Biochemical Analysis and Electron Microscopy of Human Mitral Valve Collagen in Patients with Various Etiologies of Mitral Valve Diseases

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SUMMARY

Biochemical analyses and electron microscopy of mitral valve collagen were carried out in 29 patients with various mitral valve diseases. They were divided into 3 groups: (1) rheumatic heart disease (15 patients), (2) isolated rupture of chordae tendineae (8 patients), and (3) floppy mitral valve (6 patients). Normal mitral valves obtained at necropsy from 6 patients who died from extra-cardiac causes were used for control observations. Results of the electrophoretic analysis of the collagen of normal and diseased valves showed that all valves contained collagen types I, III, and AB collagen with similar electrophoretic patterns. Electron microscopic observations and comparisons of the segment-long-spacing crystallites of each type of collagen revealed similar band patterns, irrespective of the normal or diseased valves sources. It is concluded that the composition and primary molecular structure of mitral valve collagen are usually not altered in a wide variety of disease processes affecting the mitral valve.

Additional Indexing Words:
Mitral valve collagen Segment-long-spacing crystallites

RECENT advances in the applications of biochemical analyses and electron microscopy to the study of the molecular structure of collagen have promoted a surge of interest and new understanding on collagen pathology in valvular heart disease.1-6) Although pathological findings of mitral valvular diseases with a variety of etiologies have been characterized in a number of studies, relatively little is known of the contribution of altered collagen to the
pathogenesis of mitral valve disorders, particularly the syndrome of floppy mitral valve and spontaneously isolated rupture of chordae tendineae.\textsuperscript{11,4} Since heart valves are composed predominantly of collagen proteins, the study of these proteins should lead to a better understanding of cardiac valvular pathology. To date, there is no detailed report dealing with the molecular properties of human mitral valve collagen in disease states. Therefore, it is the purpose of the present report to describe the results of a combined study using biochemical analyses and electron microscopy to investigate the molecular structure of mitral valve collagen in patients with various forms of mitral valvular disease, with particular emphasis on the role of collagen defects in the pathogenesis of a floppy mitral valve and spontaneously isolated rupture of chordae tendineae.

**Materials and Methods**

This study used tissue from 29 patients who underwent mitral valve replacement due to various mitral valve diseases. These patients were 21 men and 8 women, ranging in age from 17 to 68 years. They were divided into 3 groups according to the etiologic diagnosis, which was confirmed by operative and pathological findings. The rheumatic heart disease group included 15 patients (mitral stenosis alone in 10 and mitral steno-insufficiency in 5). The spontaneously isolated rupture of chordae tendineae group consisted of 8 patients. The remaining 6 patients had a floppy mitral valve.

The diseased mitral valves were obtained during open heart surgery, while the normal valves used as the control for comparative study were obtained at necropsy from 6 patients who died from extracardiac causes. Immediately after the excision of the mitral valves, all valves were washed with cold normal saline to remove blood and were carefully freed of muscle. Each valve was cut into small pieces, and then individually homogenized in a homogenizer (Kinematic) in 0.9 M NaCl, 50 mM Tris pH 7.5. The subsequent procedures were all performed at 4°C. The specimens were then subjected to further extraction in two changes of the same solution for 48 hours and in three changes of 0.5 M acetic acid for 72 hours to remove soluble proteins.\textsuperscript{7} The insoluble residue was spun down by centrifugation and washed 3 times with 0.5 M acetic acid. The extracted tissue was resuspended in a solution of pepsin (Sigma, twice recrystallized, 2,750 units/mg protein) in 0.5 M acetic acid to give a 1:100 (E:S) wet weight. The mixture was then stirred at 4°C for 24 hours and pepsin solubilized collagen was clarified by centrifugation at 27,000×g for 30 min.\textsuperscript{8} After centrifugation, the pellet of undigested tissue was reextracted with the same concentration of pepsin and
Fig. 1. A diagram of the isolation and purification procedures for types I, III, and V collagen.

centrifuged. Both supernatants were combined for further isolation. Subsequent procedures are outlined in Fig. 1.

