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Collagen composition of normal and myxomatous human mitral heart valves

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The collagens were studied in 13 normal and 19 myxomatous human mitral valves. The collagens of the valve were completely solubilized by using a method consisting of guanidinium chloride extraction, limited pepsin digestions and CNBr cleavage of the residue. The normal valves contained 74% type I, 24% type III and 2% type V collagen. The type I and type III collagens had similar solubility patterns, although only type I collagen was detected in the guanidinium chloride extract. Type V collagen was only detected in the first pepsin extract. The type I and III collagens had higher contents of hydroxylysine than did the same collagens from age-matched dermis. The two-dimensional electrophoretic 'maps' of CNBr-cleavage peptides showed low recoveries of the *C*-terminal α1(I) CB6 and α1(III) CB9 peptides, which are involved in forming intermolecular cross-linkages. Most of the reducible cross-linkages were present in large-M_r peptide complexes, and these complexes were shown by labelling with ^{125}I to include the tyrosine-containing α1(I) CB6 peptide. The myxomatous valves contained 67% type I, 31% type III and 2% type V collagens. There was a significant increase in the concentration of each type of collagen, which consisted of a 9% increase of type I collagen, a 53% increase of type III collagen and a 25% increase of type V collagen. The contents of hydroxylysine in type I and III collagens and the electrophoretic 'maps' of the CNBr-cleavage peptides involved in cross-linkages did not differ significantly from the results obtained from the normal valves. The biochemical findings suggest that there is an increased production of collagen, in particular type III collagen, and glycosaminoglycan as well as a proliferation of cells as part of a repair process in the myxomatous valves.

The mitral valve of the heart consists of the annulus, valve leaflets, chordae tendineae and papillary muscles (Roberts & Perloff, 1972). The collagen fibres of the mitral valve prevent the leaflets from prolapsing into the left atrium while the left ventricle is contracting. However, prolapse of the human mitral valve leaflets into the left atrium occurs as an apparently isolated abnormality in 4–6% of many populations (Procacci *et al.*, 1976; Hickey *et al.*, 1981). In its severe form, the collagen fibres of the lamina fibrosa of the prolapsed leaflets are grossly disorganized and there is an accumulation of glycosaminoglycan, which gives the leaflets a myxomatous appearance (Davies *et al.*, 1978). Hammer *et al.* (1979) described a myxomatous human mitral valve that lacked type III and type V collagens, whereas the normal valves contained type I, type III and type V collagens. In a study of ten myxomatous valves, Bonella *et al.* (1980) described an accumulation of procollagen that resembled the biochemical abnormalities reported in the type VII form of the Ehlers–Danlos syndrome, in which there is diminished activity of procollagen N-proteinase (Lichtenstein *et al.*, 1973). These reports by Hammer *et al.* (1979) and Bonella *et al.* (1980) suggest that a variety of collagen defects may exist in myxomatous mitral valves.

We have studied the collagens in 13 normal and 19 myxomatous human mitral valves. We have developed a new method for completely solubilizing the collagens of the valve, and with this method we have found a greatly increased amount of type III collagen and a smaller increase of type I and type V collagens in the myxomatous valves. We also show that the type I and type III collagens

Abbreviation used: SDS, sodium dodecyl sulphate.

from the normal and myxomatous valves contain higher contents of hydroxylysine than the same collagens from age-matched dermis and that the electrophoretic patterns of CNBr-cleavage peptides involved in cross-linkages are similar to the patterns obtained from tendon and bone.

Experimental

Materials

Ampholines were supplied by LKB Instruments, Rockville, MD, U.S.A. Dermatosparactic collagen was a gift from Dr. C. Lapiere, Liège, Belgium. Sources of other materials used have been previously described (Cole & Chan, 1981).

Human mitral valves

Normal mitral valves and skin biopsies were obtained at autopsy from 13 adults (aged 52±9 years) who had died from diseases that did not affect the mitral valve or the connective tissues. The myxomatous valves were obtained, during mitral-valve replacement surgery, from 19 adults (aged 61±7 years). The valves were immediately frozen at −70°C. The collection of these specimens was approved by the Ethics Committee of the Hospital. Five patients had first-degree relatives with prolapsing mitral valves, whereas the remaining patients appeared to be sporadic cases. None of the patients had features of the Marfan's syndrome or of any other generalized connective-tissue disorder.

Gross composition of mitral valves

Blood was washed from the surface of the valves with a solution containing 0.9% NaCl and the proteinase inhibitors 1mM-phenylmethanesulphonyl fluoride, 10mM-*N*-ethylmaleimide, 5mM-benzamidine hydrochloride and 10mM-EDTA at 4°C. The valves were blotted dry and their wet weights determined. The water contents were determined by freeze-drying the valves to constant weight. Each mitral valve was milled to a fine powder in a Spex freezer mill (Spex Industries, Metuchen, NJ, U.S.A.). The hydroxyproline contents were determined by using the method of Bergman & Loxley (1963), and the hexuronic acid contents were determined by the Bitter & Muir (1962) method. The hydroxyproline contents were converted into the collagen contents after taking into account the relative proportions and hydroxyproline contents of the different types of collagen in the valve. The DNA contents were determined by using the method of Cesarone *et al.* (1979). The values for the normal and myxomatous valves were expressed as the means±S.D. and the results were compared by using the two-tailed Student's *t* test.

