Originals

Increased rat myocardial type VI collagen in diabetes mellitus and hypertension

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Summary. Diabetic cardiomyopathy, a condition characterized by the accumulation of carbohydrate-containing material surrounding the myocardial small blood vessels, has been studied in alloxan-diabetic normotensive and hypertensive rats. Immunochemical techniques were used to monitor several extracellular matrix constituents present in extracts of cardiac tissue, namely types I, IV and VI collagen, laminin and fibronectin, as well as myosin. These studies have indicated that after induction of diabetes, type VI collagen but none of the other matrix components studied, was significantly increased (from 2.29 ± 0.04 mg/g in normal to 2.85 ± 0.18 mg/g in diabetic ventricles, $p < 0.01$). Hypertension, whether induced by the clipping of one renal artery or genetically determined (spontaneously hypertensive rats), resulted in a similar elevation in type VI collagen (2.71 ± 0.12 mg/g, $p < 0.005$ compared to normal rats). In the

presence of diabetes plus hypertension the effect was not additive, the type VI collagen level being 2.93 ± 0.15 ($p < 0.001$ compared to normal rats). Basement membrane collagen (type IV) in the myocardium appeared to be unaffected by diabetes or hypertension and the myosin contents of the hearts of the four experimental groups were similar. Quantitative determinations indicate that compared to type IV collagen, laminin or fibronectin, type VI collagen represents the major periodic acid-Schiff-reactive extracellular constituent of the rat ventricle. Its preferential increase in the heart in diabetes may provide insight into the molecular mechanisms of the diabetic microvascular disease.

Key words: Diabetes mellitus, hypertension, diabetic cardiomyopathy, type VI collagen, type IV collagen, type I collagen, laminin, myosin.

While the detrimental effects of diabetes mellitus on the kidney and eye have long been recognized, it is now clear that this disease also produces changes in the heart distinct from those caused by coronary atherosclerosis. This condition, termed diabetic cardiomyopathy [1–4] is characterized by the accumulation of periodic acid-Schiff (PAS) positive material surrounding the small blood vessels of the myocardium. A recent study from our laboratory [5] of rat myocardial glycoproteins has indicated that a major portion of the PAS reactivity of this tissue is accounted for by type VI collagen and has furthermore suggested that this molecule occurs in increased amounts in the hearts of alloxan-diabetic rats.

Although type VI collagen has been reported to occur in elevated amounts in the kidney lesions of human diabetes [6], the principal change observed in diabetic glomeruli is a substantial increase in the characteristic basement membrane collagen (type IV) which is accompanied by decreases in other macromolecular components, such as heparan sulphate proteoglycan and laminin [7, 8]. The glomerular changes are an example of the wide-

spread extracellular matrix changes occurring in diabetes [9] and are believed to represent the contribution of the epithelial, endothelial and mesangial cells which are present in these structures. It seems likely that the lesions observed at a specific site will be determined by the constellation of cell types present and their individual response to the altered milieu of the disease.

In the present study we have extended our studies of the extracellular matrix changes occurring in the diabetic myocardium and have, in addition, evaluated the effect of hypertension alone and in the presence of diabetes on myocardial content of both type VI and type IV collagen.

Materials and Methods

Animals

Male albino rats [CD strain as well as the spontaneously hypertensive rats (SHR) and their controls (WKY)] were obtained from Charles River Laboratories (Wilmington, Mass., USA). After an

Fig. 1. Characterization by immunoblotting of types IV and VI collagen in normal (N) and diabetic (D) rat hearts. Duplicate aliquots of SDS-mercaptoethanol extracts (representing 75 mg wet weight of ventricle from normal and diabetic hearts) were separated by PAGE and electroblotted prior to reaction with either anti-rat heart type VI or anti-rat type IV collagen. Although in this electrophoretogram the α_1 chain of type VI appears preferentially increased, this was not a constant finding. The migration of standard proteins (myosin, β -galactosidase, phosphorylase, bovine serum albumin, and ovalbumin) is indicated on the left

overnight fast animals were randomly selected for intrafemoral injections of alloxan monohydrate (38 mg/kg for the CD and 45 mg/kg for the WKY and SHR animals) administered under ether anaesthesia; the remainder of the animals were kept as age-matched controls. All animals had free access to food and water and experiments were performed an average of 12 weeks after the diagnosis of diabetes (range 10 to 14 weeks). Blood glucose values were determined on tail vein blood using the Glucose Analyzer (Beckman Instruments, Palo Alto, Calif., USA).

