

Biochemical characterization of individual normal, floppy and rheumatic human mitral valves

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Human mitral valves (32 floppy and 17 rheumatic) obtained at surgery were analysed and compared with 35 normal (autopsy) valves. Total amounts of collagen, proteoglycan and elastin were increased approx. 3-fold in floppy and rheumatic valves. The water content of rheumatic cusps was lower than normal. The most significant changes in floppy valves were the 59% increase in mean value of the proteoglycan content, a large increase in the ease of extractability of proteoglycans from 26.7 to 57.2% of the total and a 62% increase in mean value of the elastin content in the anterior cusps. Normal human mitral valve cusps contained a mean proportion of 29.3 (and chordae 26.6) type III collagen (as % of total types III+I collagen), the values increasing significantly to 33.2 and 36.3% respectively in chronic rheumatic disease. The ratio observed in floppy valves depended on the extent of secondary surface fibrosis, which could be demonstrated histologically; in valve cusps with considerable secondary fibrosis, the percentage of type III increased significantly (to 34.4%), whereas it decreased significantly (to 25.2%) when fibrosis was negligible. It is concluded that the ratio of collagen types in floppy valves reflects the extent of secondary fibrosis rather than the pathogenesis of the disrupted collagen in the central core of the valve.

INTRODUCTION

Two common pathological changes leading to mitral regurgitation in man are floppy or 'ballooning' valves, in which the cusps expand and thicken (Davies *et al.*, 1978), and chronic rheumatic disease, in which the cusps retract and thicken (Criley *et al.*, 1966) and cusp tissue becomes vascularized, a feature lacking in both normal and floppy mitral valves (Frable, 1969).

It has been suggested that, although an apparent accumulation of glycosaminoglycans can be demonstrated histologically in the central fibrous core of floppy valves leading to the term 'myxoid' or 'myxomatous degeneration' (Read *et al.*, 1965; McCarthy & Wolf, 1970; Davis *et al.*, 1971; Guthrie & Edwards, 1976), the primary defect is probably in the collagen moiety of the tissue (Kern & Tucker, 1972; Davies *et al.*, 1978; King *et al.*, 1982). This hypothesis is consistent with the frequent occurrence of mitral-valve prolapse in association with hereditary collagen diseases including Marfan syndrome, Ehlers–Danlos syndrome and osteogenesis imperfecta (Jeresaty, 1979; Pyeritz, 1983). In particular, the collagen-fibre integrity could be weakened in floppy valves by inefficient cleavage of aminopropeptides from procollagen I, as in dermatosparaxia (Bailey & Lapière, 1973) and Ehlers–Danlos syndrome type VII (Lichtenstein *et al.*, 1973). A pilot study supported this suggestion in pooled isolated floppy valves (Bonella *et al.*, 1980) and would be consistent with increased collagen synthesis by floppy-valve tissue in culture (Henney *et al.*, 1982).

Although estimations have been made of the collagen in mitral valves (Bashey *et al.*, 1967; Collins *et al.*, 1977; Cole *et al.*, 1984), these have not been correlated with estimations of elastin and proteoglycans in the same

valves, nor has the apparent increase in myxomatous material seen histologically in floppy valves been related to biochemical analyses of the amount of glycosaminoglycan present.

There is evidence that isolated floppy mitral valves, i.e. those in patients who do not have classic hereditary collagen diseases, may have an hereditary basis (Hunt & Sloman, 1969; Barlow & Pocock, 1975; Weiss *et al.*, 1975; Strahan *et al.*, 1983), with an autosomal dominant mode of inheritance (Schutte *et al.*, 1981; Devereux *et al.*, 1982). The possibility exists, therefore, of a slight deviation from normal in the synthesis of one of the genetic types of collagen.

