Comparative Studies of the Acid Mucopolysaccharide Composition of Rheumatic and Normal Heart Valves in Man

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SUMMARY The nature and relative proportions of acid mucopolysaccharides (AMPS) in mitral valves of normal individuals and individuals with rheumatic valvular disease were compared. The total AMPS content of valves from rheumatic patients under 21 years of age is significantly higher than that from older rheumatic patients and higher than that from age-matched normal controls. The major AMPS components of both normal and rheumatic valvular tissue are hyaluronic acid, chondroitin sulfate A/C, and chondroitin sulfate B. Valves from normal individuals in all age groups studied contain approximately 65% hyaluronic acid, 25% chondroitin sulfate A/C, and 10% chondroitin sulfate B. Valves from rheumatic patients under 40 years of age contain the same proportions of these components as do normal valves. However, valves from rheumatic patients above 40 years of age show a marked alteration in AMPS composition; they contain 35% hyaluronic acid, 47% chondroitin sulfate A/C, and 18% chondroitin sulfate B. The differences in total AMPS content between valves from young rheumatic and nonrheumatic individuals may reflect changes that are secondary to the acute inflammatory process or an abnormality in AMPS synthesis following acute injury that is peculiar to the rheumatic host. The alterations in AMPS content and composition in valves of older rheumatic patients probably reflect changes in AMPS turnover associated with fibrosis that occurs in chronic disease of valvular tissue.

ACID mucopolysaccharides are primary components of the ground substance of valvular tissue. Torii and his coworkers described the nature of the acid mucopolysaccharide composition of human valvular tissue and the changes that occur in these components during the aging process. These investigators reported that differences in acid mucopolysaccharide content are encountered in valvular tissue from young individuals and that aging is associated with a decrease in acid mucopolysaccharide content of aortic, mitral, and tricuspid valves. Morphological studies by Sell and Scully revealed that aging of human valves is associated with fibrosis and degeneration of collagen fibers, a decrease in acid mucopolysaccharides, lipid accumulation, and progressive calcification. These changes are seen in both mitral and aortic valvular tissues but are more prominent in aortic valves.

No data are available on the composition and changes in acid mucopolysaccharide content of valvular tissue in rheumatic valvular heart disease. The present study was undertaken to determine whether differences are present in the acid mucopolysaccharide composition of rheumatic valvular tissue, as compared to normal tissue, and to determine the changes in valvular tissue acid mucopolysaccharide composition that are associated with acute and chronic valvular disease in rheumatic patients. Because the mitral valve is more frequently and specifically af-

Methods

Rheumatic heart valves were obtained from patients undergoing valvectomy at this Medical Center. Confirmation of the diagnosis of rheumatic fever was established by reviewing the patient's record for a history that fulfills the modified Jones Criteria for the diagnosis of acute rheumatic fever. In addition, anatomical and histological findings in valves obtained at surgery were considered in establishing the diagnosis of rheumatic valvular disease. Normal valves were obtained at autopsy from individuals who died of accidental death. The interval between death and autopsy was 6-12 hours. All valves were quick-frozen within 2 hours after removal and stored at −80°C until used.

Fully informed consent was obtained prior to surgery, and institutional and Departments of Health, Education, and Welfare guidelines for human investigation were followed during the course of these studies.

Valves were thawed, cleaned of adventitia, cut into 2- to 3-mm pieces, and thoroughly washed with saline to remove adhering plasma. Many of the valves from older patients had gross calcific nodules. As much as possible of this material was removed from the tissue before further processing. Fragmented tissue then was rinsed several times with water and extracted by continuous shaking in chloroform-methanol (1:1, vol/vol) for 3 days at room temperature. The delipidated and dehydrated valvular tissue was then air-dried and weighed.

Valvular tissue from individual heart valves was digested with pepsin under conditions described by Kanke and Mori. The digested valvular tissue was centrifuged at
10,000 rpm for 1 hour at 4°C to remove the papain-insoluble residue, and an equal volume of 20% trichloroacetic acid (TCA) was added to the clear supernatant fluid to precipitate nucleic acids and proteins. TCA precipitation was carried out overnight at 4°C; precipitates were removed by centrifugation and washed twice with 10% TCA. The supernatant fluid and the washes were combined, dialyzed against distilled water, lyophilized, and solubilized in 1 ml of 0.04 M sodium chloride. The AMPS were precipitated by the addition of cetylpyridinium chloride and incubation of the mixture at 37°C for 2 hours.