Collagen solubility

The collagens and their CNBr-cleavage peptides were solubilized by using various methods, and the effectiveness of the procedures were assessed from the hydroxyproline contents of the extracts.

The collagens were solubilized from the valves by the method described by Jimenez & Bashey (1978). This procedure, which was carried out over 39 days, consisted of serial extractions with 0.02M-Tris/HCl buffer, pH7.4, containing 1M-NaCl (7 days), 0.5M-sodium citrate (7 days) and 0.5M-acetic acid (7 days) at 4°C, followed by three limited pepsin digestions, each for 72h at 15°C. The residue was extracted with 0.05M-Tris/HCl buffer, pH7.4, containing 0.45M-NaCl and 20mM-dithiothreitol at 4°C for 5 days. The insoluble material was digested for a fourth time with pepsin for 72h, and finally extracted with 0.5M-acetic acid for 24h. In our study, the final residue was cleaved with CNBr (Scott & Veis, 1976).

A modified form of the Jimenez & Bashey (1978) procedure was used over 5 days. The milled valves were extracted with 0.05M-Tris/HCl buffer, pH7.4, containing 1M-NaCl and proteinase inhibitors followed by 0.5M-acetic acid, each for 24h at 4°C. The residue was digested with pepsin (enzyme/substrate ratio 1:10) for 24h at 4°C and was further extracted with 0.05M-Tris/HCl buffer, pH7.4, containing 0.45M-NaCl and 20mM-dithiothreitol for 24h at 4°C. A second pepsin digestion was carried out and the final residue was cleaved with CNBr.

Milled mitral valves were also extracted with 0.15M-sodium acetate buffer, pH5.8, containing 4M-guanidinium chloride and proteinase inhibitors for 24h at 4°C. The residue was washed with water and 0.5M-acetic acid, and the washings were added to the guanidinium chloride extract. The washed residue was digested twice with pepsin for 24h at 4°C. The insoluble material was extracted with the 4M-guanidinium chloride/0.15M-sodium acetate buffer, and the final residue was cleaved with CNBr.

Cleavage with CNBr was also carried out after extractions that did not include pepsin digestions. The extractions were carried out for 24h at 4°C with the following: 0.05M-Tris/HCl buffer, pH7.5, containing 1M-NaCl followed by 0.5M-acetic acid; 0.05M-Tris/HCl buffer, pH7.5, containing 2M-urea and 0.2% (w/v) SDS; 2% (w/v) SDS; and 0.15M-sodium acetate buffer, pH5.8, containing 4M-guanidinium chloride. All solutions contained proteinase inhibitors. The extracted valves were washed three times at 4°C with 0.15M-NaCl/0.1M-NaH_2PO_4, pH7.4, and twice with acetone, and were dried under vacuum and cleaved with CNBr. To study the peptides containing reducible components, some valves were reduced with NaB^3H_4

before cleavage with CNBr (Eyre & Glimcher, 1972). To label tyrosine-containing peptides, some valves were labelled with ^{125}I before cleavage with CNBr (Glazer & Sanger, 1964).

Purification of type I and type III collagens

Pepsin-solubilized type I and type III collagens were separated by differential salt precipitation (Epstein, 1974). The collagens were separated from proteoglycans by DEAE-cellulose chromatography (Miller, 1971), and further separation of type I from type III collagens was achieved by using the differential denaturation and renaturation procedure described by Chandrarajan (1978). The purity of the preparations was confirmed by electrophoresis and amino acid analysis. Samples were hydrolysed in 6M-HCl under N_2 for 20h at 110°C, and the dried hydrolysates were analysed by using an automated Technicon amino acid analyser.

SDS/polyacrylamide-gel electrophoresis

Collagen chains were analysed on 5%-(w/v)-acrylamide separating gels with a 3.5%-(w/v)-acrylamide stacking gel. The sample preparation and electrophoresis conditions are described elsewhere (Bateman *et al.*, 1984). Type III collagen chains were separated from type I and type V collagen chains by delayed reduction of the disulphide bonds of type III collagen (Sykes *et al.*, 1976). Type V collagen was quantified from these gels by comparing the densitometry area of the α1(V) band with the densitometry areas obtained with purified type V collagen from human dermis. The ratio of α1(V) to α2(V) chains in this purified preparation of dermal type V collagen was 2.0 ± 0.1(5). Type I and type III collagens were quantified by electrophoresis of CNBr-cleavage peptides on slab gels with an exponential gradient of acrylamide from 11 to 16% (w/v) and a stacking gel of 4.5 (w/v) acrylamide (Cole & Chan, 1981). The gels, after staining with Coomassie Brilliant Blue and destaining, were scanned as previously described (Cole & Bean, 1979). The densitometry areas of the α1(I) CB8 and the α1(III) CB5 peptides were used to quantify the relative amounts of type I and type III collagens in each extract (Laurent *et al.*, 1981). The densitometry areas of these peptides were determined by using a Leitz Image Analysis system (Leitz, Wetzlar, West Germany). Linear relationships were obtained between the concentrations of the peptides applied to the gels and the areas of the marker peptides. These relationships were determined with CNBr-cleavage peptides of purified type I and type III collagens obtained from the mitral valves, and these peptides were used as standards in each electrophoretic run. Two-dimensional SDS/polyacrylamide-gel electrophoresis of the peptides showed that there were no other peptides co-migrating with either the α1(I) CB8 or α1(III) CB5 peptides. The α1(III) CB9 peptide, which had a similar molecular mass to the α1(III) CB5 peptide, is disulphide-bonded to other α1(III) CB9 peptides, and as a consequence it migrates more slowly than the α1(III) CB5 peptide (Cole & Chan, 1981). It was also shown that constant amounts of the α1(I) CB8 and α1(III) CB5 peptides were released from separate CNBr cleavages of the same, as well as different, preparations of type I and type III collagens obtained from the valves.