Hypertension was induced in normal CD animals by reducing the blood flow to one kidney by the Goldblatt technique [10, 11]; for this purpose a stainless steel microaneurysm clip (Roboz Surgical Instrument Co., Washington, DC, USA) with jaws separated by a 0.2 mm platinum wire (Fisher Scientific Co., Pittsburgh, Pa., USA) was applied to one renal artery under anaesthesia (80 mg Nembutal/kg body weight; Abbott Laboratories, North Chicago, Ill., USA). Clipped animals which were to be treated with alloxan were allowed a 1-week recovery period before its administration. Systolic blood pressure measurements were obtained by the tail cuff procedure employing a Blood Pressure Monitor (Harvard Apparatus, South Natick, Mass., USA), on animals either sedated with 60 mg Nembutal/kg body weight or calmed by exposure to low light levels. Animals classified as hypertensive had blood pressure values which exceeded by at least two standard deviations the mean value obtained at the same time for normal animals. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Characteristics of animals studied

The mean blood glucose values were 7.9 ± 0.4 mmol/l for normal rats, 8.0 ± 0.6 mmol/l for hypertensive rats, 26.1 ± 2.4 mmol/l for diabetic rats and 25.7 ± 2.9 mmol/l for hypertensive-diabetic rats, with the values for the latter two groups being significantly different from normal ($p < 0.01$). Blood pressure values compared to normotensive rats measured at the same time (1.0 ± 0.03) were 1.12 ± 0.07 for diabetic, 1.44 ± 0.07 for hypertensive and 1.65 ± 0.06 for hypertensive-diabetic rats ($p < 0.001$ for both hypertensive and hypertensive-diabetic compared to normal rats). The absolute values for normal rats were 119 ± 5 mm Hg when anaesthetized and 149 ± 7 mm Hg when calmed by low light.

Treatment of tissues

After anaesthetization of the animals with Nembutal (80 mg/kg), the chest cavity was opened, the atria were cut with a sharp scalpel blade and perfusion was accomplished by slow injection of cold 0.15 mol/l NaCl into the left ventricle until blanching of the heart was observed. The atria and large vessels were then removed by dissection and the ventricles frozen on dry ice for storage at -80°C prior to processing. For preparation of myocardial extracellular matrix components, a previously reported [12] sequential extraction procedure was employed. Briefly, minced ventricles were disrupted in 50 mmol/l Tris-acetate pH 7.4 using a Polytron ST10 homogenizer (Brinkmann Instruments, Westbury, NY, USA). The material remaining insoluble in the Tris buffer (after centrifugation at 2°C for 45 min at $100,000 \times g$ in a Beckman L5-65 instrument) was treated successively at 2°C with 4 mol/l NaCl and 0.6 mol/l KCl to extract the bulk of the tissue myosin and then with 1% (weight/volume, w/v) Triton X-100 in 20 mmol/l Tris-acetate pH 7.4 to solubilize cell membrane components. Subsequently, the tissue was treated at room temperature with 4.5 mol/l guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo, USA) followed by a wash with 50 mmol/l Tris chloride, pH 7.0, and a final extraction performed for 5 min at 100°C followed by 2 h at 37°C with 1% (w/v) sodium dodecyl sulphate (SDS) containing 5% (v/v) 2-mercaptoethanol (both Fisher Scientific Co. Pittsburgh, Pa., USA) and centrifugation at room temperature, $1,500 \times g$, for 20 min. Protease inhibitors were present in all reagents [12]. The residue remaining after the SDS-mercaptoethanol extraction was washed with 80% (v/v) ethanol and then solubilized by treatment for 6 h at 25°C with 1% (w/v) cyanogen bromide in 70% (v/v) formic acid [13].