Extraction of individual acid mucopolysaccharides was carried out according to the method of Schiller et al. The complex was extracted with 0.4 M NaCl, followed by 1.2 M NaCl, and finally 2.1 M NaCl, to solubilize selectively hyaluronic acid, chondroitin sulfates and heparin, respectively.

The estimation of chondroitin sulfate A/C and chondroitin sulfate B was carried out according to procedures described by Torri et al. To the 1.2 M NaCl extract containing the chondroitin sulfates, four volumes of 100% ethanol were added in order to precipitate AMPS. Precipitation was allowed to take place for 4 days at 4°C. The precipitated AMPS were recovered by centrifugation, dissolved in distilled water, and reprecipitated with 80% ethanol. Finally, the precipitated AMPS were dissolved in 1.0 ml of 0.1 M acetate buffer, pH 5.2, containing 0.15 M NaCl and subjected to digestion with bovine testicular hyaluronidase by adding 300 IU of enzyme per 100 mg of uronic acid. The mixture was incubated at 37°C for 8 hours and dialyzed against the same buffer. The amount of nondialyzed chondroitin sulfate B was assayed, and the amount of chondroitin sulfate A/C was calculated by subtracting the amount of chondroitin sulfate B from total chondroitin sulfates.

The physicochemical purity of the various AMPS isolated from valvular tissue was established by using the following criteria. (1) Analysis of amino sugars: AMPS samples were hydrolyzed with 4 N HCl for 4 hours at 110°C in sealed ampuls. HCl was removed by evaporation under vacuum, and the nature of amino sugars was determined by thin layer chromatography of the hydrolyzed sample, as described by Moczar et al. Glucosamine and galactosamine were used as standards. (2) Analysis of uronic acids: Uronic acid components of AMPS samples were identified by descending paper chromatography of the hydrolyzed samples, as described by Radhakrishnamurthy and Berenson. 3 D-Glucuronic acid (Sigma) and D-iduronic acid isolated from chondroitin sulfate B of porcine skin were used as standards. (3) Cellulose acetate electrophoresis: Two buffer systems were used to check the purity of individual AMPS fractions, as recommended by Hata and Nagai. Purified hyaluronic acid, chondroitin sulfates A, B, and C, heparin, and heparin sulfate (kindly supplied by Dr. B. Mathews, University of Chicago) were run in parallel with the unknown samples. The two buffer systems used were: (1) 0.1 M pyridine-0.47 M formic acid, pH 3.0; electrophoresis was carried out at a constant voltage of 140 V with a current of 1 ma/cm for 45 minutes; (2) 0.1 M barium acetate, pH 8.0; electrophoresis was run at 100 V with a current of 1 ma/cm for 4 hours. After electrophoresis, cellulose acetate membranes were stained with 0.1% toluidine-blue for 2 minutes and destained with 1% acetic acid to visualize AMPS. (4) Hyaluronidase digestion: The purity of isolated AMPS was assessed further by studying their resistance or susceptibility to digestion by bovine testicular hyaluronidase. The hyaluronidase digestion of AMPS was carried out in 0.1 M acetate buffer, pH 5.2, with 0.15 M NaCl, as recommended by Torri et al.

Analytical Methods

Acid mucopolysaccharide content of samples was estimated by assaying uronic acid in the sample, using the carbazole method described by Dische, with D-glucuronic acid as standard. Hexosamine was determined by the Elson-Morgan reaction, as modified by Boas, following hydrolysis of samples in 4 N HCl for 4 hours at 110°C, using glucosamine as standard. Sulfate content of the AMPS sample was determined by the method of Antonopoulos.

Results

Initial studies were carried out to determine the contribution of tissue calcifications to the total dry weight of valvular tissue. Representative valves were obtained from rheumatic and nonrheumatic individuals in varying age groups. These were digested with papain, and the papain-insoluble residue was retrieved and weighed. Figure 1 shows the weight of this insoluble residue in relation to the total dry weight of the valves studied. For valves obtained from individuals, both rheumatic and nonrheumatic, under the age of 60 years, 5% or less of total dry weight is papain-insoluble residue. This residue accounted for about 10% of dry weight of valves obtained from normal individuals above the age of 60 years. In contrast, the average content of this residue in valves from rheumatic in age group.