CNBr-cleavage peptides obtained from normal and myxomatous valves and dermis that had been extracted with 4M-guanidinium chloride were also analysed by using two-dimensional polyacrylamide-gel electrophoresis. Electrophoresis consisted of non-equilibrium pH-gradient electrophoresis in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension (Cole & Chan, 1981).

Results

Gross composition of mitral valves

The control mitral valves were macroscopically and microscopically normal for the age of the patients. The myxomatous valves were larger and thicker than normal and in cross-section there were mucoid areas as described by Davies *et al.* (1978). The lamina fibrosa was disorganized and there were areas where additional collagen had been deposited. The chordae tendineae were also thickened and ruptured chordae were observed in 12 of the 19 patients. All the biochemical analyses were performed with freeze-milled valves from each patient, consisting of the anterior and posterior leaflets with their attached chordae tendineae. This approach was used in order to overcome differences in sampling due to the heterogeneous nature of the pathological changes in the leaflets. The leaflets were also combined because preliminary studies showed similar pathological and biochemical abnormalities in each leaflet. The chordae tendineae were also left attached to the leaflets because the chordae merged into the leaflets without a clearly defined junction.

The control valves contained 83% water, and 67% of their dry weight was collagen (Table 1). The myxomatous valves contained significantly greater amounts of collagen, hexuronic acid and DNA, but the water content was not significantly increased.

Collagen solubility

The procedure that was successfully used by Jimenez & Bashey (1978) to solubilize completely the collagens of the bovine mitral valve extracted

Table 1. *Gross composition of normal and myxomatous mitral valves*

Experimental details are given in the text. The results are expressed as the means ± S.D. All measurements were carried out in triplicate and the numbers of valves studied are given in parentheses.

	Normal	Myxomatous
Water content (% of wet wt.)	82.8 ± 1.2 (5)	84.7 ± 2.3 (11)
Hydroxyproline (μg/mg dry wt.)	87.9 ± 6.9 (13)	100.9 ± 9.2* (19)
Collagen (μg/mg dry wt.)	673.4 ± 52.9 (13)	763.6 ± 69.7* (19)
Hexuronic acid (μg/mg dry wt.)	8.1 ± 1.3 (11)	13.8 ± 3.2* (16)
DNA (μg/mg dry wt.)	1.9 ± 0.5 (8)	3.6 ± 0.8* (10)

* Student's *t* test showed highly significant difference with $P < 0.001$.

Table 2. *Solubility of collagen from normal human and bovine mitral valves by using the Jimenez & Bashey (1978) procedure*

Experimental details are given in the text. The results are expressed as percentages of the hydroxyproline content of the valves. The values for the normal human valve were the means of the result obtained from two separate valves. The results for the normal bovine valve were taken from Jimenez & Bashey (1978).

	Normal human	Normal bovine
NaCl extraction	1.0	1.03
Citrate extraction	Trace	0.09
Acetic acid extraction	1.1	0.47
First pepsin extraction	11.8	22.2
Second pepsin extraction	4.9	14.3
Third pepsin extraction	2.6	12.4
Dithiothreitol extraction	19.3	24.2
Fourth pepsin extraction	8.6	13.1
Amount extracted	49.3	87.8
CNBr cleavage of residue	22.2	—
Final residue	25.3	—
Recovery	96.8	87.8

Table 3. *Solubility of collagen from normal and myxomatous mitral valves by using a modified Jimenez & Bashey (1978) procedure*

Experimental details are given in the text. The results are expressed as the means ± S.D. for percentages of the hydroxyproline contents of the valves. All measurements were carried out in triplicate and the numbers of valves studied are given in parentheses.

	Normal (7)	Myxomatous (9)
NaCl extraction	1.2 ± 0.2	1.1 ± 0.3
Acetic acid extraction	0.7 ± 0.2	0.5 ± 0.1
First pepsin extraction	14.8 ± 3.1	14.5 ± 2.3
Dithiothreitol extraction	4.4 ± 0.8	4.4 ± 1.9
Second pepsin extraction	15.0 ± 3.0	18.6 ± 5.9
CNBr cleavage of residue	16.4 ± 5.8	24.3 ± 6.2
Total extracted	52.5 ± 7.7	63.4 ± 8.9*
Final residue	30.2 ± 4.8	16.4 ± 8.3
Recovery	82.7 ± 1.9	79.8 ± 5.9

* Student's *t* test showed significant difference with $P < 0.05$.