Normal human mitral valves contain collagen types I, III and V (Hammer *et al.*, 1979; Lee *et al.*, 1983; Cole *et al.*, 1984). In one case report, the redundant mitral valve removed at surgery contained no pepsin-extractable type III and V collagens (Hammer *et al.*, 1979). Lee *et al.* (1983), however, found all three collagen types in normal, floppy and rheumatic valves, although only the pepsin-extractable collagens were identified and these were not quantified. The study of a family of type IV Ehlers–Danlos syndrome proband demonstrated a clear association between mitral-valve prolapse and reduced biosynthesis of type III collagen by cultured skin fibroblasts from each affected member (Jaffe *et al.*, 1981). Aumailley (1982) found a single family in which isolated mitral-valve prolapse was associated in a similar way with reduced type III collagen production by skin fibroblasts. In contrast with these findings, a study by Cole *et al.* (1984) demonstrated an increase of type III and V collagens in floppy valves. In view of these conflicting results the present study was designed to quantify the collagen types and relate the findings to the biochemical composition and histology of the valve

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cusps and chordae tendineae. Floppy valves removed at surgery because of mitral regurgitation were compared with age-matched controls (autopsy) or with normal valves from hearts obtained from transplant patients, and with surgically removed valves rendered stenotic as a result of chronic rheumatic disease.

EXPERIMENTAL

Materials

Human embryonic foreskin fibroblasts (passage no. 17), Dulbecco's modification of Eagle's medium, foetal bovine serum and antibiotics were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Sterile plastic culture flasks and Nystatin were from Gibco Biocult, Uxbridge, Middx., U.K.

The enzymes hyaluronidase (EC 3.2.1.35) from bovine testes type IV-S (750 units/mg of protein), pepsin (EC 3.4.23.1) from porcine stomach mucosa (2500–3200 units/mg of protein) and papain (EC 3.4.22.2) from papaya latex type III (16–40 units/mg of protein) were from Sigma, Poole, Dorset, U.K. High-purity collagenase (EC 3.4.24.3) from *Clostridium histolyticum* (1300 units/mg) was from Lome Laboratories, Twyford, Berks., U.K. Rabbit bone collagenase was generously given by Dr. G. Murphy and Dr. J. Reynolds, Strangeways Research Laboratory, Cambridge, U.K.

Pepstatin, soya-bean trypsin inhibitor, CNBr and acetonitrile were from Sigma. Acrylamide, *NN'*-methylenebisacrylamide and Kenacid Blue R-250, all Electran grade, were from BDH, Dagenham, Essex, U.K. Millers elastin stain, acid fuchsin and picric acid were from Raymond Lamb, North Acton, London NW10 6JL, U.K. All other chemicals were of analytical grade or of the best available grade.

Methods

Human mitral valves. Mitral valves (32 floppy and 17 non-calcified, predominantly regurgitant rheumatic) were obtained from valve-replacement surgery. None of the patients had evidence of a major generalized connective-tissue disease such as Marfan syndrome or Ehlers–Danlos syndrome. Most of the valves were used immediately; those which had been stored at -70°C were not used for procollagen estimation. Normal mitral valves were obtained at autopsy from individuals with no evidence of cardiac disease and from cardiac-transplant-recipient hearts without delay.

Processing of mitral valves. A section was taken from valve annulus to free edge from the centres of both the anterior and the posterior medial cusps for histology. Each valve was separated into cusps and chordae tendineae for further treatment. The samples were homogenized (2 ml/mg of sample) in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 M-NaCl and the proteinase inhibitors 20 mM-EDTA, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-*o*-phenanthroline, with a Silverson standard-model tissue disintegrator using the Micro (diameter 16 mm) head. After extraction at 4°C for 18 h the suspension was centrifuged at 8000 *g* for 30 min. Portions of the supernatant were taken for hydroxyproline, protein and glycosaminoglycan analysis and the rest used for procollagen identification. The precipitate was extracted

at 4°C overnight in 50 mM-Tris/HCl buffer, pH 7.5, containing 4 M-guanidinium chloride with the proteinase inhibitors described above. The pellet, after centrifugation at 8000 *g* for 30 min, was washed several times in deionized water, freeze-dried and stored at -70°C for measurement of collagen and proteoglycan contents and for collagen type analysis.