Collagen isolation and subsequent purification:

Pepsin solubilized collagen was filtered through a thin layer of pyrex wool (Corning). Collagen was precipitated by dialysis into 20 mM Na₂HPO₄ at 4°C. The precipitates were redissolved in 0.2 M acetic acid and dialyzed into 0.86 M NaCl in 50 mM Tris-HCl (pH 7.5), according to the method described by Brown et al. After removal of precipitates, the supernatant was precipitated by further dialysis into 1.5 M NaCl and 2.6 M NaCl in 50 mM Tris-HCl (pH 7.5) to obtain type III and type I collagen, respectively. The supernatant was then dialyzed against 3.2 M NaCl in 50 mM Tris-HCl (pH 7.5) to remove contaminating type I collagen. A further dialysis into 4.0 M NaCl precipitated AB collagen. All of the precipitations were repeated 3 times. The precipitates which formed on dialysis were redissolved in 0.1 M acetic acid, desalted (by dialysis extensively against 0.1 M acetic acid) and lyophilized.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis:

Collagen proteins were analysed on a 5% SDS-polyacrylamide slab gel.
Fig. 2. SDS-polyacrylamide gel electrophoresis of pepsin-soluble mitral valve collagen showed similar banding patterns and peaks in the normal control and diseased groups.

SDS = sodium dodecyl sulfate.
A: normal control, B: rheumatic heart disease, C: isolated rupture of chordae tendineae, D: floppy mitral valve.
1: \( \beta_{1-1} \), 2: \( \beta_{1-2} \), 3: \( \beta_{2-2} \), 4: \( \alpha B \), 5: mixed bands of \( \alpha_1 (I) \), \( \alpha_1 (III) \), and \( \alpha A \), 6: \( \alpha_2 (I) \).

Samples of lyophilized native collagen were dissolved in a Tris (10 mM), boric acid (6 mM) solution at pH 8.5, containing 2 M urea, 0.2% SDS, and 10% glycerol. They were denatured at 100°C for 3 min either with or without 50 mM dithiothreitol. The samples were run at 30 mA at room temperature until the bromophenol blue tracing dye reached the end of the gel (approximately 3 1/2 hours). Both the gel and electrode buffer were Tris (50 mM), and boric acid (30 mM) at pH 8.5. The slab was then sliced to remove the stacking gel and stained overnight in a solution of 50% methanol (V/V), and 10% acetic acid (V/V) containing 0.05% Coomassie Brilliant Blue G 250 (Bio-Rad). The gel was destained in a solution of 7.5% acetic acid and 7.5% methanol until clear. Bands of proteins were scanned at 595 nm by using Clini Scanner (Helena Laboratories).

*Segment-long-spacing (SLS) crystallites:*

SLS crystallites of each type of collagen were prepared by dialysis of each
Fig. 3. SDS-polyacrylamide gel electrophoresis of each collagen type, obtained from the normal and diseased mitral valves by differential salt precipitation. Banding patterns of each type of collagen were identical in the normal control and diseased groups.

SDS = sodium dodecyl sulfate.
A: normal control, B: rheumatic heart disease, C: isolated rupture of chordae tendineae, D: floppy mitral valve.
I: Type I collagen, III: Type III collagen, V: Type V collagen.

Collagen solution against 0.4% ATP in 0.1 M acetic acid for 72 hours (two changes), according to the method described by Timpl et al. SLS crystallites were then negatively stained with phosphotungstic acid (0.4%, pH 3.5) and uranyl acetate (1%, pH 4.5), and examined with a Hitachi H 500 electron microscope.

RESULTS

Polyacrylamide gel electrophoresis:

The electrophoretic analyses of the pepsin-soluble collagen of mitral valves from normal control and diseased groups are presented in Fig. 2. The values from all groups contained types I, III, and AB collagen. The individual electrophoretic patterns of the collagen were similar in the normal and diseased groups. Further purification of each type of collagen from the normal and diseased valves was achieved by differential salt precipitation. Polyacrylamide gel electrophoresis of each collagen type also resulted in similar band patterns in the normal and diseased groups (Fig. 3).
Fig. 4. Segment-long-spacing crystallites prepared from type I collagen of the normal and diseased mitral valves showing identical band patterns in the normal control and diseased groups. The bands have been numbered according to Bruns et al.\textsuperscript{12} and Wiedemann et al.\textsuperscript{13} Horizontal bar=20 nm (×500,000).

A: normal control, B: rheumatic heart disease, C: isolated rupture of chordae tendineae, D: floppy mitral valve.

