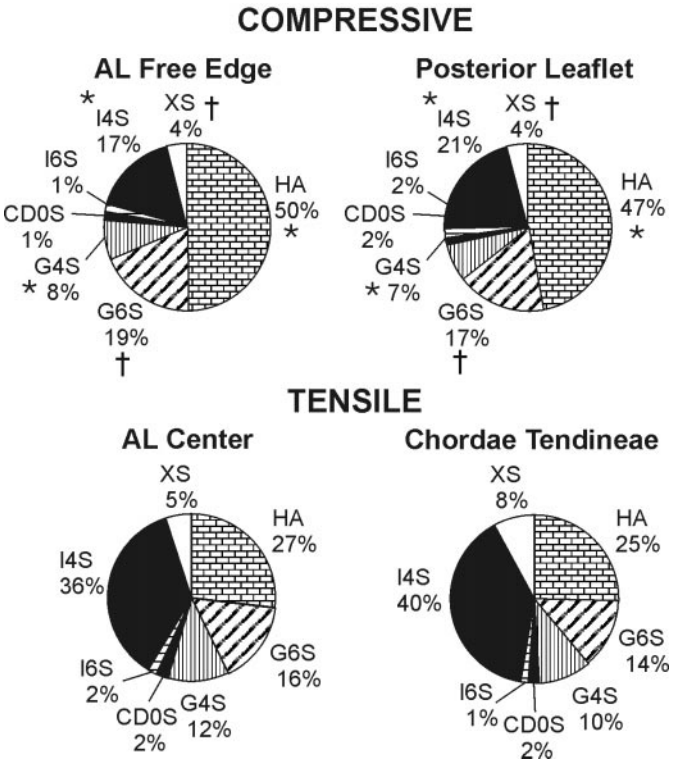

Fig. 7. Pie charts showing GAG compositional analysis in the compressive (upper two charts) and tensile (lower two charts) regions of the mitral valve. Data are given in percentage of specific GAGs normalized to the total GAG content measured by FACE.
$^{*}p < 0.01$ versus anterior leaflet center and versus chordae tendineae.
$^{\dagger}p < 0.01$ versus chordae tendineae. G6S, G4S, I6S, I4S are as in Figure 6, and XS indicates a combined group of ΔDi4,6S, ΔDi2,6S, and ΔDi2,4,6S.

The two compressive regions contained similar GAG profiles, although the free edge of the anterior leaflet had significantly more glcA-6S-galNAc ($p = 0.01$) and unsulfated CS/DS (ΔDi0S, $p = 0.01$) and slightly more ΔDiHA ($p = 0.08$) than the posterior leaflet (Figure 6). In contrast, the central region of the anterior leaflets contained mainly iduronate with 4-sulfated galNAc (idoA-4S-galNAc, 36% of total GAGs), less glcA relative to idoA ($p < 0.001$), and greater amounts of 4-sulfation overall ($p < 0.001$) as compared to the other leaflet samples (Table I, Figures 6 and 7). Quite notably, this central portion of the anterior leaflet contained significantly less glcA-6S-galNAc ($p < 0.001$) and ΔDiHA ($p < 0.001$) than

the adjacent anterior leaflet free edge. The most predominant GAG in chordae was also idoA-4S-galNAc (40% of total GAGs). Chordae contained the greatest proportions of idoA relative to glcA and of 4-sulfation relative to 6-sulfation, as compared with the compressive leaflet regions (Table I, $p < 0.001$).

The FACE gels also demonstrated bands corresponding to 6- and 4-sulfated galNAcs (the nonreducing termini of the CS/DS chains, with a 4S:6S ratio of 5:1) for all the valve samples. It has been demonstrated in aggrecan that the termini of CS/DS chains consist of 85%–90% nonreducing galNAcs and 10%–15% nonreducing disaccharides (Plaas *et al.*, 1997). Therefore to identify nonreducing disaccharides that would run in the ΔDi6S and ΔDi4S band positions, another set of samples were digested with HABC, then treated with mercuric acetate to cleave the uronate from the hexosamine. This treatment showed that there were no detectable nonreducing disaccharides; after the reducible ΔDi6S and ΔDi4S bands had migrated to the sulfated galNAc band positions, only residual 2-aminoacridone hydrochloride remained underneath the ΔDi6S and ΔDi4S bands.