Further digestion with testicular hyaluronidase (EC 3.2.1.35) was required to obtain clear backgrounds on the 11%-(w/v)-polyacrylamide gel used to separate the CNBr peptides. Testicular hyaluronidase (250 units/mg dry wt. of tissue sample) was incubated with a suspension of the valve tissue in 0.1 M-sodium acetate buffer, pH 5.5, containing 0.15 M-NaCl, 5 mM-pepstatin and 0.01% (w/v) soya-bean trypsin inhibitor at 37°C for 72 h, then centrifuged at 8000 *g* for 30 min. Valve tissue samples were not cleaved efficiently by CNBr under normal conditions and required preincubation in water at 60°C for 30 min with agitation to encourage heat denaturation of the collagen. Formic acid was added to a final concentration of 70% (v/v), followed by CNBr (2 mg/mg of tissue) dissolved in acetonitrile. The sample was incubated under N_2 at 40°C for 4 h, centrifuged at 8000 *g* for 30 min, and the supernatant diluted 10-fold with deionized water and freeze-dried. The pellet was used for elastin estimation.

SDS/polyacrylamide-gel electrophoresis. CNBr cleavage peptides were separated by electrophoresis on 11% (w/v) acrylamide gels with 5% (w/v) stacking gels using a modification of the method of Laemmli (1970), similar to that of Light (1982). The gels were stained with Coomassie Brilliant Blue and scanned with a Joyce–Loebl Chromoscan 3 microdensitometer at a wavelength of 530 nm, and the areas under the peaks of the peptides $\alpha 1(\text{III})$ CB5 and $\alpha 1(\text{I})$ CB8 were integrated and used to calculate the ratio of type III to types (III+I) collagens (Laurent *et al.*, 1981). A standard mixture of composition verified by amino acid analysis was prepared from pepsin-extracted type III collagen purified by the method of Bailey & Sims (1976) and acid-soluble type I collagen (Kang *et al.*, 1966), both from human placenta.

Procollagens and collagens were precipitated from the neutral salt extracts of the valves by adding $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation (Byers *et al.*, 1974; Nusgens *et al.*, 1980), freeze-dried and separated before and after reduction with mercaptoethanol, on a 6%-(w/v)-acrylamide slab gel.

Procollagen standard was synthesized by a confluent culture of human embryonic foreskin fibroblasts using the method of Booth *et al.* (1980). Samples of both standard and valve-derived procollagens were incubated with pepsin (100 $\mu\text{g}/\text{ml}$), bacterial collagenase (200 $\mu\text{g}/\text{ml}$) and rabbit bone collagenase (2 units/ml), and the digestion products were separated by electrophoresis on 6% gels.

Analytical procedures. Protein concentrations were estimated in triplicate by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and hexuronic acid concentrations by the method of Bitter & Muir (1962), with glucuronolactone as standard. Estimations of sulphated glycosaminoglycans were made by a modified method of Kanwar *et al.* (1981) after extraction by papain digestion of the proteoglycan components at 65°C for 12 h in 50 mM-phosphate buffer, pH 6.5,

containing 5 mM-cysteine, 5 mM-EDTA and 250 μ g of papain/5 mg dry wt. of valve or 25 μ g/100 μ l of valve extract. Hydroxyproline contents were estimated by the method of Burleigh *et al.* (1974); recovery was checked by the addition of an internal hydroxyproline standard before hydrolysis in 6 M-HCl, and the reproducibility of the assay was within 5%. The conversion factor used to determine collagen concentration in valve samples depended on the relative concentrations of type I and type III collagen. In our assay, type I collagen contained 9.7% (w/w) and type III 11.5% (w/w), so the conversion factors used were 10.3 and 8.7 respectively.

