Semiquantitative analysis of collagen types in the hypertrophied left ventricle

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ABSTRACT

Cardiac fibrosis is a characteristic feature of left ventricular hypertrophy. The aim of this study was to develop a simple and accurate method to analyse collagen accumulation, taking into account the variation in cardiac muscle fibre orientation and nonuniform collagen distribution. This technique was used to determine the amount and types of collagen that accumulate during pressure overload cardiac hypertrophy. These data were correlated with myocyte size, and with the diastolic stress–strain relationship of the intact myocardium. Myocyte size was significantly increased in the hypertrophied hearts, compared with age and sex matched controls (control 363 ± 25 μm² vs experimental 244 ± 12 μm²; mean ± S.E., $P < 0.05$). No overall collagen accumulation was observed in the hypertrophied hearts, but a significant increase in collagen I was found with a reduction in the amount of collagen III in experimental animals. Since no increase in diastolic stiffness of the hearts was observed, these results indicate that an increase in the overall collagen content of the heart, rather than the upregulation of a specific type, may be necessary to cause diastolic dysfunction.

Key words: Heart; collagen; left ventricular hypertrophy.

INTRODUCTION

Despite advances in cardiac research, heart failure still remains one of the dominant causes of death worldwide. Recent evidence has implicated left ventricular hypertrophy as one of the most important prognostic factors associated with heart failure and sudden cardiac death (Lenfant, 1996). Left ventricular hypertrophy often leads to diastolic dysfunction, resulting from a diminished rate of cardiac relaxation and diastolic filling. The primary cause of diastolic dysfunction associated with hypertension was assumed to lie within the myocytes (Badeer, 1964). It is now more widely recognised that, whereas cardiac myocytes and the coronary vasculature are central to the contractile function and viability of the myocardium, so too is the extracellular matrix, or cardiac interstitium (Weber, 1989). For the heart to function optimally, contractile, vascular and interstitial compartments must be in equilibrium with each other, based on their structural integrity and arrangement, relative proportions and biochemical characteristics (Weber et al. 1987). A disproportionate accumulation of fibrillar collagen within the heart leads to cardiac fibrosis (Weber et al. 1995).

Numerous biochemical and histological studies investigating collagen accumulation in the hypertrophied heart have demonstrated a correlation between increased collagen deposition and diastolic stiffness (Jalil et al. 1989; Janicki, 1992; Weber, 1989; Weber & Brilla, 1991). One method of determining the amount of collagen present in the heart is by assessing the hydroxyproline concentration in a sample of cardiac tissue (Stegemann & Sadler, 1967). This method is not particularly effective when attempting to correlate diastolic stiffness with collagen content as it overlooks 2 important factors. First, it is not merely the quantity of collagen that affects myocardial stiffness but the arrangement of the collagen bundles, which cannot be assessed using this technique. Secondly, it has been shown that the collagen content of the vessel wall and of the perivascular spaces may be markedly increased in experimental hypertension (Staubesand & Fischer, 1980). This connective tissue

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contributes significantly to the overall collagen content but confers little to mechanical stiffness of the heart and cannot be distinguished from the interstitial collagen by a hydroxyproline assay (Thiedemann et al. 1983).

Histological quantification of cardiac collagen content involves staining sections of heart, usually using sirius red, and assessing random frames of connective tissue using image analysis (Jalil et al. 1988). This method has an advantage over biochemical analysis in that perivascular fibrosis can be distinguished from interstitial collagen. However, in order to be accurate, histological methods must take into account the great variation in cardiac muscle fibre orientation and the nonuniform distribution of collagen throughout the heart. One technique that overcomes this problem is to compare collagen deposition in the subendocardium, mesocardium and subepicardium in experimental hearts individually with the corresponding regions in control hearts (Ho et al. 1996). However, the majority of studies investigating cardiac fibrosis fail to take either the variation in muscle fibre orientation or nonuniform collagen distribution into account (Doering et al. 1988; Jalil et al. 1991; Sabbah et al. 1995). The aim of this study was to devise a simple and accurate semiquantitative method to analyse collagen in the subendocardium of the left ventricle.

It is not only the increase in total collagen in the cardiac tissue that may create a pathological state, but also changes in the ratio of collagen types. It is well documented that cardiac fibrosis results from an accumulation of collagen types I and III (Eghbali et al. 1989; Weber, 1989; Weber & Brilla, 1991). Type III fibres frequently coexist with type I, and together they constitute approximately 90% of the total collagen content of the heart. Both types are fibrillar in nature, but type I forms thick fibres as opposed to type III that creates a fine reticular network. Type I fibres have extremely high tensile strength and largely determine the stiffness of the myocardium. Type III collagen fibres possess a resilience that is suitable for maintaining structural integrity and distensibility of the collagen network (Weber, 1989).