Chain lengths

The number of disaccharides in the CS/DS chains was estimated from the ratio of the total amount of the CS/DS Δdisaccharides to the total amount of saccharides at the nonreducing termini of the chains. The average lengths of the CS/DS chains were 82 ± 24 and 83 ± 28 disaccharides in the posterior leaflet and the free edge of the anterior leaflet, respectively. Chain length in the center of the anterior leaflet was slightly less ($p = 0.081$) at 69 ± 19 disaccharides. The shortest chain lengths were found in chordae, with an average of 56 ± 27 disaccharides ($p < 0.001$ versus posterior leaflet and free edge of anterior leaflet). Regions of compression therefore had longer chain lengths than regions of tension.

Influence of subject demographics

The mitral valves used in this study were discarded from autopsies of normal humans who had no known or suspected history of any cardiac, cardiovascular (i.e., hypertension), or valvular dysfunction. This subject population was 46.4 ± 15.3 years old, 71% male/26% female (1 gender unknown), and 60% Caucasian/40% black. There were no significant differences in age between different genders or different races. Moreover, there were identical distributions of race and gender above and below the mean age. Likewise, the mean body surface area was identical for subgroups younger and older than the mean age and for difference races.

Because the age of the normal human autopsy subjects has been previously reported to influence the concentration of GAGs in heart valves (Baig *et al.*, 1978; Murata, 1981; Torii *et al.*, 1965), the individual GAG class concentrations were analyzed with respect to subject demographics. Subject age had a considerable influence on the content of the individual GAG classes in the tensile loading regions. Significant negative correlations were found between subject age and the concentration of ΔDiHA, glcA-6S-galNAc, glucuronate with 4-sulfated galNAc (glcA-4S-galNAc), idoA-4S-galNAc, total GAGs, and water (correlation coefficients and *p*-values in Table II, Figure 8). When the proportions of the different GAG classes were calculated relative to the total GAG quantity as measured by FACE, however, the relative proportions of idoA-4S-galNAc and idoA-4S-galNAc were positively correlated with age in the tensile loading regions. The proportion of ΔDiHA in tensile regions decreased slightly with age.

Table II. Correlation coefficients with respect to age

	R	*p*-value[a]
Tensile tissues		
Water concentration	−0.289	<0.001
ΔDiHA concentration	−0.396	<0.001
G6S concentration	−0.257	0.013
G4S concentration	−0.196	0.061
I4S concentration	−0.466	<0.001
Total GAG concentration	−0.409	<0.001
ΔDiHA proportion	−0.178	0.086
I6S proportion	0.764	0.002
I4S proportion	0.597	0.031
Chordae tendineae		
Chain length	−0.199	0.079
Compressive tissues		
Water concentration	−0.215	0.086
G6S concentration	−0.287	0.036
G4S concentration	−0.355	0.009
Total GAG concentration	−0.245	0.075
Chain length	−0.270	0.047

[a]All correlations for which $p < 0.10$ are shown.
[b]G6S and other abbreviations are defined at the end of the text.

In compressive tissues (the posterior leaflet and the free edge of the anterior leaflet analyzed together), there were significant negative correlations between age and the concentration of glcA-6S-galNAc and glcA-4S-galNAc (Table II). Age was correlated somewhat with the concentration of total GAGs and the concentration of water. The length of the CS/DS chains was significantly reduced with age in the compressive tissues, and was slightly reduced with age in chordae tendineae.

Subject gender was a significant factor in the content of individual GAG classes in the tensile loading regions, particularly in chordae. The concentration of ΔDiHA in tensile regions was 27% less in females than in males ($p = 0.05$). The concentration of idoA-4S-galNAc and total GAGs, respectively, were 35% and 28% greater in females ($p < 0.01$ for both). Furthermore the proportions of ΔDiHA and glcA-6S-galNAc in the tensile regions were 25% less ($p = 0.009$) and 17% less ($p = 0.034$) in females. The only significant gender difference in the compressive loading regions was a 16% lower proportion of glcA-6S-galNAc in females ($p = 0.032$).