Elastin was estimated by the method of Lansing *et al.* (1951). A portion of the final residue was analysed for hydroxyproline content to check the efficiency of elastin digestion; values of 1–2% (w/w) hydroxyproline were accepted as being derived from elastin only (Ross, 1973).

Amino acid analyses were kindly determined by Dr. M. Francis (Nuffield Department of Orthopaedic Surgery, University of Oxford, Headington, Oxford, U.K.) on a Rank-Hilger Chromospec two-channel high-pressure amino-acid analyser (Gage *et al.*, 1986).

RESULTS

Soluble procollagen and collagen from valves

The relative amounts of procollagen and salt-soluble collagen extracted from each type of valve are expressed as total hydroxyproline-containing material (Table 1). The amount of collagen-precursor material extracted from floppy and fresh transplant valves was very similar, representing approx. 4% of total valve collagen. In contrast, extracts from fresh rheumatic and from autopsy valves contained significantly decreased amounts. If fresh transplant valves or floppy valves were frozen and kept for at least 24 h at -70°C , negligible hydroxyproline-containing material could be extracted by neutral salt solutions. Confirmation that the freezing (or, more likely, rethawing) process caused the loss was made by bisecting a fresh transplant valve to include posterior- and anterior-cusp material in both halves, freezing one half before extraction. The frozen half contained negligible salt-soluble hydroxyproline, whereas immediate extraction yielded 0.04% (wet wt.) hydroxyproline.

The neutral-salt extracts from normal and floppy valves were characterized by separation of non-reduced and reduced samples on SDS/6% -polyacrylamide gels. Both standard procollagens and those extracted from fresh normal or floppy valves were converted by pepsin into corresponding α 1(I) and α 2 collagen chains migrating at distances equivalent to those of standard collagen chains. The resulting collagens were completely digested by collagenase from *Clostridium histolyticum*, and cleaved three-quarters of the way along the helix by rabbit bone collagenase.

Biochemical analysis of mitral valves

The wet weights of both floppy and rheumatic valves and their chordae showed a significant 3-fold increase over normal values (Table 2). The mean water content of normal chordae was lower than that of normal cusps, although floppy chordae contained almost as much water as normal or floppy cusps. In contrast, the mean water content of rheumatic valves was significantly decreased.

The total collagen content of cusps and chordae from normal valves as well as floppy and rheumatic valves varied greatly, independently of patient age, with no significant difference between groups. The increase in mean collagen content which occurred in floppy and rheumatic chordae was significant only in floppy chordae. The concentration of elastin in normal valve cusps, however, was constant, apart from a small increase with age. In contrast, the concentration in cusps from floppy and rheumatic valves was very variable, with a high proportion of floppy cusps having an increased elastin content. The rise in elastin content of the anterior floppy cusps was significantly high ($15.4 \pm 4.9\%$ dry weight cf. $9.5 \pm 1.1\%$ in normal), but little change was observed in the chordae.

Rheumatic valves showed a wide range of concentration of glycosaminoglycan determined as hexuronic acid, with only a small increase over normal. In floppy-valve cusps the hexuronic acid content was significantly increased, this alteration being more apparent in posterior cusps (18.2 ± 6.1 cf. 9.9 ± 1.0 μ g/mg dry wt. in normal) than in anterior cusps (14.5 ± 8.0 cf. 10.5 ± 1.2 μ g/mg dry wt. in normal).

When proteoglycan content was estimated as sulphated glycosaminoglycan using chondroitin sulphate as standard, results for floppy valves showed a small increase in

Table 1. Content of hydroxyproline-containing material in neutral-salt extracts of mitral valves

The content of hydroxyproline and total protein in each valve extract was calculated as a percentage of the initial wet weight for that valve; the data from each set of valves were then used to calculate the mean \pm s.d. The number of valves in each set is given in parentheses.