Other less abundant collagen types, such as IV, V and VI, are also likely to play an integral part in the preservation of normal cardiac structure. Type IV is a basement membrane protein produced predominantly by endothelial cells and myocytes (Eghbali et al. 1989). It does not form fibres, but is laid down in the basement membrane as an open network where it plays a role in the regulation of molecular transport and cell adhesion (Madri et al. 1980). Type V is found coexisting with type IV in the basement membrane and interspersed with type I and III in the interstitium (Madri et al. 1980). It appears closely associated with the cell surface where it may contribute to cell shape by forming an exocytoskeleton (Amenta et al. 1986). Collagen type VI also resides in the interstitium, where it coats the surface of the fibrillar collagens, possibly regulating fibre size (Amenta et al. 1986). It has not yet been determined what effects pathological conditions, such as cardiac hypertrophy, have on these less prevalent collagen types. As these collagen types are involved both in regulating the structure of the heart and the transportation of molecules, it is likely that any alterations in their distribution or turnover could have substantial repercussions on cardiac function.

In this study, the effect of left ventricular hypertrophy on collagen deposition in the heart has been investigated. The amount and distribution of the different collagen types has been analysed in both the normal and hypertrophied hearts and the results correlated with diastolic stiffness.

MATERIALS AND METHODS

Animal model

Animals were maintained, handled and operated upon in accordance with the guidelines laid down by the Home Office of Great Britain and Northern Ireland. Pressure overload cardiac hypertrophy was induced in adult male Sprague–Dawley rats (Charles River) (230–280 g) by constriction of the abdominal aorta interrenally as described previously by Boateng et al. (1998). Anaesthesia was induced by an intraperitoneal injection of ketamine, medetomine and sterile water (ratio 2.4:1:9). A laparotomy was performed and the abdominal aorta exposed and partially ligated between the left and right renal arteries using a 0.7 mm OD blunted needle. The antibiotic, amphipen (30 mg/kg) and antisedan, (1.4 mg/kg) an anaesthetic reversal agent, were administered postsurgery. Sham operated controls were subjected to the same procedure as experimental animals, without the constriction of the abdominal aorta. Animals were maintained for 9 wk postsurgery and allowed food and water ad libitum.

Physiological study

At 9 wk postsurgery, animals were anaesthetised with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg bodyweight). Heparin (1000 iu/kg) was

administered via the femoral vein. The heart was quickly removed, the thoracic aorta cannulated and perfused in a modified Langendorff isovolumic mode (Ogino et al. 1996). Perfusions were carried out using Krebs-Henseleit buffer (37 °C) gassed with 95% O_2/5% CO_2 at a constant head of pressure (85 cm H_2O). A PVC balloon, connected to a pressure transducer, via a fluid filled line, was inserted into the left ventricle. The balloon volume was increased in 0.02 ml increments over a left ventricular diastolic pressure of 0–35 mmHg and the resulting pressure traces recorded using a Maclab recorder system (AD instruments). To assess myocardial stiffness, stress (δ) and strain (ϵ) for the midwall of the left ventricle were calculated assuming spherical geometry (Doering et al. 1988).

$$\text{Stress} = \frac{[1.36 \cdot \text{LVEDP} \cdot \text{V}^{2/3}]}{[(\text{V} + 0.943 \cdot \text{LVW})^{2/3} - \text{V}^{2/3}]}$$

$$\text{Strain} = \frac{[\text{V}^{1/3} + (\text{V} + 0.943 \cdot \text{LVW})^{1/3}]}{[\text{V}_0^{1/3} + (\text{V}_0 + 0.943 \cdot \text{LVW})^{1/3}] - 1}$$

V = volume (ml); V_0 = volume at end diastolic pressure of 0 mmHg; LVW = left ventricular weight (g); LVEDP = end diastolic pressure (mmHg).

The degree of cardiac hypertrophy was determined from the heart weight to tibial length ratio (Yin et al. 1982).

Tissue analysis

Hearts were arrested in diastole using St Thomas' Hospital cardioplegic solution. The major coronary vessels and atria were dissected away and the left and right ventricles kept intact and weighed. The volume of the ventricles was calculated by displacement and the length measured from apex to base. Hearts were precooled in 2-methylbutane and frozen in liquid nitrogen. The precise location of the equator of each heart was calculated and 12 µm cryostat sections were cut at this point. The sections were stained and used to determine the extent of cardiac myocyte hypertrophy and the total amount and distribution of collagen in a reproducible region.