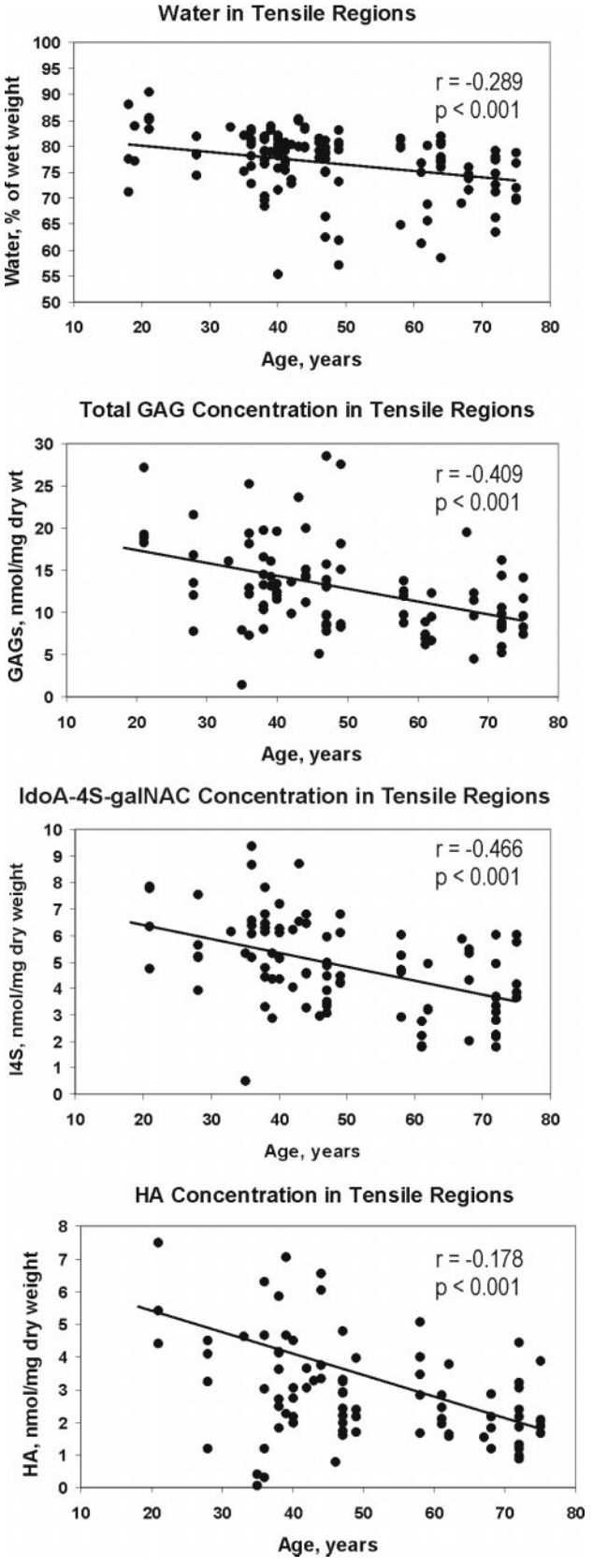

Fig. 8. Selected significant correlations between age and water or GAG composition in tensile regions of the mitral valve (see Table II). Water concentration was normalized to tissue wet weight, whereas GAG concentrations were normalized to tissue dry weight.

The influences of race and body surface area were also examined. Black subjects had an 89% greater concentration and 47% greater relative proportion of glcA-4S-galNAc in the posterior leaflet ($p = 0.013$ for both), and a 20% greater concentration of idoA-4S-galNAc in their chordae ($p = 0.05$). Caucasian subjects had a 42% greater concentration ($p = 0.045$) and a 25% greater proportion ($p = 0.049$) of glcA-6S-galNAc in the anterior leaflet free edge. There were no significant correlations between any biochemical variable and the subject body surface area.

PG measurements

Immunoblotting was used to confirm the identity of guanidine HCl–extracted, purified PG core proteins from different regions of the mitral valve. The core proteins for decorin (43 kDa doublet) and biglycan (43 kDa) were found in blots from all valve tissues. Versican core protein was also present in all valve tissues as lightly visible V0 (~450 kDa) and abundant V1 (~400 kDa) isoforms, in a 3:1 V1:V0 ratio. Because all the specimens from each valve were run together on the same gel, trends between regions of compressive and tensile loading were examined by normalizing band intensities from each PG to the band intensity from the anterior leaflet free edge for each individual valve (Figure 9). These ratios of band intensities generally agreed with our predicted findings based on the GAG results. Decorin was most abundant in the center of the anterior leaflet (tensile), present in lower quantities in the free edge of the anterior leaflet (compressive) and least abundant in the chordae tendineae (tensile) and posterior leaflet (compressive). Biglycan was again most abundant in the center of the anterior leaflet, less abundant in the chordae tendineae and anterior leaflet free edge, and least abundant in the posterior leaflet.

An ANOVA found a statistical difference between valve regions for the decorin and biglycan (both $p < 0.001$). In contrast, versican was present in greater quantities in the posterior leaflet and free edge of the anterior leaflet (compressive regions) with lower quantities in the chordae tendineae and center of the anterior leaflet (tensile regions), although this trend was not a significant difference, likely due to the small sample size. The decorin and biglycan samples were analyzed eight times to establish intraobserver variability as 3.9% for band quantitation, 5.5% for lane-lane repeatability from the same precipitated volume, and 9.7% for repeatability in separately precipitated samples from the same extraction volume. There were no statistical differences between data from different gel runs, precipitations, and GelPro analyses (paired t-tests, two-tailed $p > 0.05$). Due to the large volume of sample required to visualize the versican bands, the versican samples were not run in duplicate.