![Figure 1: Proportion of insoluble residue remaining following papain digestion of normal and diseased valves from patients in various age groups.](https://example.com/figure1.png)
mative individuals older than 60 years is about 36%; in some cases it is as high as 80% of the dry weight.

Limited studies were performed to determine the nature of this papain-insoluble residue. An analysis of the material revealed that it was composed of approximately 40% calcium and did not contain any uronic acid residues. These results support the “calcific” nature of the insoluble residue and show that papain digestion effected complete solubilization of all acid mucopolysaccharides in the tissue.

Addition of TCA to the papain-digested valvular tissue resulted in complete recovery of acid mucopolysaccharides in the TCA-soluble fraction, as indicated by the absence of uronic acid-containing material in the TCA-insoluble material. Dialysis of the TCA-insoluble fraction against distilled water followed by analysis of acid mucopolysaccharide content showed no appreciable loss of acid mucopolysaccharides from the dialysants. The precipitation of acid mucopolysaccharides by cetylpyridinium chloride as acid mucopolysaccharide-cetylpyridinium chloride complex was complete, as revealed by absence of uronic acid containing material in the cetylpyridinium-soluble fraction. The aqueous acid mucopolysaccharide extract was devoid of protein and nucleic acid contaminants, as determined by absorption at 280 and 260 nm.

**Total Acid Mucopolysaccharide Content**

As can be seen in Figure 2, there is a gradual decrease in the total acid mucopolysaccharide content of valvular tissue with increasing age in both rheumatic and nonrheumatic individuals. Differences in total acid mucopolysaccharide content among the various age groups and between normal and rheumatic valves were not significant, except for the valves obtained from individuals under the age of 21 years. Valves obtained from rheumatic patients in this age group contained a mean total so of 914 ± 144 µg/100 mg dry tissue of AMPS, as compared to 628 ± 74 µg/100 mg (mean ± sd) for valves from age-matched normal controls. This difference was highly significant (P < 0.001).

**Identification of Individual Acid Mucopolysaccharides**

The qualitative nature and purity of the individual acid mucopolysaccharides solubilized at various salt concentrations from extracts of rheumatic and nonrheumatic valvular tissues were examined.

Analysis of hexosamine and uronic acid components of the 0.4 M NaCl-soluble AMPS revealed the presence of D-glucosamine and D-glucuronic acid only, suggesting that the component solubilized was hyaluronic acid. The purity of the hyaluronic acid recovered was confirmed by cellulose acetate electrophoresis of the 0.4 M NaCl soluble AMPS in the two-buffer systems. This step resulted in the detection of a single component with electrophoretic mobility similar to that of the reference purified hyaluronic acid. Incubation of 0.4 M NaCl-soluble AMPS with bovine testicular hyaluronidase resulted in the disappearance of the hyaluronic acid band in cellulose acetate electrophoresis, and complete loss of material following dialysis indicated the complete digestion by hyaluronidase.

Prior to subfractionation of the 1.2 M NaCl-soluble AMPS into chondroitin sulfate A/C and chondroitin sulfate B, the nature of amino sugars present in this fraction was investigated. Only D-galactosamine was found, suggesting that the 1.2 M NaCl-soluble fraction was free from hyaluronic acid and heparin contaminants.

The hyaluronidase resistant fraction from the 1.2 M NaCl soluble fraction also was subjected to qualitative sugar analysis and cellulose acetate electrophoresis to establish its purity. The only uronic acid found in this fraction was L-iduronic acid, and D-galactosamine was the only amino sugar detectable. A single component was detected on cellulose acetate electrophoresis in the two-buffer system. This component was similar in mobility to purified chondroitin sulfate B. Although chondroitin sulfates A and/or C never were isolated as such, their presence in the 1.2 M NaCl-soluble fraction was shown by the following findings: (1) Cellulose acetate electrophoresis of 1.2 M NaCl-soluble AMPS fraction resulted in the detection of two separate components. One component was distinct and similar in its electrophoretic mobility to chondroitin sulfate C. (2) Qualitative analysis of the uronic acid contents of the 1.2 M NaCl-soluble AMPS revealed the presence of L-iduronic acid as well as D-glucuronic acid. These results suggest that 1.2 M NaCl-soluble fraction contained chondroitin sulfate B and chondroitin sulfate A and/or C. (3) Treatment of 1.2 M NaCl-soluble fraction with hyaluronidase followed by cellulose acetate electrophoresis resulted in the complete disappearance of components which migrated with chondroitin sulfate A/C.