Myocyte size

Sections of tissue were fixed in formol saline for 10 min, dehydrated through graded alcohols and immersed in eosin for 1 min. The samples were then dehydrated in 95% and absolute alcohol, cleared and mounted.

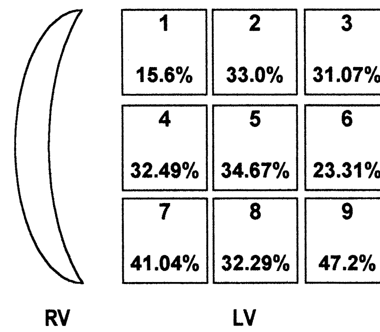

Fig. 1. Diagrammatic representation of the variation in myocyte hypertrophy in different regions of an equatorial cross-section of the subendocardium of the left ventricle.

Myocyte diameter and perimeter were assessed using the trace facility of the image analyser (Sonata II, Seescan). Ten cells selected randomly in 9 specific regions of the subendocardium of the left ventricle were measured (Fig. 1). Myocyte area was calculated using the smallest diameter of the cell, thus allowing for variation in muscle fibre orientation within the heart (Gerdes, 1997). Assessing myocyte dimensions in 9 fixed regions allowed the extent and variation of hypertrophy throughout the left ventricle to be evaluated.

Connective tissue distribution

Total amount and distribution of collagen were determined using sections stained with 0.1% solution of sirius red F3BA in saturated aqueous picric acid (Sweat et al. 1964). Addition of fast blue to the staining procedure prevented uptake of sirius red into the cytoplasm (Williams et al. 1998). This created greater contrast between the collagen and intracellular elements and thus facilitated image analysis. Sections were dehydrated, cleared and mounted.

To analyse collagen accumulation, taking into account the variation in cardiac muscle fibre orientation and nonuniform collagen distribution, a simple but accurate method of image analysis was developed. Sections stained with sirius red were analysed using the image analysis thresholding facility. The threshold level was adjusted so the amount and distribution of red pixels corresponded to the interstitial collagen. The collagen content of the subendocardium was assessed frame by frame, plotting the amount of collagen in each frame, thereby creating a collagen map (Fig. 2).

The total area of the subendocardium was measured, the number of frames in this region calculated and thus the total collagen content de-