Discussion

This study showed that the components of the mitral valve apparatus that experience predominantly tensile loads, such as the central portion of the anterior leaflet and the chordae, contain relatively less water and less hyaluronan. The most abundant GAG was iduronate with 4-sulfated galNAc, and

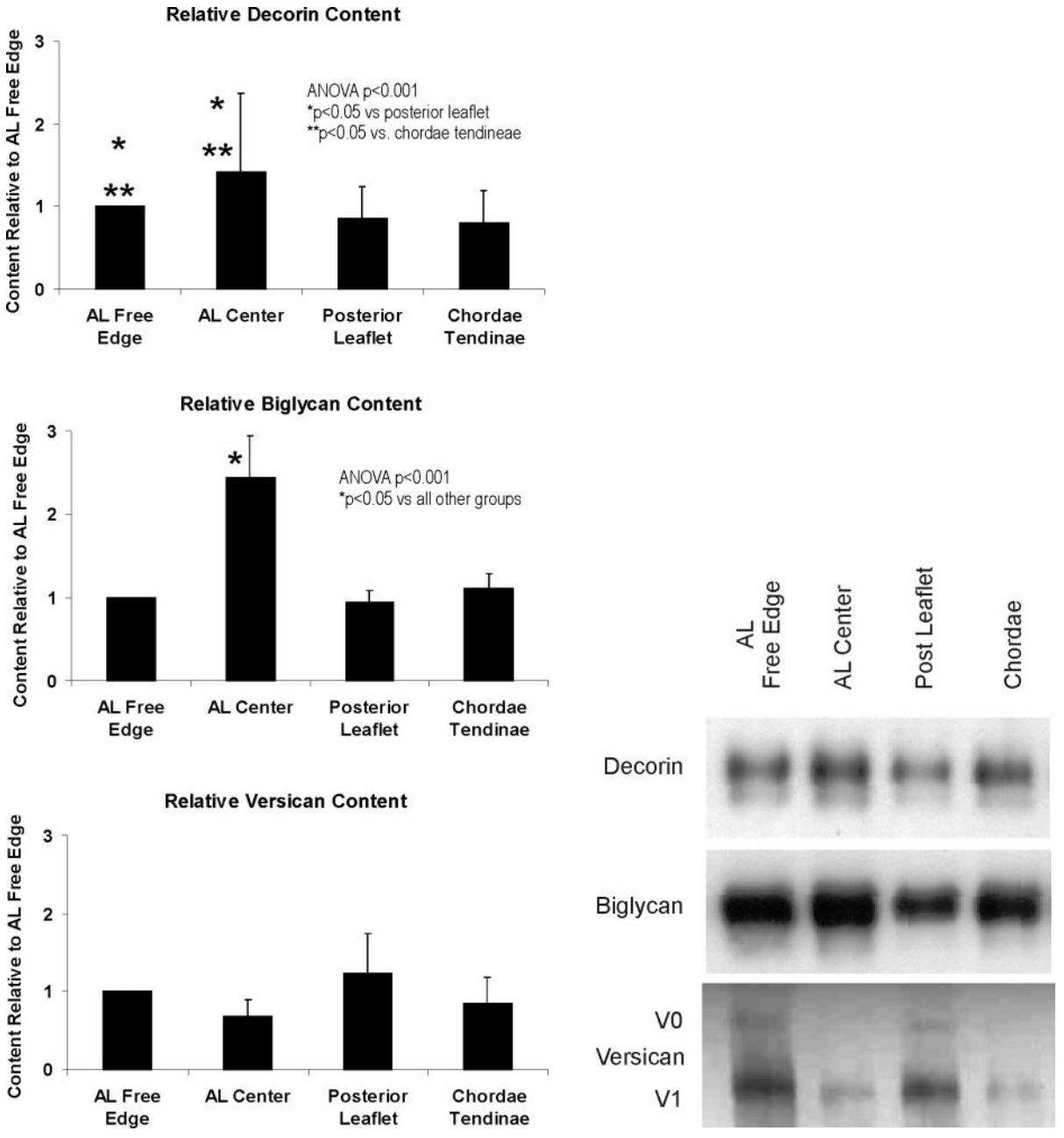

Fig. 9. (**a**) Relative abundances of the proteoglycans decorin, biglycan, and versican in different regions of the mitral valve, normalized to the specific proteoglycan content in the free edge of the anterior leaflet (mean ratios ± SD, $n = 5$, see text for explanation). Compressive regions = posterior leaflet and free edge of the anterior leaflet. Tensile regions = chordae tendineae and center of the anterior leaflet. (**b**) Representative western blots of the proteoglycans in these same leaflet regions.