Valve	Total hydroxyproline extracted (% of initial wet wt.)	Total protein extracted (% of initial wet wt.)	Extracted hydroxyproline (as % of total protein)
Transplant normal (15)	0.03 ± 0.02	1.77 ± 1.2	1.63 ± 1.06
Autopsy normal (10)	$0.004 \pm 0.003^*$	1.68 ± 1.0	$0.24 \pm 0.38^*$
Floppy (23)	0.04 ± 0.05	1.54 ± 1.1	2.55 ± 1.28
Rheumatic (11)	$0.003 \pm 0.003^*$	1.46 ± 1.2	$0.40 \pm 0.41^*$

* Student's *t* test showed significant differences when compared with data for transplant normal valves ($P < 0.001$).

Table 2. Analysis of the cusps and chordae from normal, floppy and rheumatic valves

Experimental details are given in the text. All assays were done in triplicate and results are expressed as means \pm S.D., with the number of samples in each group given in parentheses. Abbreviation: GAG, glycosaminoglycans.

Parameter	Part of valve	Normal	Floppy	Rheumatic
Wet wt. (g)	Cusps	1.4 \pm 0.4 (26)	*4.4 \pm 2.7 (24)	*3.3 \pm 1.2 (16)
	Chordae	0.2 \pm 0.09 (22)	†0.6 \pm 0.6 (25)	*0.6 \pm 0.4 (16)
Water content (% of wet wt.)	Cusps	81.8 \pm 2.6 (19)	82.7 \pm 3.8 (21)	*76.4 \pm 3.6 (13)
	Chordae	74.7 \pm 4.7 (18)	†80.0 \pm 4.3 (19)	73.9 \pm 5.4 (12)
Collagen content (% of dry wt.)	Cusps	57.0 \pm 7.5 (40)	59.2 \pm 15.9 (32)	60.5 \pm 15.6 (17)
	Chordae	64.5 \pm 12.6 (32)	*72.7 \pm 11.1 (28)	75.6 \pm 8.8 (16)
Elastin content (% of dry wt.)	Cusps	10.0 \pm 1.7 (33)	12.7 \pm 5.8 (28)	11.0 \pm 5.4 (16)
	Chordae	9.7 \pm 2.1 (31)	11.4 \pm 4.9 (28)	10.7 \pm 3.9 (14)
Hexuronic acid content (μ g/mg dry wt.)	Cusps	9.8 \pm 0.9 (20)	*15.6 \pm 4.2 (21)	10.4 \pm 5.8 (13)
	Chordae	9.0 \pm 1.4 (19)	13.2 \pm 6.7 (18)	10.9 \pm 3.8 (13)
Sulphated GAG content (μ g/mg dry wt.)	Cusps	18.9 \pm 3.6 (16)	23.0 \pm 6.7 (30)	15.0 \pm 4.8 (13)
	Chordae	15.5 \pm 5.4 (16)	16.4 \pm 9.1 (23)	16.5 \pm 4.5 (23)

*,† Student's *t* test showed significant difference from the normal cusp value: **P* < 0.001; †*P* < 0.005.

Table 3. Proportion of initial hexuronic acid and sulphated glycosaminoglycan which could be readily extracted from mitral valves

The concentrations of hexuronic acid and sulphated glycosaminoglycans in the neutral-salt and 4 M-guanidinium chloride extracts were estimated as described in the text, the two results for each valve being added and calculated as a percentage of the total hexuronic acid or sulphated glycosaminoglycan in that valve (results in Table 2). The data for each set of cusps or chordae are expressed as means \pm S.D. and results from 'pathological' valves compared with corresponding results from normal valves.