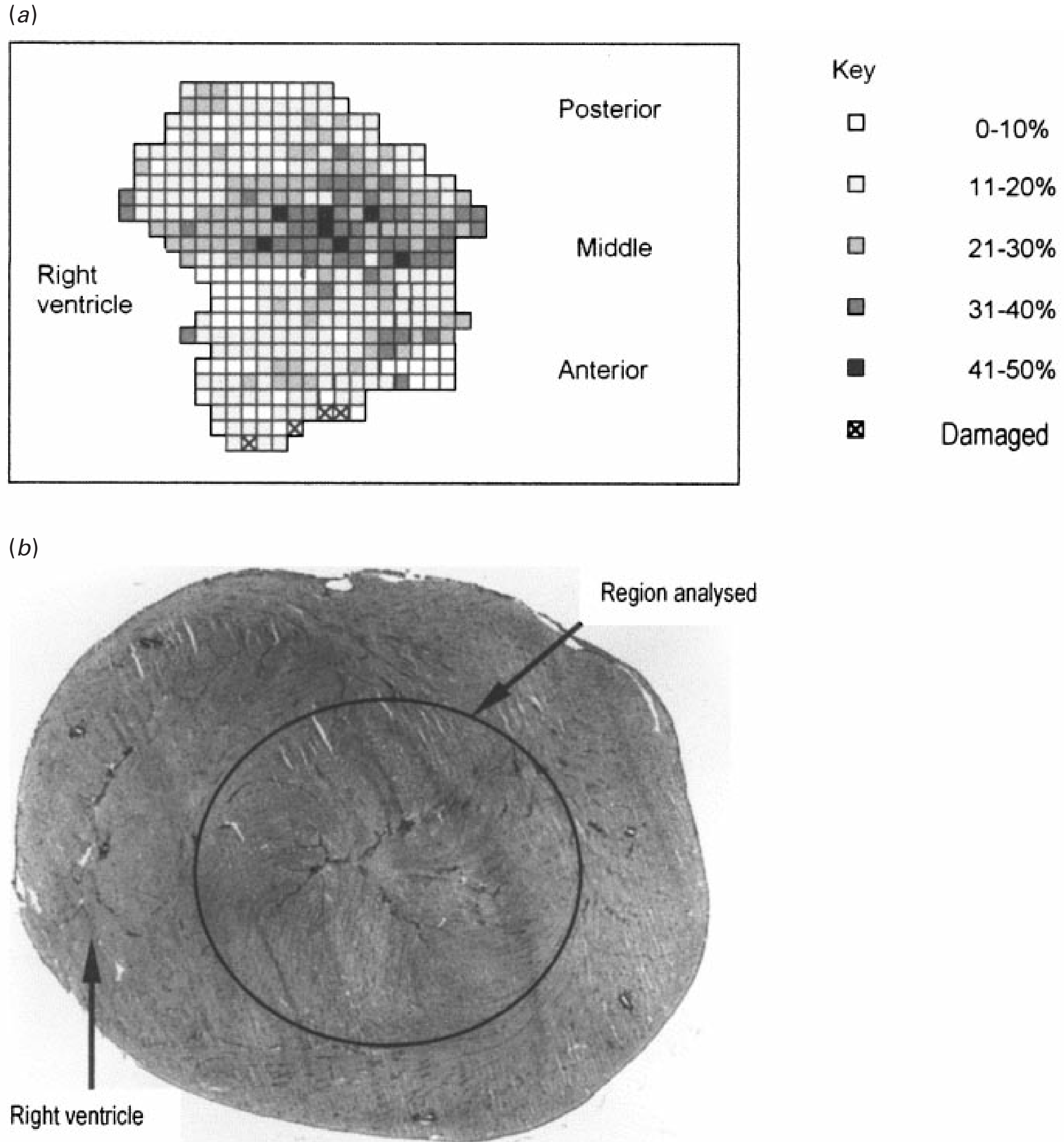

Fig. 2. (*a*) Diagrammatic representation of the collagen distribution in the subendocardium of the left ventricle of a control rat heart. This map reveals a higher concentration of collagen in the middle of the subendocardium compared with the anterior or posterior regions (*b*). Frozen section taken from the equator of the heart. Section illustrates the region in the heart in which collagen distribution was assessed to create the collagen map shown in *a*.

Table 1. *Comparison of the accuracy of 2 techniques of image analysis used to determine the amount of collagen in a control heart*

Technique	Total collagen area ($\mu m^2 \times 10^6$)
Collagen mapping	3.9
15 frame analysis	3.6

termined. A second analysis was then performed on the same section, analysing only 5 frames from the anterior, middle and posterior regions of the cross section. The result from this second analysis was compared with those from the collagen map and no difference was observed, either in total collagen content or distribution (Table 1). This confirmed that the second, less time consuming method, was an accurate means of measuring collagen in cardiac tissue.

Collagen types

The primary antibodies used for this study were polyclonal goat antihuman collagen types I, III, IV, V

Table 2. *Gross anatomical effects resulting from* 9 *wk of abdominal aortic banding*

	Control (n = 7)	Experimental (n = 7)
Body weight (g)	574±33	538±21
Ventricular weight (g)	1.53±0.10	1.75±0.07
Tibial length (cm)	4.66±0.03	4.59±0.05
Ventricular weight/ tibial length (g/cm)	0.32±0.02	0.38±0.02*
Left ventricle length (cm)	1.6±0.03	1.7±0.04
Ventricular volume (cm^2)	2.36±0.18	2.21±0.21
Myocyte area (μm^2)	244±12	363±25*
Right kidney/left kidney weights	0.97±0.02	1.34±0.05*

Values are mean±S.E. * = $P < 0.05$ vs controls.

and VI (Cambridge Biosciences). All incubations were carried out at room temperature and sections were washed with Tris buffer between incubations. Staining for collagen I was enhanced by the exposure of the epitopes using microwaves. Slides were immersed in a 0.1% solution of sodium citrate at pH 6.1 (Sigma) and heated for 8 min in a 800 W microwave on full power (Miller, 1996). Immunostaining for all collagen types was then as follows. The sections were incubated for 30 min in 4% hydrogen peroxide to completely block endogenous peroxide activity. The sections were treated with normal swine serum (1:5) (Dako) for 20 min to prevent any nonspecific binding to the primary antibody. The serum was tapped off and the primary antibody (1:10) was applied and incubated for 1 h. Amplification of the primary antibody signal was carried out by 2 individual 30 min incubations with the secondary antibodies, rabbit antigoat HRP (1:200) and swine antirabbit HRP (1:100) (Dako). The peroxidase complexes were exposed by the application of 3,3′-diaminobenzidine (Sigma). Sections were dehydrated in graded alcohols, cleared and mounted.