the average chain length was 50–70 disaccharides. In the regions that experience predominantly compressive loads, such as the leaflet free edges and most of the posterior leaflet, there were longer GAG chains and elevated concentrations of water, ΔDiHA, and glucuronate with 6-sulfated galNAc. These data are supported by immunoblots that confirm the presence of versican, decorin, and biglycan in these valve tissues.

Although the division of the mitral valve into regions that experience tensile and compressive loading certainly simplifies its very complex loading regime, a rationale for this segregation is provided by the valve's mechanical and microstructural heterogeneity. For example, the elastic moduli of the center region and of the chordae tendineae are higher than those of the anterior leaflet free edge and the posterior leaflet (Clark, 1973; Kunzelman and Cochran, 1992; May-Newman and Yin, 1995). In addition, both the central portion of the anterior leaflet (adjacent to the annulus) (Cochran *et al.*, 1991) and the chordae (Millington-Sanders *et al.*, 1998) contain collagen fibers that are highly

oriented in the main loading direction, whereas the posterior leaflet and the free edge of the anterior leaflet have less collagen fiber alignment (Kunzelman, 1991) and a thicker GAG-rich spongiosa (Kunzelman *et al*., 1993).

Our findings agree well with previously published data, despite the fact that many of these previous studies did not differentiate between the 6-sulfated and 4-sulfated forms of galNAc. The GAG profiles of the compressive loading regions (predominantly ΔDiHA) match the published data from adult humans (Baig *et al*., 1978; Murata, 1981; Torii *et al*., 1965), although the data here contain a greater proportion of glcA-6S-galNAc than reported previously, likely due to our sectioning of the tissue into different loading regions. The profiles found in the tensile regions (dominated by idoA-4S-galNAc) resemble the reported profile of chordae tendineae (von Figura *et al*., 1973), although that study did not differentiate between sulfation patterns. The findings of this present study show better agreement overall with previous studies in human adults (Baig *et al*., 1978; Murata, 1981; Torii *et al*., 1965) than with studies of valves from young animals (Castagnaro *et al*., 1997; von Figura *et al*., 1973). These similarities show that FACE, a much more sensitive and convenient method, can be used in place of earlier techniques.

The correlations between GAG content and subject age confirmed or expanded on previous reports. The total concentration of GAGs in mitral valves decreased with age (Baig *et al*., 1978; Moretti and Whitehouse, 1963; Murata, 1981; Torii *et al*., 1965), as did the concentrations in the many GAG subclasses. When the GAG class concentrations were converted into proportions of the total GAG content, the proportion of idoA in the tensile regions of the mitral valve increased with age, as previously noted for heart valves overall (Moretti and Whitehouse, 1963; Murata, 1981; Torii *et al*., 1965). In the compressive regions, the reduction of glcA with age also confirms other reports (Moretti and Whitehouse, 1963; Torii *et al*., 1965). Although prior studies found that the proportion of HA either increased (Murata, 1981) or decreased with age (Moretti and Whitehouse, 1963; Torii *et al*., 1965), this study found no significant correlation between HA proportion and age in any tissue component of the normal mitral valve. Furthermore the contents of the GAG classes were not correlated with the subject body surface area, but gender and race had a mild effect. Although the relevance of these demographic influences is unknown, it is notable that the incidences of certain valvular cardiac diseases are also associated with age, gender, and race.