Table 4. *Solubility of collagen from normal and myxomatous mitral valves by using the guanidine/pepsin/CNBr procedure*

Experimental details are given in the text. The results are expressed as the means ± S.D. for percentages of the hydroxyproline contents of the valves. All measurements were carried out in triplicate and the numbers of valves studied are given in parentheses.

	Normal (5)	Myxomatous (5)
First guanidine extraction	8.9 ± 1.1	9.2 ± 1.4
First pepsin extraction	30.8 ± 1.2	31.8 ± 1.7
Second pepsin extraction	18.5 ± 1.8	15.0 ± 4.1
Second guanidine extraction	6.8 ± 1.0	7.8 ± 1.9
CNBr cleavage of residue	24.3 ± 1.2	25.9 ± 4.5
Total extracted	89.1 ± 1.4	89.6 ± 1.7
Final residue	1.9 ± 1.2	1.8 ± 2.3
Recovery	91.0 ± 2.6	91.4 ± 2.3

significantly less collagen from the normal human mitral valve (Table 2). The relative solubilities of the human collagens in the various extracting solutions were similar to those reported for the bovine valves, and the greatest amount of collagen was also extracted with the buffer containing dithiothreitol. However, the Jimenez & Bashey (1978) procedure was not used for the major part of our study as only 49% of the hydroxyproline was released by the prolonged extractions of the human valves and only a further 22% of the hydroxyproline was released by CNBr cleavage.

The modified Jimenez & Bashey (1978) procedure released less collagen from the mitral valves, but because it was shorter it was more suitable for the study of collagen solubility in pathological samples (Table 3). The solubility patterns from the normal and myxomatous mitral valves were similar, but very little collagen was released with the buffer containing dithiothreitol. Overall, a significantly greater percentage of the hydroxyproline ($P < 0.05$) was extracted from the myxomatous valves, which was mainly due to the greater proportion of the collagen that was cleaved by CNBr. When the concentrations of hydroxyproline in the samples were considered it was observed that 43.7 ± 8.2(7) μg of hydroxyproline/mg dry wt. was extracted from the normal

valves, and 63.4±9.4 (9) μg of hydroxyproline/mg dry wt. from the myxomatous valves ($P < 0.001$).

The collagens in the normal and myxomatous mitral valves were almost completely solubilized by using the guanidine/pepsin/CNBr procedure (Table 4). When the valves were extracted with guanidinium chloride a greater proportion of the collagen was released by the following pepsin digestions than was observed with the other methods of extraction used in this study. The first guanidinium chloride extraction did not permanently denature the insoluble collagen. Over 95% of the hydroxyproline that was solubilized by the following pepsin digestions was recovered as collagen molecules from the 1.2M-NaCl precipitates. The second guanidinium chloride extraction solubilized very little additional collagen, and when this step was deleted the residue was still almost completely solubilized by CNBr cleavage. When the concentrations of hydroxyproline in the samples were considered it was observed that 77.8±4.6(5) μg of hydroxyproline/mg dry wt. was extracted from the normal valves, and 92.0±7.1(5) μg of hydroxyproline/mg dry wt. from the myxomatous valves ($P < 0.001$).

The amount of collagen released as peptides by CNBr cleavage varied with the methods used to extract the valves initially. Thus 46% of the hydroxyproline was released by CNBr cleavage after extractions with solutions containing 1M-NaCl and 0.5M-acetic acid, 36% after 2M-urea and 0.2% (w/v) SDS, and 40% after 2% (w/v) SDS. After guanidinium chloride extraction 66.5±12%(5) of the hydroxyproline was released by CNBr cleavage. Similar results were obtained from the myxomatous valves. The amount of collagen released by CNBr cleavage after guanidinium chloride extraction of the normal or myxomatous valves was not increased by denaturation of the residues in water containing proteinase inhibitors at 60°C for 30min (Light, 1982).

The amount of hexuronic acid released by the extracting solutions used before pepsin digestion or CNBr cleavage was determined. It was found that 39% of the hexuronic acid was solubilized by solutions containing 1M-NaCl, 15% by 2M-urea and 0.2% (w/v) SDS, 14% by 2% (w/v) SDS, and 64% by 4M-guanidinium chloride. The proportions of hexuronic acid solubilized from the myxomatous valves were not significantly different.