The total amount and distribution of the various collagen types were assessed by image analysis, employing the same method used to analyse the connective tissue.

Statistical analysis

Differences between the experimental and control group were tested for significance ($P < 0.05$) using Student's *t* test, with the exception of myocyte area, which was evaluated by a nonparametric Kruskal–Wallis test.

RESULTS

Morphology

Experimental animals displayed an increase in heart weight to tibial length ratio, the standard indicator of the presence of hypertrophy (Table 2) (Yin et al. 1982). Myocyte area increased by an average of 38% following 9 wk of banding in comparison with the control group, confirming the presence of significant cardiac hypertrophy in the experimental group (Fig. 4). The extent of myocyte hypertrophy was not uniform throughout the left ventricle and the variation

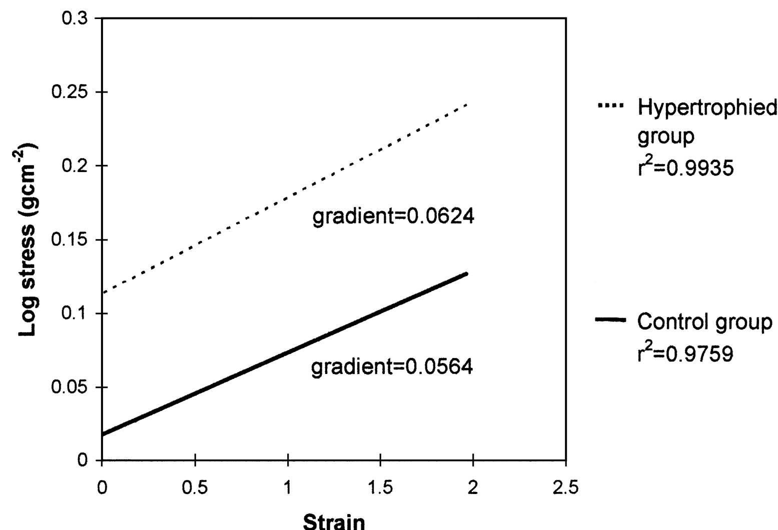

Fig. 3. Diastolic stiffness constant of the heart as determined by the gradient of the log [stress] strain correlation. No significant difference between the diastolic stiffness of hearts from control (n = 6) and hypertrophied hearts (n = 6) is observed.