To hypothesize the type of PGs found in the tensile and compressive loading regions of the mitral valve, the GAG class concentrations and fine structure characteristics measured from mitral valves were compared with the reported characteristics of PGs found in cardiovascular soft tissues. The compressive loading regions contained high concentrations of water, ΔDiHA, and glcA, an average chain length of 83 disaccharides, and an average glcA-6S-galNAc/glcA-4S-galNAc ratio of ~2.7, which are similar to the characteristics of the PG versican. Versican, also known as PG-M, is a large PG that is synthesized by arterial smooth muscle cells (Chang *et al*., 1983; Schonherr *et al*., 1991), and has been found in aorta (Yao *et al*., 1994), skin (Zimmermann *et al*., 1994), and malignant tumors (Isogai *et al*., 1996). Versican consists of 15–20 CS chains (Schonherr *et al*., 1991) branching off a 430–460-kDa core protein (Chang *et al*., 1983; Schonherr *et al*., 1991; Yao *et al*., 1994). Versican CS chains are reportedly 43–45 kDa (85–100 disaccharides) in length (Chang *et al*., 1983) and composed of 60%–80% glcA (Schonherr *et al*., 1991) with a glcA-6S-galNAc/glcA-4S-galNAc ratio of at least 2.0 (Chang *et al*., 1983; Schonherr *et al*., 1991). Furthermore the versican GAG chains create macromolecular domains encompassing large volumes of solvent (Hardingham and Fosang, 1992), and versican itself aggregates with HA (Chang *et al*., 1983; Schonherr *et al*., 1991; Zimmermann and Ruoslahti, 1989). These macromolecular constituents are ideally suited to distribute changes in compressive load as a consequence of repetitive changes in strain.

The tensile regions, in contrast, contained a high concentration of idoA, an overall 6S/4S ratio of ~0.34, and an average chain length of 58 disaccharides, which are more indicative of the PGs decorin and biglycan. These two small PGs contain one and two GAG chains, respectively, of 25–40 kDa in length (50–80 disaccharides) (Fisher *et al*., 1989; Oldberg *et al*., 1996; Toole and Lowther, 1968; Wight *et al*., 1991). These PGs contain ~70% idoA with an overall 6S/4S ratio of 0.35 for idoA and glcA combined (Chang *et al*., 1983; Roughley and White, 1992). DS and, more specifically, decorin have been frequently found in tissues with an abundance of type I collagen, such as tendon (Junqueira and Montes, 1983; Toole and Lowther, 1968) and pericardium (Simionescu *et al*., 1988). Decorin binds tightly to type I collagen fibers and, based on the tensile forces, governs fibril formation and fibril diameter. Biglycan is similar to decorin but contains two GAG chains and may also have functional roles in highly collagenous tissues (Ameye *et al*., 2002). Furthermore, a small collagen-associated DS PG that was likely decorin was found in bovine valve tissues by Toole and Lowther (1965). The data from this study are also supported by the work of Daniel and Mills (1988), in which cells grown from the tensile region of rabbit flexor tendon produced small PGs that were only 48% susceptible to enzymatic digestion by chondroitinase AC, indicating a high proportion of idoA. Cells grown from the compressive region of the tendon, in contrast, produced large PGs that were wholly digestible by both chondroitinases ABC and AC, indicating that the GAGs present uniformly contained glcA. These data are very similar to our results. In addition, the concentration of collagen in heart valve tissues increases with age (Trnavsky *et al*., 1965), as does the proportion of idoA-4S-galNAc measured here, suggesting that these GAGs may be components of collagen-associated decorin and biglycan.

The hypothesized differential abundances of these PGs in tensile versus compressive loading regions of the mitral valve was partially confirmed by our immunoblotting of valve extracts. It was evident that versican, decorin, and biglycan were present in some quantity throughout the valves, which agreed with the earlier results showing that all regions had a wide distribution of GAGs. There were significant regional differences, however, in the relative amounts of decorin and biglycan, and versican was slightly more abundant in the compressive loading regions.

The difference in the GAG profiles and relative PG composition of the compressive as compared to tensile loading regions of the valve reflects the structural and functional differences of these regions. The long, multiple glcA-6S-galNAc chains on the PG versican extend away from the core protein to minimize electrostatic interactions. The resulting large hydrodynamic volumes are ideal to respond to variable compressive loads and to withstand high pulsatile forces (Wight *et al.*, 1991). Versican would thus allow the loosely layered appositional surfaces of the mitral valve to reversibly buffer the considerable impact and shearing deformations that occur during valve opening and closing. The core protein of versican also contains a HA-binding region (LeBaron *et al.*, 1992; Zimmermann and Ruoslahti, 1989) that anchors the PGs on long strands of hyaluronan and thereby retains them in the tissue. The combined versican-HA aggregate has been suggested to perform a lubrication and antiadhesive role in tissues by preventing ligand–receptor interactions between cells and matrix (Lemire *et al.*, 1996; Yamagata *et al.*, 1989). This lubrication could also aid in viscous dissipation for the impact forces during valve closure (von Figura *et al.*, 1973). In contrast, several characteristics of the GAG profile suggest the presence of decorin and biglycan in the more solid tensile loading structures of the valve. Decorin in particular is abundant in connective tissues with high concentrations of type I collagen (Bianco *et al.*, 1990), in which it stabilizes and orients collagen fibrils (Hedbom and Heinegard, 1989; Toole and Lowther, 1968). Decorin therefore may play a role in the overall strength of high load–bearing tissues. Biglycan, which typically does not colocalize with decorin, also contributes to collagen fibril diameter and tissue strength (Ameye *et al.*, 2002).