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Samples for electrophoresis were dried by lyophilization and desalted by two extractions with 80% ethanol with the samples being allowed to sit 2 h at -20°C prior to centrifugation; recovery was complete under these conditions. The desalted samples were then solubilized at 100°C in 4% (w/v) SDS containing 5% (v/v) 2-mercaptoethanol. After electrophoresis in SDS on 1.5 mm vertical slab gels (4–12% acrylamide gradient) by the procedure of Laemmli [14], the proteins were transferred to nitrocellulose [15] using a Trans-Blot Cell (Bio-Rad, Richmond, Calif., USA). Proteins used as standards included rat heart type VI collagen [5]; rat type IV collagen (prepared by SDS-mercaptoethanol solubilization of guanidine-extracted rat glomerular basement membrane); laminin and fibronectin (both from Calbiochem, San Diego, Calif., USA). Molecular weight markers were obtained from Sigma.

Immunoassays

All antisera were from rabbits. The preparation of anti-rat glomerular basement membrane type IV collagen [7] and anti-rat heart type VI collagen [5] have previously been described. Myosin, which was

Fig. 2. Effect of diabetes and hypertension on rat heart type VI collagen. After separate electrophoresis of the guanidine and SDS-mercaptoethanol extracts of normal (N), diabetic (D), hypertensive (H), and hypertensive-diabetic (HD) hearts, immunoblotting with anti-rat heart type VI collagen was performed. The results represent a summation for each animal of the $\alpha 3$ and $\alpha 1$ plus $\alpha 2$ bands in both extracts and are expressed per g of wet weight of the ventricle. CD (●), WKY (■), and SHR (▲) represents the strains of rats employed. No difference was found between CD and WKY normal rats; significant differences observed for other groups were as follows: CD diabetic compared to CD normal rats, $p < 0.02$; CD hypertensive compared to CD normal rats, $p < 0.001$; SHR hypertensive compared to WKY normal rats, $p < 0.05$; SHR hypertensive-diabetic compared to WKY normal rats, $p < 0.01$.

isolated from the 4 mol/l NaCl extract of rat heart by gel filtration on Bio-Gel A-5M (Bio-Rad) and its purity monitored by SDS-PAGE, was used for antibody production by the multiple intradermal technique [16]; antibodies against rat type I collagen (Calbiochem) were prepared similarly. Antibodies to laminin and fibronectin were obtained from Calbiochem.

For quantitative determination of types VI and I collagen as well as laminin and fibronectin, the nitrocellulose sheets obtained after electroblotting were treated in sequence [17] with 0.15 mol/l sodium chloride in 0.05 mol/l sodium phosphate, pH 7.4, containing 0.1% (w/v) of bovine serum albumin (PBS-BSA), diluted goat serum, specific antisera and finally with Protein A (Calbiochem) which had been labelled with ^{125}I (Amersham, Arlington Heights, Ill., USA). For dilution of antisera and washing of the nitrocellulose sheets, 0.5 ml/l of Tween 20 was added to the PBS-BSA. After exposure to X-Omat AR film (Kodak, Rochester, NY, USA) at -70°C , the bands detected were cut from the nitrocellulose and their radioactivity measured in a 1290 GammaTrac counter (TM Analytic, Elk Grove, Ill., USA) and compared to the values obtained for known amounts of standard proteins electrophoresed on the same gel. For type VI collagen the $\alpha 3$ (205 kDa) and $\alpha 1$ plus $\alpha 2$ chains (150 kDa) were studied (Fig. 1); for laminin only the 220 kDa chain was present

Table 1. Effect of diabetes and hypertension on rat myocardial extracellular matrix constituents and myosin

	Type VI collagen	Type IV collagen	Laminin	Myosin
	(mg/g ^a)	(mg/g)	($\mu\text{g/g}$)	(mg/g)
Normal ^b rats	2.29 ± 0.04 (18)	0.89 ± 0.08 (21)	112 ± 22 (5)	8.1 ± 0.61 (12)
Diabetic rats	2.85 ± 0.18 (12) ^c	1.14 ± 0.12 (17)	106 ± 24 (6)	7.0 ± 0.7 (7)
Hypertensive rats	2.71 ± 0.12 (10) ^d	0.85 ± 0.16 (9)	ND	7.9 ± 0.6 (9)
Hypertensive-diabetic rats	2.93 ± 0.15 (7) ^e	0.93 ± 0.14 (8)	ND	9.1 ± 0.6 (8) ^f