	Proportion (% of total)	
	Cusps	Chordae
(a) Hexuronic acid		
Normal	26.7 \pm 7.7 (20)	29.7 \pm 15.0 (20)
Floppy	57.2 \pm 17.4 (22)*	52.3 \pm 20.2 (21)*
Rheumatic	38.7 \pm 17.0 (14)	27.2 \pm 11.3 (13)
(b) Sulphated GAG†		
Normal	24.8 \pm 10.0 (16)	26.5 \pm 12.0 (16)
Floppy	63.1 \pm 16.2 (30)*	41.6 \pm 19.7 (23)
Rheumatic	42.6 \pm 24.0 (13)	28.0 \pm 24.6 (13)

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

† Abbreviation: GAG, glycosaminoglycans.

mean value compared with normal valves, whereas those for rheumatic-valve cusps were consistently low. Neither assay indicated any consistent changes in proteoglycan content with age.

The most significant alteration in floppy valves compared with normal ones was the increased proportion of the initial proteoglycan which could be readily extracted (Table 3). The change was more significant for both cusps and chordae when proteoglycan was estimated by hexuronic acid content.

Amino acid composition

Analysis of four normal valves showed the amino acid distribution in valvular collagen to be almost identical with that of normal chest skin, with the exception of higher threonine and valine contents. Although the hydroxylysine content of valves was not higher than that of skin, the ratio of hydroxylated lysine to lysine was 0.27 compared with 0.17 for skin.

The differences seen between eleven floppy and the four normal valves were not significant and could be accounted for by the presence of variable amounts of elastin and differing proportion of type III relative to type I collagen.

Ratio of collagen type III to types (III + I) in the mitral valve

Cleavage with CNBr solubilized 85 \pm 3% of the total valve collagen from normal and floppy valves and chordae and 76 \pm 4% from rheumatic valves. Approx. 60% of the residue was elastin. Reproducibility of the SDS/polyacrylamide-gel-electrophoresis technique and densitometric quantification of the α 1(I) CB8 and α 1(III) CB5 peptides was assessed by running one digest from a normal valve ten times on different gels; the mean value was 29.0 \pm 2.6% (S.D.; range 27.0–30.0%).

Anterior and posterior cusps from normal valves and their chordae all showed similar type III/(III + I) collagen ratios to that shown by normal whole cusps. There was a significant increase over normal values in the proportion of type III collagen present in rheumatic cusps and especially chordae (36% increase) (Table 4). The mean values for floppy cusps and chordae, although higher than normal, showed no significant difference, owing to a large variation in values for individual samples. Analysis of separated floppy cusps revealed a higher proportion of type III in the anterior cusp than in the posterior, but the difference was not significant. The finding that the proportion of type III collagen tended to be higher in anterior cusps from floppy valves compared with the corresponding posterior cusps, together with the more frequent observation of secondary surface fibrosis in anterior cusps, prompted a comparison of type III

Table 4. Type III collagen as a percentage of types (III + I) collagens in mitral valves

Experimental details are given in the text. Results are expressed as means \pm S.D., with the number of measurements in each group given in parentheses. Abbreviation: F, fibrosis.

Valve	Proportion (%) of type III collagen [= III/(III + I) \times 100]			
	Cusps	Floppy cusps + F	Floppy cusps - F	Chordae
Normal	29.3 \pm 2.1 (37)			26.6 \pm 2.6 (20)
Floppy	32.7 \pm 8.7 (24)	34.4 \pm 7.5 (26)*	25.2 \pm 2.8 (10)*	30.2 \pm 5.8 (26)
Rheumatic	33.2 \pm 2.75 (17)*	—	—	36.3 \pm 5.6 (17)*

* Student's *t* test showed significant differences from the corresponding values for normal valves ($P < 0.001$).

collagen content in cusps with and without appreciable secondary fibrosis determined by observation of elastic-van Geisen-stained sections (Table 4). Comparison with the proportion of type III collagen in normal cusps indicated a significant increase in floppy cusps with fibrosis, but, in contrast, a significant decrease in floppy cusps where negligible fibrosis was observed.