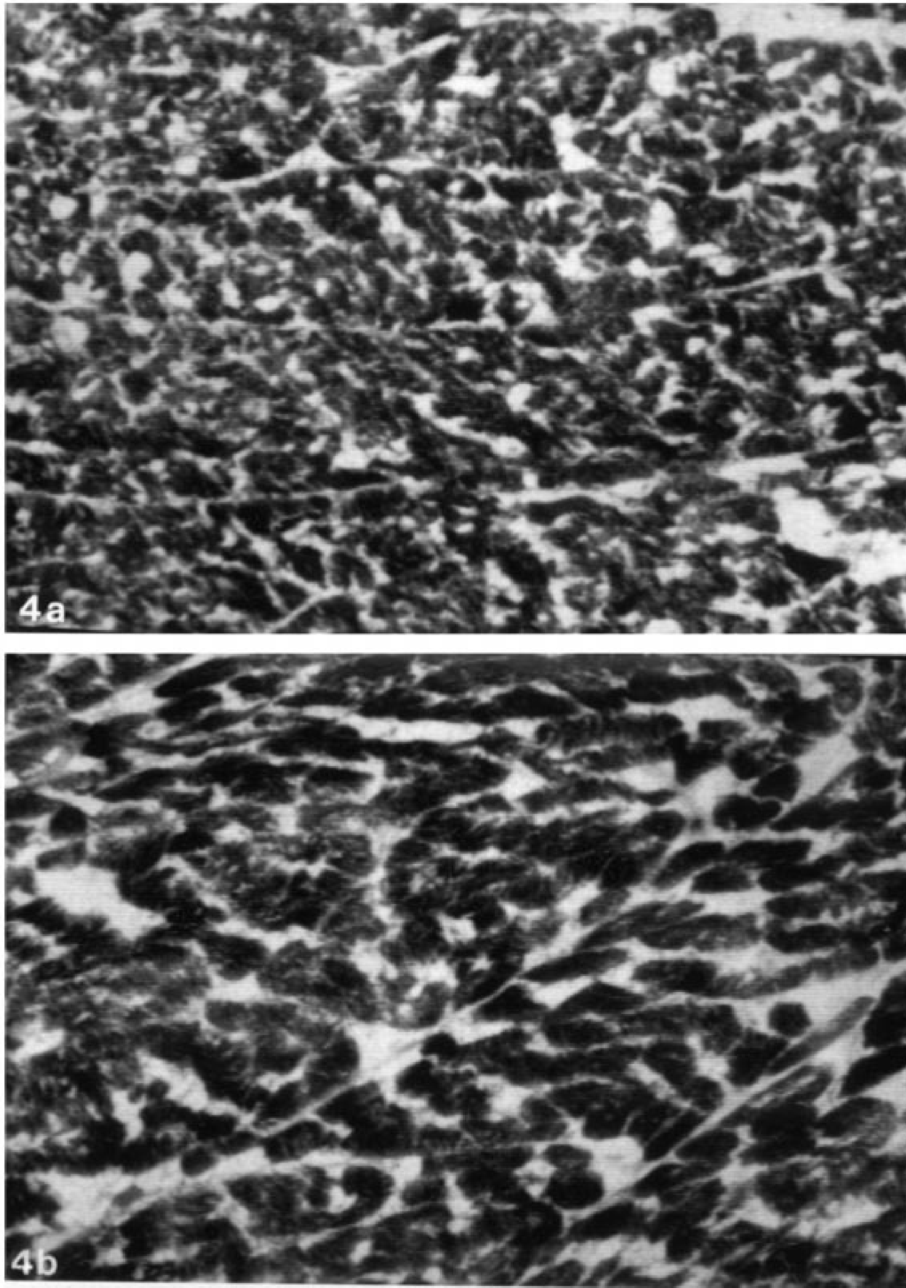

Fig. 4. Eosin-stained sections of control (*a*) and experimental (*b*) hearts. ×25. Following 9 wk of pressure overload myocyte area increased by an average of 38 % in experimental hearts.

Table 3. *Quantification of collagen in the left ventricle*

Collagen area	Control (n = 7) ($\mu m^2 \times 10^6$)	Banded (n = 7) ($\mu m^2 \times 10^6$)
Total	4.30±0.45	3.67±0.61
Type I	2.94±0.54	5.55±0.52*
Type III	5.29±0.89	3.45±0.69
Type IV	7.49±1.00	7.99±0.99
Type V	4.98±0.97	5.85±0.56
Type VI	6.93±1.00	6.78±0.87

Values are mean±S.E. * = $P < 0.05$.

in myocyte size in different regions is displayed in Figure 1.

Connective tissue

There was no significant difference between the total collagen content in experimental and control hearts (Table 3). Examination of the anterior, middle and posterior regions of the subendocardium revealed no apparent changes in the total collagen content between experimental and control groups. In the experimental