Characteristics of the valve collagens

Similar complex mixtures of proteins, which were mainly non-collagenous proteins, were extracted from the normal and myxomatous valves with solutions containing the following: 1M-NaCl;

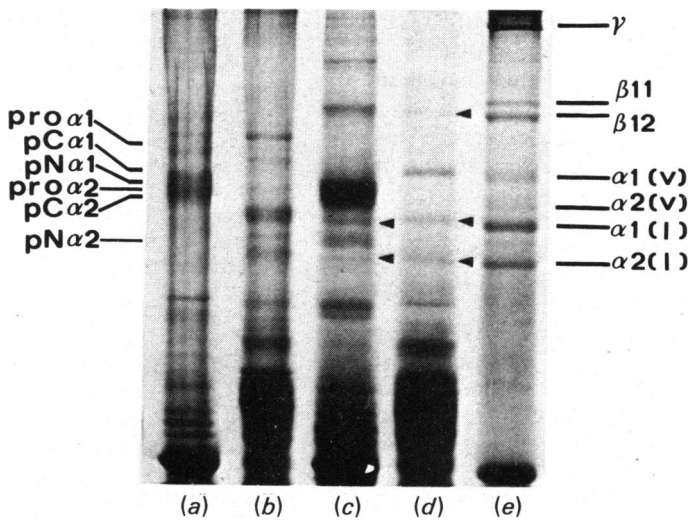

Fig. 1. *Electrophoresis of guanidine extract of normal mitral valve*

Experimental details are given in text. (*a*) Proteins precipitated by dialysis against 0.5M-acetic acid; (*b*) sample (*a*) after reduction with 2-mercaptoethanol; (*c*) soluble proteins after dialysis against 0.5M-acetic acid; (*d*) sample (*c*) after reduction with 2-mercaptoethanol; (*e*) collagens released by limited pepsin digestion of valve after guanidine extraction. The migration positions of type I collagen chains [α1(I), α2(I), dimeric β11 and β12] and type V collagen chains [α1(V) and α2(V)] are shown. γ refers to collagen molecules containing three α chains. The migration positions are also shown for type I procollagen chains (pro-α1 and pro-α2), and for the partially processed pN-type I collagen chains (pNα1 and pNα2) and pC-type I collagen chains (pCα1 and pCα2). ◀ indicates the only proteins in the guanidine extract that were digested with purified bacterial collagenase.

0.2% (w/v) SDS and 2M-urea; 2% (w/v) SDS; or 4M-guanidinium chloride. The same electrophoretic patterns were obtained after rapid (2min) or slower (24h) extractions, and all solutions contained proteinase inhibitors. The electrophoretic patterns obtained from a guanidinium chloride extract (24h) are shown in Fig. 1. After dialysis of the guanidinium chloride extract against 0.5M-acetic acid, a precipitate formed, but the precipitate did not contain any detectable proteins that were susceptible to cleavage by purified bacterial collagenase (Peterkofsky & Diegelmann, 1971). In the supernatant, α1(I), α2(I), β11 and β12 chains of type I collagen were detected, and these chains were cleaved by purified bacterial collagenase. Other types of collagen were not detected in these extracts. Many of the other protein bands co-migrated with serum proteins, and the extracts were shown by immunodiffusion on Ouchterlony plates to contain serum albumin.

The electrophoretic patterns obtained from the initial extracts were also compared with procollagen standards, because of the report by Bonella *et al.* (1980) of procollagen chains in normal and myxomatous human mitral valves. The standards from fibroblast cultures included type I and type III procollagens, pN-α1(I) and pN-α2(I) chains (type I procollagen chains lacking *C*-terminal propeptides), as well as pC-α1(I) and pC-α2(I) chains (disulphide-linked type I procollagen chains lacking *N*-terminal propeptides). In addition, pN-α1(I) and pN-α2(I) chains from dermatosparactic sheep and bovine dermis were also used as standards. The migrations of the procollagen and partially processed procollagen chains are shown in Fig. 1. Some of the protein bands from the valve extracts had similar migrations to these chains, but they were not cleaved by purified bacterial collagenase. After reduction of disulphide bonds, many of the protein bands migrated beyond the positions expected for chains from type I, type III, type IV or type V procollagens.

The normal mitral valves contained type I, type III and type V collagens (Fig. 2). The first pepsin digest, obtained with any of the methods used in the present study, contained type I, type III and type V collagens, but type V collagen was not detected in the following pepsin digests or in the CNBr-cleavage peptides of the final residue. The α1(V) and α2(V) chains of type V collagen were present in the ratio of $2.2 \pm 0.2(7)$ from normal valves and $2.3 \pm 0.3(9)$ from myxomatous valves. These ratios are in agreement with the chain composition of $[\alpha 1(V)]_2$ α2V described by Bentz *et al.* (1978). The ratio of α1(I) to α2(I) chains was $1.96 \pm 0.3(7)$ from normal valves and $1.9 \pm 0.2(9)$ from myxomatous valves, as expected in type I collagen. Selective NaCl precipitates of the pepsin-solubilized collagens did not provide any electrophoretic evidence of type I-trimer $[\alpha 1(I)]_3$ or type IV collagen.