DISCUSSION

Although the alterations in the relative proportions of the biochemical constituents of pathological valves are small and very variable, it should be emphasized that, because of the 3-fold increase in net weight of these valves, the total contents of each component are also increased 3-fold. This finding is consistent with the raised synthetic activity of such valves in organ culture (Henney *et al.*, 1982).

The concentrations of collagen and elastin estimated in individual normal valves were similar to those reported by Bashey *et al.* (1967), with confirmation of the slight increase in elastin with donor age, whereas those of hexuronic acid were in agreement with the data of Torii *et al.* (1965) for human valves and Moretti & Whitehouse (1963) for bovine valves. The anterior and posterior cusps from individual normal valves showed no significant differences in composition, whereas chordae differed from cusps in having higher collagen and lower water contents.

The present data indicate that both the biochemical composition and the histology of individual floppy valves is very variable. The significant rise in hexuronic acid content of floppy valve cusps (but not chordae) was also found by Cole *et al.* (1984) and is consistent with the increased proteoglycan demonstrated histologically (Guthrie & Edwards, 1976). The increase of proteoglycan alone is insufficient to account for the leaflet thickening, in contrast with the view expressed by Wynne (1986); our data show that collagen, and in particular elastin, also increase.

The most striking biochemical difference observed between normal and floppy valves was the increase from 26.7 to 57.2% respectively in the extractability of the proteoglycans by neutral salt and guanidinium chloride solutions. This loosening of the interaction between proteoglycans and collagen fibres in floppy valves could be a consequence of a primary disruption and disorganization of the collagen fibres, or could reflect a

change in the nature of the proteoglycan population or its interaction with the collagens.

A simple explanation for the pathogenesis of floppy mitral valves would have been a failure to cleave the *N*-terminal propeptides of type I procollagen, as previously suggested (Bonella *et al.*, 1980). Accumulation of procollagen has been shown to interfere with normal fibre formation in degenerative cartilage in dogs, resulting from osteoarthritis (Miller & Lust, 1979). The present data do not support this concept. Levels of neutral-salt-soluble collagen and procollagen in floppy valves were similar to those in normal valves provided that the latter were freshly obtained (from the hearts of transplant patients) and extracted immediately. The valves from transplant patients were always checked for normal pathology before being included as controls. The failure of others (Cole *et al.*, 1984) to demonstrate procollagen in floppy valves resulted from storage of the valves at -70°C before extraction, a procedure shown here to cause loss of procollagen.

A different possible cause of structural weakness in floppy valves could be an inefficient maturation of collagen fibres due to a deficiency in cross-linking. Similar macroscopic changes occur in the valves of rats made experimentally lathyrictic by a diet of β -aminopropionitrile (Oka *et al.*, 1966; Jeresaty, 1973). The predominant reducible cross-link in porcine heart valve is Δ^6 -dehydro-5,5'-dihydroxylysino-leucine, derived from two hydroxylysine residues (Collins *et al.*, 1977) and normally found in connective tissues with high tensile strength, such as bone and tendon (Bailey *et al.*, 1974). This finding is consistent with the high hydroxylysine/lysine ratio found in bovine (Bashey *et al.*, 1972) and human mitral valves (Cole *et al.*, 1984). The contents of hydroxylysine in both type I and III collagens and the mobility of CNBr-derived peptides normally involved in cross-linking were no different in floppy valves from normal (Cole *et al.*, 1984). Bonella (1984) found no significant differences in either reducible cross-links or the mature cross-link pyridinoline between normal- and floppy-valve collagens.