In conclusion, these data on GAGs and PGs from human mitral valves indicate that the regions in compression are rich in versican and contain abundant glucuronate and 6-sulfated galNAc, with little iduronate. Regions in tension are rich in decorin and biglycan and demonstrate the opposite pattern, with abundant iduronate (and almost exclusively 4-sulfated galNAc) but less glucuronate. The compositional patterns of mitral valve GAGs and PGs thus provide new insight into the roles of these molecules in load-bearing tissues.

Materials and methods

Normal mitral valves ($n = 35$) were obtained at autopsy from persons who died of noncardiac-related causes and were harvested after less than 24 h in cold storage. Our laboratory has determined that valves kept at 4°C retain their matrix-based mechanical properties for at least 5 days (Patel, 2003). We therefore believe that the matrix in these cadaveric valves was not measurably degraded during the postmortem period.

All chemicals were obtained from either Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA), except for glycerol, guanidine HCl, and proteinase-K (Invitrogen, Carlsbad, CA); hyaluronidase SD, chondroitinases ABC and ACII, and 2-B-1 antibody for versican (Seikagaku America, Falmouth, MA); Q-Sepharose Fast Flow beads and peroxidase-linked secondary antibodies (Amersham, Uppsala, Sweden); Triton X-100 (Roche, Indianapolis, IA); and 2-aminoacridone HCl (Molecular Probes, Eugene, OR). Monosaccharide electrophoresis running buffer and the preformed monosaccharide gels were purchased from Glyko (Novato, CA). The polyclonal antibodies LF136 for decorin and LF51 for biglycan were generously provided by Larry Fisher at the National Institute of Dental and Craniofacial Research, NIH.

Sample preparation

Mitral valves were prepared by first removing the chordae then sectioning the valve into anterior and posterior leaflets (Figure 1). The anterior leaflets were sectioned into the central region and the free edge. The chordae were trimmed from the leaflets at their point of attachment, and divided into four groups: anterior basal, anterior marginal, posterior basal, and posterior marginal. The wet weights of the leaflet samples and chordal samples (chordae were pooled into the four groups) were determined. The water content was calculated after lyophilizing the samples for 16 h and then weighing again to obtain the dry weight (Figure 2). The dried samples (typically 20–200 mg each) were stored in microcentrifuge tubes at −20°C prior to analysis.

GAG analyses

For subsequent analysis, each sample was minced with fine scissors and 1 ml of 100 mM ammonium acetate buffer (pH 7) added. A 100-μl aliquot of proteinase-K solution (10 mg/ml) was added to each sample followed by incubation for 16 h at 60°C. The completely solubilized digests were then heated at 100°C to inactivate the proteinase-K. An aliquot (100 μl) of each digest was taken to measure the quantity of uronic acid to estimate total GAG content (Figure 2) (Blumenkrantz and Asboe-Hansen, 1973) and hence to determine the volume required for the FACE analysis.

The samples were analyzed by FACE to quantify the different classes of GAGs (Calabro *et al.*, 2000b). Two aliquots containing 5 μg uronic acid were diluted to 100 μl in buffer and then incubated with either (1) 2 μl hyaluronidase SD + 3 μl chondroitinase ABC (each 10 mU/μl, termed HABC), or (2) 3 μl chondroitinase ACII (10 mU/μl, termed ACII) for 3 h at 37°C. The HABC treatment provides a measure of the minimum glcA content, allowing the maximum idoA contents to be estimated by subtracting the ACII data from the HABC data. One HABC digested aliquot was further treated with mercuric acetate to cleave the unsaturated hexuronate from the hexosamines and to determine the identity and quantity of any nonreducing terminal disaccharides. After digestion, the samples were dried, fluorotagged, mixed with glycerol and an internal standard (see later description), and electrophoresed on a monosaccharide gel as previously described (Calabro *et al.*, 2000a,b). The gel bands were imaged and analyzed using Gel-Pro (Media Cybernetics, Silver Spring, MD).

Known serially diluted quantities of a 2-sulfated chondroitin sulfate disaccharide (not found in valves) were added to individual samples to provide an internal calibration curve for fluorescence intensity (Figure 3). 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 density of the band(s), the calibration curve, and the aliquot volume used for the enzyme digest (Calabro *et al.*, 2000a,b).