The almost complete extraction of the valve collagens by using the guanidine/pepsin/CNBr procedure enabled the relative percentages and concentrations of type I, type III and type V collagens to be determined (Table 5). The normal

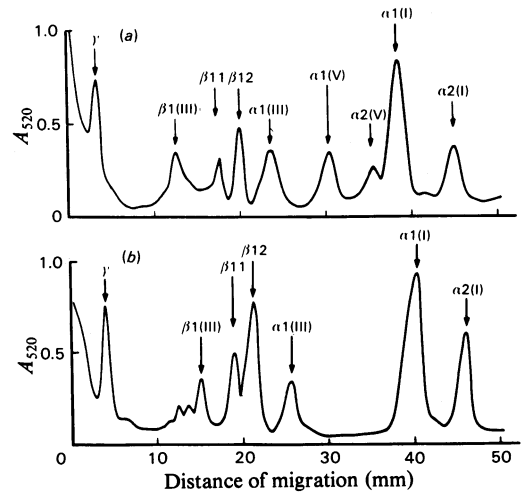

Fig. 2. *Electrophoretograms of pepsin-solubilized collagens of normal mitral valve and dermis*

Samples were electrophoresed on 5%-(w/v)-acrylamide gels using the delayed reduction procedure of Sykes *et al.* (1976). (*a*) Collagens precipitated from the first limited pepsin digest of normal mitral valve; (*b*) collagens precipitated from limited pepsin digest of age-matched dermis. The migration positions of type I collagen chains [α1(I), α2(I), dimeric β11 and β12], type III collagen chains [α1(III) and dimeric β1 (III)] and type V collagen chains [α1(V) and α2(V)] are shown. γ refers to collagen molecules containing three α chains.

Table 5. *Types of collagen in normal and myxomatous mitral values*

Experimental details are given in the text. The results are expressed as the means ± S.D. for μg of collagen/mg dry wt. of valve. All measurements were carried out in triplicate and the numbers of valves studied are given in parentheses.

Collagen	Normal (10)	Myxomatous (12)
Type I	489.7 ± 32.5	532.5 ± 50.1*
Type III	160.7 ± 17.9	247.0 ± 34.5†
Type V	13.8 ± 1.4	18.2 ± 2.4†

* Student's *t* test showed significant difference with $P < 0.05$.

† Student's *t* test showed highly significant differences with $P < 0.001$.

valves contained 74% type I, 24% type III and 2% type V collagen, whereas the myxomatous valves contained 67% type I, 31% type III and 2% type V collagen. In the myxomatous valves there was a significant increase in the concentration of each type of collagen, this consisting of a 53% increase of type III collagen and a 9% increase of type I collagen. The concentration of type V collagen was increased by 25%, but there was only a small amount of type V collagen in the valves.

The pepsin-solubilized chains and the CNBr-cleavage peptides of type I collagen obtained from normal and myxomatous valves migrated more slowly on SDS/polyacrylamide-gel electrophoresis than did the type I collagen chains and peptides obtained from the dermis of the same patient or age-matched controls (Figs. 2 and 3). The slower migration of the chains and peptides from the valves was probably due to their increased content of hydroxylysine. Of the lysine residues of type I collagen from normal and myxomatous valves 27% were hydroxylated, compared with 9% of the lysine residues from age-matched dermal type I collagen. The type III collagen chains obtained from the normal and myxomatous valves migrated a little more slowly than did the type III collagen chains from age-matched dermis, but the major α1(III) CB5 and α1(III) CB8 peptides did not show this difference (Fig. 3). It was found that 21% of the lysine residues were hydroxylated in type III collagen from normal valves, compared with 18% from myxomatous valves and 10% from age-matched dermis.

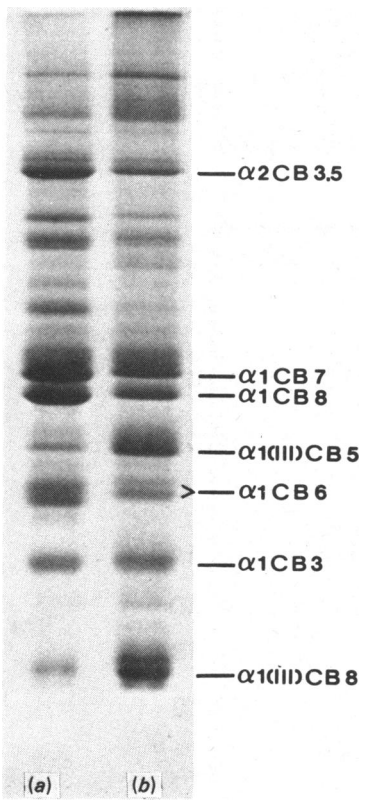

Fig. 3. *Electrophoresis of type I and type III collagen CNBr-cleavage peptides*

Experimental details are given in the text. (*a*) CNBr-cleavage peptides of age-matched dermis; (*b*) CNBr-cleavage peptides of normal mitral valve. The samples were not reduced with 2-mercaptoethanol. The major peptides of the α1(I) and the α2(I) chains of type I collagen and the α1(III) chains of type III collagen are marked.

The recovery of the *C*-terminal α1(I) CB6 peptide, which takes part in intermolecular cross-linkages (Light & Bailey, 1979), was greatly diminished in the normal and myxomatous valve samples when compared with age-matched dermal samples (Fig. 4) and soluble type I collagen (Cole & Chan, 1981). The *C*-terminal α1(III) CB9 peptide, which is also involved in intermolecular cross-linkages (Nicholls & Bailey, 1980), was not detected in either the dermal or the valve samples. Most of the radioactivity, after reduction of valves with NaB^3H_4, was associated with peptides that were located at the top of the gel (results not shown). Similarly, after ^{125}I-labelling of tyrosine residues, most of the radioactivity was observed in large-M_r peptides at the top of the gel, and only a relatively small amount of radioactivity was noted in the tyrosine-containing α1(I) CB6 and α2(I) CB3.5 peptides (results not shown).