The results of these studies indicate that valvular tissue is composed of a limited number of acid mucopolysaccharides which include hyaluronic acid, chondroitin sulfate A/C, and chondroitin sulfate B. A trace amount of material, which contained less than 1% of the total salt-extractable acid mucopolysaccharide, also was extracted from both normal and rheumatic valvular tissue in the 2.1 M NaCl fraction. Because of its low yield, this fraction could not be identified. Its solubility in 2.1 M NaCl suggests that it might be heparin. These results reveal...
that identical acid mucopolysaccharides are found in normal and rheumatic valvular tissue.

**Proportions of Individual Acid Mucopolysaccharides**

The relative proportions of individual acid mucopolysaccharides found in mitral valves of rheumatic and nonrheumatic individuals are outlined in Table 1. Normal valves from all age groups showed a similar AMPS composition, consisting of 64–66% hyaluronic acid, 21–27% chondroitin sulfate A/C, and 10–14% chondroitin sulfate B. The AMPS composition of mitral valves from rheumatic individuals up to 40 years of age was similar to their age-matched normal controls. Although valves from young rheumatic patients, under 21 years of age, were richer in their total AMPS content than normal controls, they were similar in their AMPS composition. In contrast, a marked difference in AMPS composition between rheumatic and nonrheumatic valves was apparent in individuals more than 40 years old. This change is characterized by a highly significant decrease in hyaluronic acid content and a rise in chondroitin sulfates. As noted, valves of rheumatic individuals older than 40 years contain about half the amount of hyaluronic acid and twice as much chondroitin sulfates as was present in normal valves.

**Discussion**

This study reveals that calcific changes and alterations in acid mucopolysaccharide composition occur with advancing age in mitral valve tissue of patients with rheumatic valvular disease. These age-associated changes are similar but more pronounced than those reported previously for nonrheumatic individuals.2,3 Two additional differences between rheumatic and nonrheumatic valves also were encountered. The total acid mucopolysaccharide content of valves from young rheumatic patients was higher than their age-matched controls, and a significant alteration in the proportions of individual acid mucopolysaccharides occurred with advancing age in valves of rheumatic individuals but did not occur in valves of nonrheumatic controls.

Torii et al.2 reported that the acid mucopolysaccharide content of valvular tissue from young “normal” individuals was significantly higher than that of valves from older individuals. Although we were not able to confirm this finding, our data show that such a difference exists between rheumatic individuals. The mean total acid mucopolysaccharide content of valves from rheumatic patients 20 years old and younger is significantly higher than the value obtained for rheumatics in the 21- to 40-year age group (914 ± 144 µg/100 mg vs. 606 ± 130 µg/100 mg of dried tissue; P < 0.03). In addition, the mean value for the total acid mucopolysaccharide content in rheumatics under 21 years of age is significantly higher than their age-matched normal controls (P < 0.001). No significant difference was observed in the total acid mucopolysaccharides of valves of older rheumatic and nonrheumatic individuals.

One of the variables that could influence the total acid mucopolysaccharide content, as expressed in this study, is the degree of calcification present in the tissue. The results presented by Torii et al.2 do not include data on calcific content of the dried tissue. Based on morphological and microscopic changes, Sell and Scully3 showed that calcification was detectable in valvular tissue of normal individuals at the age of 41 years, remained at the same level until the age of 60 years, and became more apparent after the 6th decade of life. In our study, the amount of calcific material found in normal and rheumatic valves was determined quantitatively. Our results show that calcific residue in normal mitral valves remains at a low level until the age of 60 years and, as described by Sell and Scully,3 increases appreciably thereafter. This pattern also is seen in rheumatic valves. However, the amount of calcific residue encountered in valves of rheumatic individuals older than 40 years is approximately 3-4 times greater than that of age-matched normal controls. Thus, significant differences in calcification of tissue occur only in older individuals, and the changes in total acid mucopolysaccharide content encountered in young individuals should not be influenced by calcific changes in these valves.