**Electron microscopy of segment-long-spacing crystallites:**

In order to compare the number, location, and width of the SLS band patterns, the individual dark bands were identified and numbered according to Bruns et al.\textsuperscript{12} and Wiedemann et al.\textsuperscript{13} The most distinct dark bands, which are bands 15 and 37–40 in type I and type III molecules, were used as the corresponding regions for matching the distribution of stained dark bands and unstained light bands in the SLS crystallites of each collagen type prepared from normal and diseased valves. Representative SLS crystallites prepared from each collagen type of normal and diseased valves are shown in Figs. 4–6. In general, the individual bands in well-formed crystallites were clearly defined in type I and type III collagen molecules. A comparison of the band patterns appearing in well-resolved crystallites of type I molecules revealed no significant differences between the control and diseased groups (Fig. 4). The band patterns of type III crystallites were also virtually identical in the normal and diseased groups (Fig. 5). The individual bands of
AB collagen molecules were usually not defined in a satisfactory manner. However, detailed observation and comparison of cross-striation patterns of AB collagen crystallites showed no differences between normal and diseased groups (Fig. 6). In summary, although minor variations in the staining intensity of bands may exist in the SLS crystallites of each collagen type, we have generally confirmed the similarity of the band patterns in each type crystallite by examining and comparing SLS crystallites prepared from normal and diseased valves.

**DISCUSSION**

Although there have been a few reports concerning the role of altered collagen composition in the development of mitral valve dysfunction, a systemic analysis of the molecular properties of the collagen of diseased mitral valves has not been attempted. The present study has used biochemical analyses and electron microscopy to evaluate the molecular basis of the con-
Fig. 6. A comparison of the band patterns in segment-long-spacing crystallites prepared from type V collagen of the normal and diseased mitral valves showing no significant differences between normal control and diseased groups. Note that the band patterns between the arrows are more clearly identified.

Horizontal bar=20 nm (×500,000).
A: normal control, B: rheumatic heart disease,
C: isolated rupture of chordae tendineae, D: floppy mitral valve.

DISTRIBUTION OF COLLAGEN DEFECTS TO THE PATHOGENESIS OF MITRAL VALVE DISORDERS, PARTICULARLY THE FLOPPY MITRAL VALVE AND THE SPONTANEOUSLY ISOLATED RUPTURE OF CHORDAE TENDINEAE. The six control valves (which were considered normal) consistently demonstrated the presence of types I, III, and AB collagen in polyacrylamide gel electrophoresis. Regardless of the diseased group, the electrophoretic patterns of the mitral valve collagen were similar to those in the control group; all three collagen species were found in the diseased valves. Further analyses of each types of collagen molecules by electrophoresis also revealed no significant differences in the banding patterns between the normal and disease valves. Thus, there were no differences in the collagen compositions of the normal or diseased valves.

The application of electron microscopy to the amino acid sequence analysis of collagen is possible because of the unique properties of the collagen molecules, which align in perfect transverse register to form crystallites in the presence of adenosine triphosphoric acid at low pH.8)12)–16) When stained with solutions of phosphotungstic acid and/or uranyl acetate, the crystallites, segment-long-spacings that are the same length as the collagen molecule,
reveal a characteristic banding pattern which reflects the distribution of clusters of charged and uncharged amino acids along the molecule.\textsuperscript{12,13} The locations of dark bands along the segment indicate the distribution of polar amino acids along the molecule. Therefore, the banding pattern of the SLS crystallites has been used primarily in a qualitative manner for the study of the molecular structure of collagen.\textsuperscript{12,13} In the present study, a detailed comparison of the banding patterns of each type of collagen molecule showed no significant differences in the number, location, and width of the band patterns along the SLS crystallites of the normal and diseased valves. Recently, Hammer et al\textsuperscript{4} reported the absence of two collagen species in a patient with a prolapsed mitral valve and ruptured chordae tendineae, and suggested that an altered collagen composition contributed to the development of his mitral valve disorder. However, our patients with a floppy mitral valve and/or ruptured chordae tendineae did not display alterations in the collagen composition. Thus, the report of Hammer et al\textsuperscript{4} is a particular case which should not be construed to suggest that all mitral valve prolapse and/or ruptured chordae tendineae are a result of altered collagen composition. In summary, both normal and diseased mitral valves contained types I, III, and AB collagen in electrophoretic analyses, and had similar electrophoretic patterns for each collagen type. Electron microscopic observation and comparison of the SLS crystallites of each collagen type revealed similar banding patterns in the normal or diseased valves. Thus, it is suggested that the composition and primary molecular structure of mitral valve collagen are usually not altered in a variety of disease processes affecting the mitral valve.

\textbf{REFERENCES}