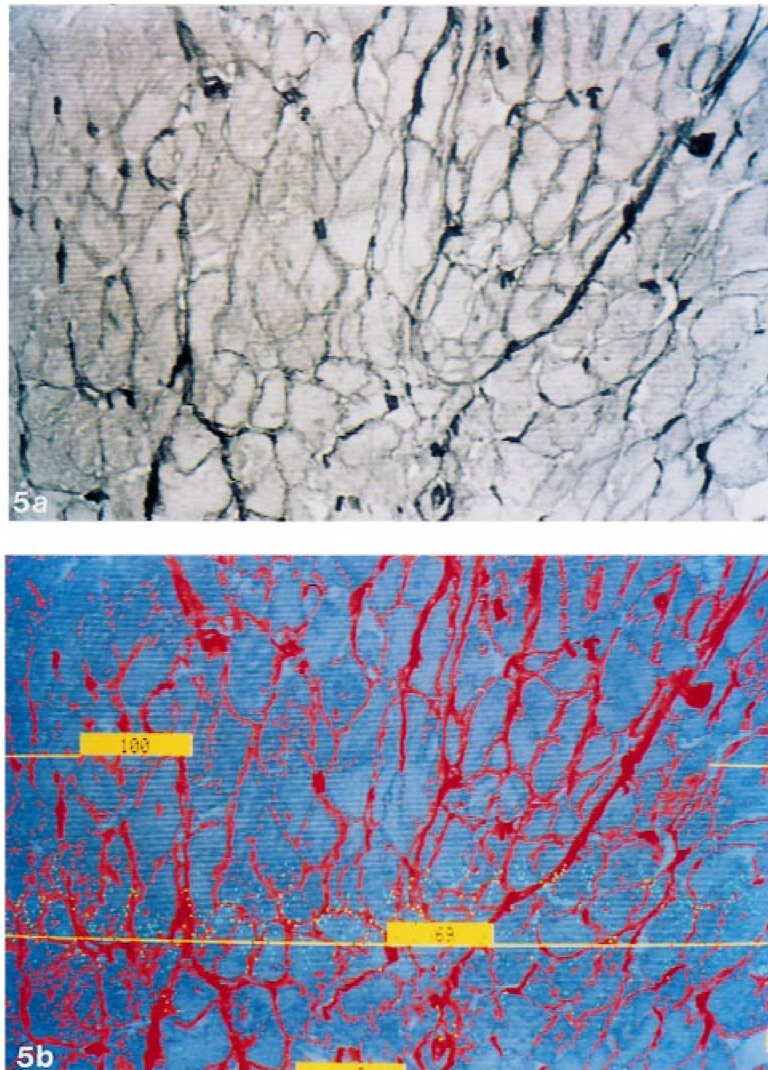

Fig. 5. (*a*) Frozen section of hypertrophied heart stained with sirius red to display connective tissue (*b*). Identical section displayed on the image analyser, with the threshold levels set to incorporate all connective tissue (red). ×25.

group, there was a significant increase in the total amount of collagen I present with a reduction in the amount of collagen III (Figs 5, 6). No significant difference was observed in the other collagen types.

Diastolic stiffness

Following perfusion, the diastolic stiffness constant of the hearts was calculated from the gradient of the log [stress] strain correlation (Fig. 3). No significant difference ($P = 0.5920$) was observed in the diastolic stiffness constant of experimental hearts ($n = 6$) compared with those from the control group ($n = 6$).

DISCUSSION

This work describes a semiquantitative method for analysing collagen deposition in the hypertrophied heart. This method of image analysis permits both the determination of global collagen accumulation within the left ventricle and the assessment of collagen in defined regions within the subendocardium. The map of the total collagen distribution in a control heart revealed a higher concentration of collagen in the middle of the left ventricle than in the anterior or posterior regions (Fig. 2). This result emphasises the nonuniform collagen distribution within the left ventricle, and therefore the importance of using comparable regions of the heart when investigating changes in collagen deposition associated with cardiac hypertrophy.

Compensating for myocyte hypertrophy when quantifying cardiac fibrosis is a major problem. No method has yet been devised to overcome this issue satisfactorily. Expressing the amount of collagen per unit of tissue does not compensate for myocyte hypertrophy. Myocytes in a hypertrophied heart occupy a higher proportion per unit of tissue than