### Table 1  Acid Mucopolysaccharide Composition of Normal and Rheumatic Mitral Valves

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Source</th>
<th>No. analyzed</th>
<th>Hyaluronic acid (mean ± SD)</th>
<th>Chondroitin sulfate A/C (mean ± SD)</th>
<th>Chondroitin sulfate B (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;21</td>
<td>Normal</td>
<td>7</td>
<td>65 ± 5</td>
<td>21 ± 5</td>
<td>14 ± 5</td>
</tr>
<tr>
<td></td>
<td>Rheumatic</td>
<td>5</td>
<td>69 ± 8</td>
<td>21 ± 5</td>
<td>12 ± 8</td>
</tr>
<tr>
<td>21-40</td>
<td>Normal</td>
<td>8</td>
<td>64 ± 6</td>
<td>24 ± 4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>Rheumatic</td>
<td>3</td>
<td>57 ± 19</td>
<td>29 ± 11</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>41-60</td>
<td>Normal</td>
<td>7</td>
<td>66 ± 2†</td>
<td>23 ± 2†</td>
<td>12 ± 2†</td>
</tr>
<tr>
<td></td>
<td>Rheumatic</td>
<td>6</td>
<td>33 ± 7</td>
<td>47 ± 4</td>
<td>21 ± 9</td>
</tr>
<tr>
<td>&lt;60</td>
<td>Normal</td>
<td>4</td>
<td>64 ± 7†</td>
<td>27 ± 4†</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>Rheumatic</td>
<td>6</td>
<td>37 ± 3</td>
<td>47 ± 7</td>
<td>15 ± 5</td>
</tr>
</tbody>
</table>

*The recovery of acid mucopolysaccharides following fractionation was better than 95%.

†P < 0.001 compared to rheumatic.
The data on the nature of the individual acid mucopolysaccharides and their relative proportions in normal valvular tissue are also similar to those reported by Torii et al.2 Calcification of valvular tissue, unlike its effect on total acid mucopolysaccharide content, should not affect the comparative data on differences in the relative proportions of the individual acid mucopolysaccharides encountered between normal and rheumatic valvular tissue. As also reported by the above investigators, our results indicate that aging does not induce any alteration in the nature of these components or their relative proportion in normal valvular tissue. No difference in the relative proportion of individual acid mucopolysaccharides was encountered between rheumatic and normal valvular tissue in individuals under 40 years of age. However, significantly lower hyaluronic acid content and proportionately higher chondroitin sulfate content were found in older rheumatoid valvular tissue as compared to valvular tissue of age-matched normal controls.

A factor that could contribute to the observed difference in total AMPS and relative AMPS composition between normal and diseased valves is the difference in the method of procurement of tissue. Normal valves were removed and frozen at varying intervals following death, and diseased valves were obtained and frozen within minutes following surgical resection. To assess whether the interval between death and necropsy could have affected the total AMPS or the individual composition of the valves, the values obtained for total AMPS and hyaluronic acid content were correlated with the time interval between death and necropsy. No significant correlation was obtained between either total AMPS and hyaluronic acid content and time interval between death and valve removal ($r = 0.1$ and 0.13; $P = 0.45$ and 0.56). The close similarity between the acid mucopolysaccharide composition values for normal valves obtained by us and by Torii et al.5 further indicate that the interval between death and valve resection does not appreciably influence these values.

The results of this study indicate that mitral valves of young individuals with rheumatic valvulitis contain significantly more acid mucopolysaccharides than age-matched nonrheumatic controls. With advancing age, this difference disappears. However, a change in the relative proportions of individual acid mucopolysaccharide components is encountered in older individuals. The latter differences are, in all probability, secondary to the chronic inflammatory process involving the diseased valves. The differences encountered in the valves of younger individuals could reflect either tissue changes that are the consequence of acute inflammation, an intrinsic abnormality in acid mucopolysaccharide composition of rheumatic valvular tissue, or both; that is, there may be an abnormality in the synthesis of tissue ground substance following acute tissue injury that is peculiar to rheumatic individuals. Such an abnormality would be similar to that observed for collagen synthesis in keloid and hypertrophic scar formation of human skin.13 Recent studies report differences in collagen synthesis in sclerotic areas of mitral valves, as compared to nonsclerotic areas of the same tissue.14 A pertinent extension of this observation would be the study of collagen content and synthesis, as well as the synthesis of acid mucopolysaccharides in rheumatic and nonrheumatic valvular tissues. The finding of differences would suggest that an abnormality in the synthesis of ground substance and collagen may play a role in the evolution of chronic valvular disease in rheumatic individuals.

References

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