The type I and type III collagens that were solubilized by pepsin digestion of the valves contained normal amounts of the α1(I) CB6 and α1(III) CB9 peptides (Cole & Chan, 1981). Similar recoveries of these peptides were also observed after CNBr cleavage of the valves that had undergone limited pepsin digestion.

Discussion

In order to study the types and characteristics of the collagens in normal and myxomatous human mitral valves it was essential to be able to solubilize all or a substantial amount of the collagen. Nearly complete solubilization of the collagen was achieved by using a method consisting of guanidinium chloride extraction, limited pepsin digestions and CNBr cleavage of the residue. It is likely that intermolecular collagen cross-linkages and interactions between collagen and other components of the valve were important factors in determining the solubility of the valve collagens.

Collins *et al.* (1977) showed that type I collagen from bovine valves contained higher contents of

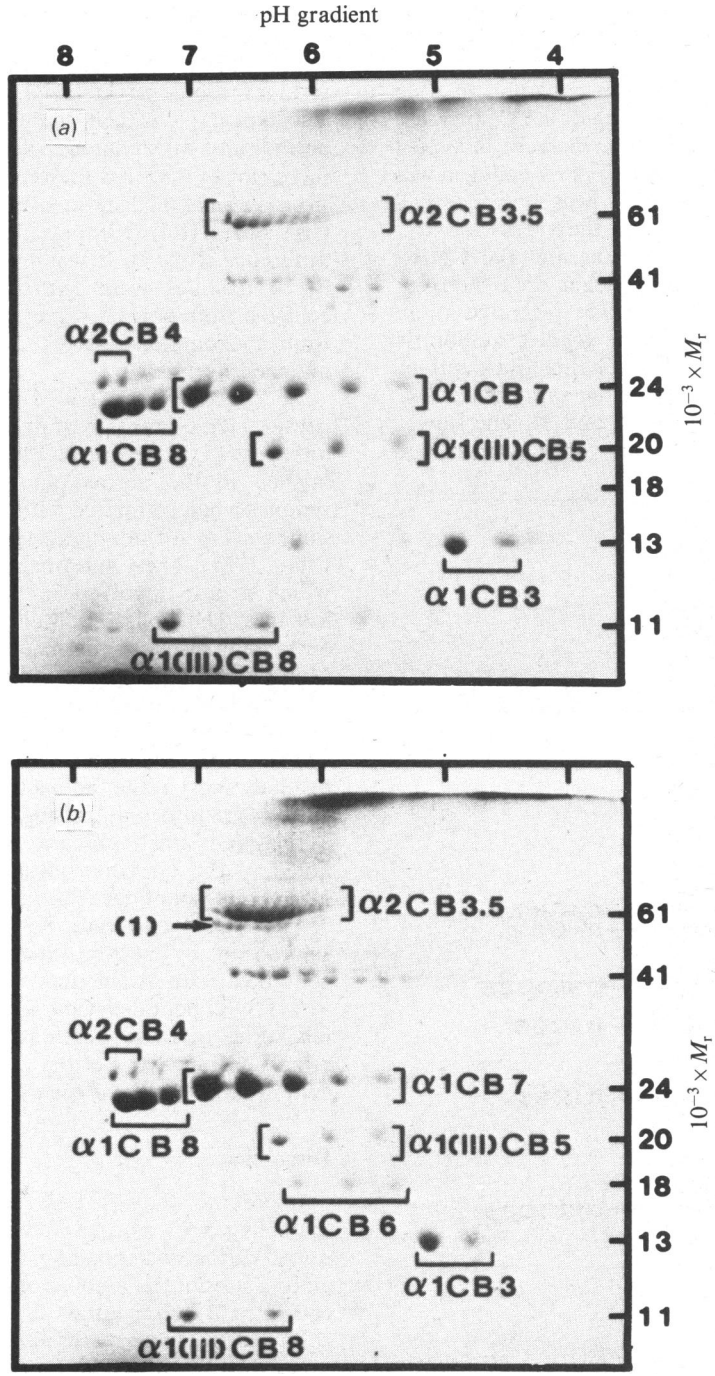

Fig. 4. *Two-dimensional electrophoresis of CNBr-cleavage peptides of normal mitral valve and dermis*
Samples (100 μg) of CNBr-cleavage peptides obtained from (*a*) normal mitral valve and (*b*) age-matched dermis were resolved by non-equilibrium pH-gradient gel electrophoresis and SDS/polyacrylamide-gel electrophoresis as described in the text. The major α1(I), α2(I) and α1(III) peptides are marked. The peptides marked (I) in (*b*) represent cross-linked peptides.