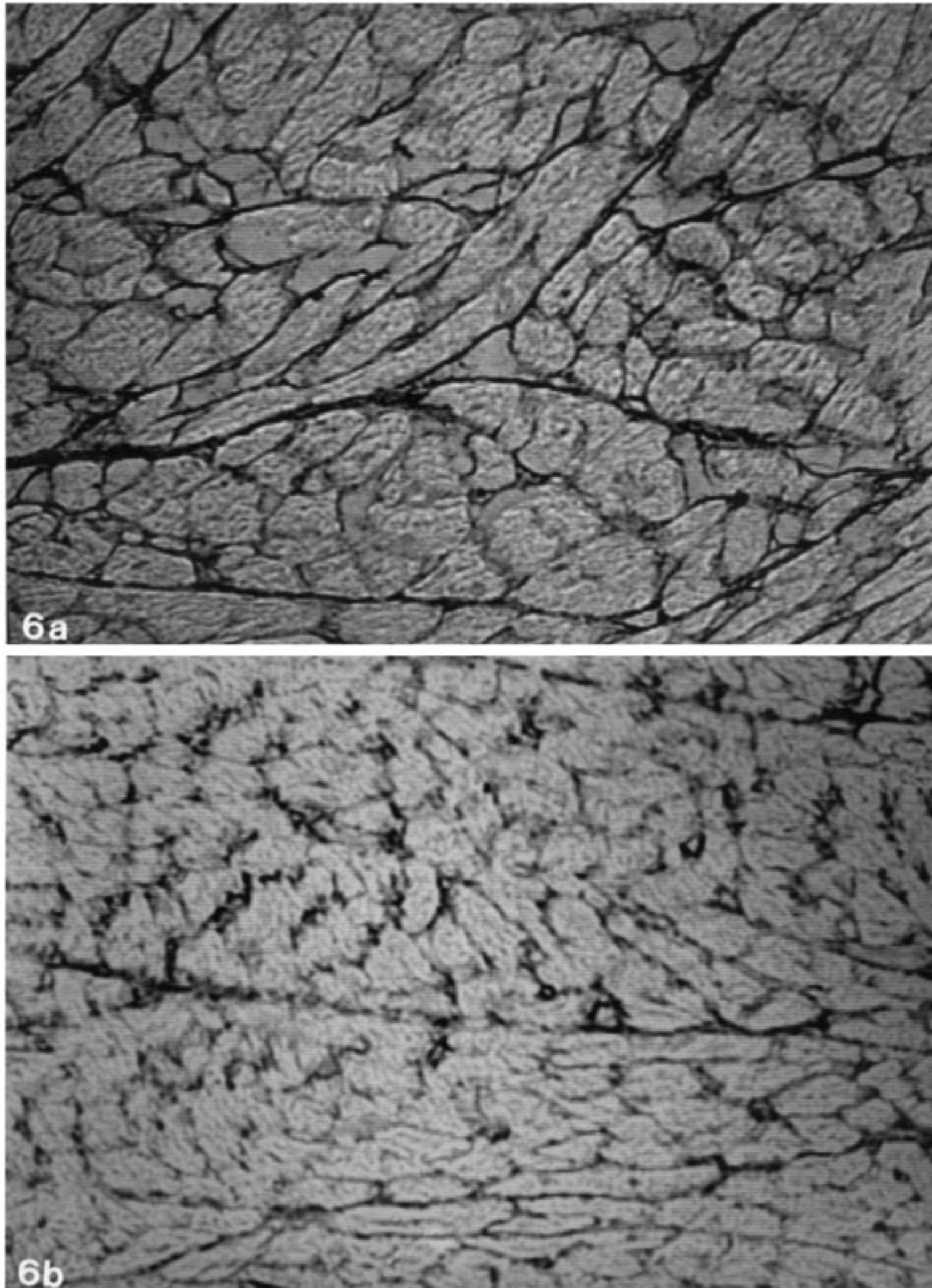

Fig. 6. Frozen sections of hypertrophied heart stained for (*a*) collagen I and (*b*) collagen III. ×25.

in a control heart. This means that even when collagen deposition is increased, there may be a smaller amount of collagen per unit of tissue in hypertrophied hearts compared with controls. In this study, collagen deposition was expressed globally to overcome this problem.

Using a pressure overload model of cardiac hypertrophy, significant cardiac myocyte hypertrophy was produced. The extent of myocyte hypertrophy was not uniform throughout the subendocardium (Fig. 1). Myocytes in the anterior lateral region of the subendocardium hypertrophied to a greater degree than those in the posterior medial region. It is conceivable that due to their position in the free wall of the left ventricle; myocytes in the anterior lateral region are exposed to higher pressures during the cardiac cycle than those in the posterior medial region.

In experimental animals, the total collagen content did not change but a significant increase in collagen I was observed with a reduction in collagen III. A similar change in proportions of collagen type I and III has been observed in hearts with dilated cardiomyopathy (Marijianowski et al. 1995). It has been

suggested that the deposition of collagen I is limited by the presence of collagen III (Contard et al. 1993; Fleischmajer, 1986; Romanic et al. 1992). In normal hearts collagen III is laid down onto a core of collagen I, and thus might inhibit further deposition (Marijianowski et al. 1995). In this study, the decrease in collagen III observed in experimental hearts may have prevented the inhibition of collagen I production, and thus led to a significant increase in collagen I.

The thick fibres of collagen I provide the heart with its tensile strength, and a small increment in the concentration of this inelastic element adds substantially to myocardial stiffness (Weber et al. 1995). Collagen III fibres maintain the structural integrity and distensibility of the heart (Weber, 1989), thus a reduction in the amount of collagen III would be expected to cause a less compliant, or stiffer heart (Mays et al. 1988). It is therefore surprising that neither a significant increase in the amount of collagen I, nor a reduction in collagen III, caused a concomitant increase in the diastolic stiffness constant in the experimental group. It is possible that an increase in the overall collagen content of the heart, in addition to the upregulation of a specific type, is necessary to cause diastolic dysfunction. Alternatively, the use of a PVC balloon to monitor diastolic function may have been a relatively insensitive technique and unable to detect small alterations in diastolic stiffness.

Both haemodynamic overload and the upregulation of the renin-angiotensin-aldosterone system (RAAS) are contributory factors in the development of myocyte hypertrophy and cardiac fibrosis (Weber & Brilla, 1991). However, the relative contribution of these 2 factors is not clearly understood. As pressure overload and the RAAS are linked, it is difficult to determine which is the primary stimulus for hypertrophy or fibrosis. The model of pressure overload used in this study has produced significant myocyte hypertrophy without the increase in total collagen deposition characteristic of cardiac fibrosis, and thus may prove to be a useful tool in clarifying the role of haemodynamic and humoral factors in the progression of cardiac hypertrophy.

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