The resulting GAG profiles were used to calculate several factors specific to particular PGs. The proportions of ΔDiHA to total CS/DS, as well as the ratio of ACII to HABC digestion products, assessed the regional balance of GAG classes and glcA/idoA (Figures 4–5). The ratios of 6-sulfation to 4-sulfation of the galNAcs were calculated for glcA- and idoA-containing disaccharides together and separately (Table I). The ratio of glcA to idoA was also calculated. Finally, the number average lengths of the CS/DS chains were estimated by calculating on a lane-by-lane basis the total amount of the CS/DS Δdisaccharides (except the 2S internal standard) and dividing by the total amount of saccharides at the nonreducing termini of the chains (Figure 3).

The measured GAG class contents from each sample were divided by the sample dry weight to estimate the tissue concentrations (Figure 6). The proportions of the different GAG classes were then calculated relative to the total GAG quantity as measured by FACE (Figure 7). Statistical comparisons between GAG class concentrations, proportions, or chain lengths in the different valve regions were calculated using a repeated measures ANOVA, followed by Tukey tests as needed, with two-tailed significance accepted at $p < 0.01$ (reduced due to numerous statistical comparisons between regions). Two-group comparisons (different genders and race) were conducted using *t*-tests with two-tailed significance accepted at $p < 0.05$. Correlation coefficients between the GAG class concentrations/proportions and subject age and body surface area (Figure 8) were calculated using linear regression with significance accepted at $p < 0.05$.

PG extraction and purification

Five fresh mitral valves were dissected into regional samples as described, lyophilized overnight, and reweighed to obtain dry weight. Each sample was minced with fine scissors in a 2-ml centrifuge tube and then agitated in extraction buffer overnight at 4°C (4 M guanidine HCl, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.05 M ammonium acetate, 0.01 M ethylenediamine tetra-acetic acid (EDTA), 0.1 M 6-aminohexanoic acid, 0.08% benzamidine HCl, 10 mM N-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 ml per 25 mg tissue dry weight). After extraction, the samples were centrifuged (13,000 rpm) and the supernatant dialyzed four times against 7 M urea buffer (containing 2 mM EDTA, 0.05 M Tris, 0.5% Triton X-100, pH 7.5) to remove the guanidine. After dialysis, volumes of extract solutions containing equivalent proportions of starting dry mass were mixed with Q-Sepharose beads, and the beads were rinsed with 40 column volumes of 7 M urea buffer containing 0.25 M NaCl. The bound purified PGs were eluted with six column volumes of 7 M urea buffer containing 3 M NaCl.

SD–PAGE and western blotting

Equivalent aliquots of the purified PG samples were mixed with water to a final volume of 300 μl and precipitated by adding 1 ml 95% ethanol/1.3% potassium acetate and incubating at −20°C for 2 h. The precipitate was suspended in 20 μl of enzyme digest solution (containing 2.5 mU/μl chondroitinase ABC, 0.01% bovine serum albumin (BSA), 0.05 M Tris, 3 mM Na acetate, 8 mM 6-aminohexanoic acid, 0.42 mM benzamidine HCl, and 0.08 mM PMSF) and incubated at 37°C for 3 h. Samples were then mixed with an equivalent volume of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and run on a 4%–12% SDS–PAGE gel at constant 180 V. The gel was transferred to a 0.2-μm nitrocellulose membrane at 100 V (1 h for decorin and biglycan, 3 h for versican). The membrane was blocked in Tris-buffered saline (TBS) with 0.1% Tween-20 and 2% BSA overnight at 4°C, then treated with primary antisera to decorin, biglycan, or versican (1:6000 dilution in TBS/Tween containing 2% fetal bovine serum) overnight at 4°C. After four washes in TBS/Tween, the membrane was treated with horseradish peroxidase–linked secondary antibodies (1:20,000 dilution in 2% BSA) for 2 h at room temperature, then washed six times more. Proteins were detected using chemiluminescent exposure to radiographic film. PG bands were identified by comparison with positive controls and quantified using densitometry. Each PG band (decorin, biglycan, or versican) was normalized by the content of the corresponding band from the anterior leaflet free edge sample run on the same gel (Figure 9).

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Abbreviations

ACII, chondroitinase ACII; ANOVA, analysis of variance; BSA, bovine serum albumin; CS, chondroitin sulfate; DS, dermatan sulfate; EDTA, ethylenediamine tetra-acetic acid; FACE, fluorophore-assisted carbohydrate electrophoresis; GAG, glycosaminoglycan; HA, hyaluronan; HABC, hyaluronidase SD combined with chondroitinase ABC; PG, proteoglycan; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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