hydroxylysine than did type I collagen from bovine dermis. They also showed that Δ^6-dehydro-5,5'-dihydroxylysinonorleucine, a collagen cross-link derived from two hydroxylysine residues and ordinarily found in tendon and bone, was the predominant cross-link in the heart valve. The type I and type III collagens of the human mitral valves also contained similar high contents of hydroxylysine. The two-dimensional electrophoretic 'maps' of the CNBr-cleavage peptides from the human mitral valve were also similar to the 'maps' obtained previously from bone (Cole & Chan, 1981). In both tissues, as well as in aging tendon (Light & Bailey, 1979), there was a low recovery of the α1(I) CB6 peptide, which was probably due to the involvement of this *C*-terminal peptide in intermolecular cross-linkages. This view is supported by our finding that most of the incorporated radioactivity after NaB^3H_4 reduction of cross-linkages was found in large-M_r peptide complexes. In addition, most of the incorporated ^{125}I was also observed near the origin of the electrophoretic gels, which suggested that the tyrosine-containing α1(I) CB6 peptide was involved in forming these large-M_r cross-linked peptides (Light & Bailey, 1979). The α1(III) CB9 peptide was not detected in the mixture of CNBr-cleavage peptides from the human mitral valve, and it is likely that this *C*-terminal peptide of the α1(III) chain was also involved in forming intermolecular cross-linkages (Nicholls & Bailey, 1980).

Interactions between collagen and other components of the valve also influence the solubility of the collagen. Jimenez & Bashey (1978) showed that more collagen was solubilized from bovine valves when disulphide bonds were reduced with dithiothreitol. They proposed that the valves contained a disulphide-bonded matrix that was responsible for the insolubility of the collagen. A similar effect of dithiothreitol was noted with the human valves, although this was not observed with a shorter form of the Jimenez & Bashey (1978) method. Extraction of the proteoglycan with 4M-guanidinium chloride was shown to increase greatly the amount of collagen that was subsequently released by limited pepsin digestion or CNBr cleavage of the human valve. The recovery of normal amounts of the α1(I) CB6 and α1(III) CB9 peptides from the pepsin-digested valves showed that the pepsin digestions had released these *C*-terminal peptides from their involvement in collagen cross-linkages. As a result, it is likely that the collagens remaining in the tissue after pepsin digestion were involved in interactions with other components of the valve. These interactions may have involved glycosaminoglycans, as Lowther *et al.* (1967) have shown that dermatan sulphate is strongly bound to the insoluble collagen of the heart valve.

The development of the guanidine/pepsin/CNBr procedure enabled us to study most of the collagen in the valve. We agree with Hammer *et al.* (1979) that the human mitral valves contained type I, type III and type V collagens, although only 30% of the collagen was solubilized in their study. We showed that the normal human mitral valves contain 74% type I, 24% type III and 2% type V collagen. It is likely that the type I and type III collagens were closely associated in the fibres of the human mitral valve, because these collagens had similar solubility patterns with low recoveries of the *C*-terminal CNBr-cleavage peptides that are involved in forming intermolecular cross-linkages. Both types of collagen have been shown to contain similar types of cross-linkages (Nicholls & Bailey, 1980), and cross-linkages between the α1(I) chains of type I collagen and the α1(III) chains of type III collagen have been isolated from other tissues (Henkel & Glanville, 1982). Type V collagen was only detected in the first pepsin extract, and this finding may be due to the pericellular location of type V collagen (Gay *et al.*, 1981).

The myxomatous mitral valves contained an increased amount of type I, type III and type V collagens. The concentration of type III collagen was increased the most, and this finding suggests that the fibroblasts of the valve were producing additional collagen of a similar composition to that observed in other tissues undergoing repair or fibrosis (Light, 1982). The increased amount of type V collagen may reflect an increase in the number of cells as the DNA content of the valves was increased. The increased amount of hexuronic acid in the myxomatous valves is also in keeping with a repair process.

When the results from the different myxomatous valves were compared with each other we did not observe any significant differences. We did not observe any myxomatous valves lacking type III and type V collagens, as was reported in one valve with ruptured chordae tendineae by Hammer *et al.* (1979). In the same study, Hammer *et al.* (1979) found normal relative amounts of type I, type III and type V collagens in five other myxomatous valves, although only 30% of the collagen was extracted for these studies. We did not confirm the report by Bonella *et al.* (1980) that normal and myxomatous human mitral valves contain procollagen and partially processed procollagen chains. However, we did observe that the initial extracts from the valves contained mainly plasma proteins and only a small amount of type I collagen.

The pathological and biochemical changes observed in the myxomatous mitral valves could have resulted from a variety of primary defects. Genetically determined defects of type I or type III collagens underlie the valvular disorders in some

cases of the Marfan's syndrome (Scheck *et al.*, 1979) and the Ehlers–Danlos syndrome (Jaffe *et al.*, 1981). However, the primary defect underlying the changes in the myxomatous mitral valves that we have studied is uncertain. It is possible that the primary defect was a malformation of the valve, which led, as a result of cyclical loading, to disruption of the collagen fibres (King *et al.*, 1982). Our biochemical and pathological findings suggest that a repair process is also involved with the production of additional collagen, in particular type III collagen, and glycosaminoglycans as well as the proliferation of cells.

The guanidine/pepsin/CNBr-cleavage procedure developed for solubilizing the collagens of the human mitral valve should facilitate further studies of genetically determined and acquired disorders of heart valves.

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