Our data for the proportion of collagen type III in floppy valves reflect the extent of secondary surface fibrosis observed histologically. This finding may in part explain the difference between our results and those of Cole *et al.* (1984), who demonstrated a surprisingly consistent increase in the percentage of type III collagen in floppy valves, from 24% in normal valves to 31%, without histological observation of the valves. The lower

value obtained for the proportion of type III collagen in normal valves, compared with our value of 29%, also contributes to the discrepancy. This might be explained by the different extraction methods used: Cole *et al.* (1984) used CNBr digestion after sequential pepsin and dithiothreitol treatments (Jimenez & Bashey, 1978), whereas in the present study CNBr acted directly on the valve tissue. In those valve cusps in our study in which little or no fibrosis was observed, the proportion of type III was significantly reduced, though never absent, as in one patient described by Hammer *et al.* (1979). Our finding, that type III collagen is increased in those valves or valve cusps in which secondary fibrosis occurs, does not necessarily imply that all this new fibrotic collagen is type III; there is an increase in both types of collagen, but proportionally more type III than type I. The fibrosis occurs as a result of trauma to the hypermobile expanded cusps and chordae, and such secondary fibrosis often contains a high proportion of type III collagen (Light, 1982), as it quickly forms a 'scaffold' owing to retention of disulphide bridges (Nimni, 1983). Excess, but uncontrolled, production of collagen, proteoglycan and elastin on the valve surface continues to be stimulated by the persistent trauma of mechanical impact (Marshall & Shappell, 1974), leading to a net increase in size of the floppy valves, despite loss of core collagen from both cusps (Davies *et al.*, 1978) and chordae (Bittar & Sosa, 1968; McKay & Yacoub, 1973).

A small amount of fibrosis on the atrial surface at the line of apposition on the anterior cusps was present in all normal valves investigated, even in young adults, in agreement with the results of a more extensive investigation by Pomerance (1967). In floppy valves an additional pattern of secondary fibrosis occurs on the atrial, ventricular and even chordal surfaces, as observed previously (Trent *et al.*, 1970; Guthrie & Edwards, 1976; Shrivastava *et al.*, 1977) and consistent with our finding of significant net increase in the collagen component of the floppy chordae.

The proliferation of collagen in floppy mitral valves occurring in the form of surface fibrosis, despite destruction of core collagen, contrasts strongly with the thickening of core collagen and lack of surface fibrosis in rheumatic cusps and chordae observed in this series; collagen fibres in rheumatic chordae appear normal in the electron microscope (Sung *et al.*, 1981). The elastin and collagen moieties both contribute to this increase in mean value, although the change in each component is not significant because of the great variation in results from individual valves. Both types of collagen, but particularly type III, contribute to the thickening which, with the lower water content, causes a stiffening of both cusps and chordae, leading to mitral stenosis whether or not calcification occurs (Pomerance, 1972; Fenoglio & Wagner, 1973; Rippe *et al.*, 1980). A further contrast between the two pathological valves was the low amount of collagen precursors in rheumatic valves, suggesting a low rate of collagen turnover compared with normal and floppy valves.

It is difficult to understand the initial reason for, and the mechanism of, collagen loss in the core of the cusps and chordae of floppy valves, although this is presumably enzymic breakdown (Caulfield *et al.*, 1971). Degradation by mammalian collagenase seems improbable, since floppy and normal valves fail to synthesize any active or latent collagenase in culture, even when the tissue is

subjected to potent stimuli of collagenase production, although the tissue inhibitor of metalloproteinases ('TIMP') is present (A. M. Henney, unpublished work). Electron-microscopic studies have been reported in which collagen fragments have been observed in membrane-bound cellular inclusions, possibly secondary lysosomes (Renteira *et al.*, 1976; Gravanis & Campbell, 1982), and collagen degradation can be initiated by cathepsin B (Burleigh *et al.*, 1974). Once the initial destruction of collagen has occurred, attempts at compensatory synthesis by fibroblasts may be rapid and lack normal regulation, leading to overproduction of collagens, elastin and proteoglycans. Our conclusion is that the collagen type ratio reflects contributions by this process and by traumatically induced secondary fibrosis in valves which have deteriorated to the extent necessitating surgical replacement; hence the early stage of pathogenesis could only be studied in an animal model such as the dog, which can also develop floppy mitral valve (Wagner, 1968; Pomerance & Whitney, 1970).

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