^a Values are expressed as mg or μg of protein per g wet weight of ventricle. Type VI collagen and laminin were measured by immunoblotting (for type VI, summation of $\alpha 3$ plus $\alpha 1$ and $\alpha 2$ chains of both guanidine and SDS-mercaptoethanol extracts was used; for laminin the guanidine extract was measured); type IV collagen and myosin were determined by solid phase radioimmunoassay on the SDS-mercaptoethanol and NaCl extracts, respectively, as described in the Materials and methods section.

^b The normal group contains both CD and WKY animals (see Fig. 2) except in the case of laminin for which only CD normal and diabetic animals were used.

^c $p < 0.01$, ^d $p < 0.005$, ^e $p < 0.001$ vs normal rats; ^f $p < 0.025$ diabetic vs hypertensive diabetic rats. ND, Not determined. *n* for all groups is given in parentheses

in rat heart; for type I collagen, immunoreactive cyanogen bromide fragments with approximate M_r values of 70 and 50 kDa were observed and measured.

Type IV collagen, which was present in rat heart as the $\alpha 1$ and $\alpha 2$ chains [$M_r = 200$ and 180 kDa, respectively (Fig. 1)] was determined by the solid phase radioimmunoassay technique [17]; the samples, which contained SDS-mercapto ethanol, were allowed to dry in the wells overnight and the detergent was removed by extraction with 50% methanol before reaction with specific antiserum and ^{125}I -labelled protein A. Radioactivity bound to the wells was compared to that obtained from standard rat type IV collagen. Rat heart myosin, present predominantly in the NaCl extract, was determined similarly after being allowed to bind to the immunowells overnight at 2°C .

Chemical analyses

Protein was determined by the Peterson modification of the Lowry procedure [18]. Amino acids were determined after hydrolysis in constant boiling hydrochloric acid as their phenylthiocarbamyl derivatives by high performance liquid chromatography [19].

Statistical analysis

Data were evaluated with the use of Student's *t*-test; results are expressed as the mean $\pm$ SEM.

Results

Effect of diabetes on rat heart type VI collagen. Although qualitatively similar electrophoretic patterns were observed for both types VI and IV collagen in normal and

diabetic hearts (Fig. 1), increased amounts of the former were present in the diabetic myocardium. In normal hearts from both CD and WKY strains, the type VI collagen was found to be 2.29 mg per g of wet weight of ventricle (representing the sum of the 205 and 150 kDa bands present in both guanidine and SDS-mercaptoethanol extracts (Fig. 2 and Table 1). After 12 weeks of diabetes, the type VI content had increased to 2.85 mg/g ($p < 0.02$ compared to CD normal rats). Although several attempts were made to induce alloxan diabetes in the WKY animals, only transient hyperglycaemia was ever achieved by this treatment; resistance to streptozotocin in these animals has been previously reported [20, 21].

Type VI collagen in hearts of hypertensive and hypertensive-diabetic rats. Regardless of whether hypertension occurred as a result of a genetic predisposition (SHR animals) or had been induced by partial occlusion of a renal artery (in CD rats), this condition led to an increase in the type VI content of the ventricle (Fig. 2). The mean value for CD hypertensive rats was 2.67 mg/g ($p < 0.001$ compared to CD normal rats) while for spontaneously hypertensive rats it was 2.72 mg/g ($p < 0.05$ compared to WKY rats). The presence of both hypertension and diabetes did not have an additive effect on the type VI collagen and the amount, 2.93 mg/g ($p < 0.01$ compared to normals), was comparable to those found in animals with either diabetes or hypertension alone (Fig. 2 and Table 1).

Effect of diabetes and hypertension on other extracellular matrix constituents. The amount of type IV collagen, which is the principal component of basement membranes, was found by solid phase immunoassays to be approximately 0.9 mg per g of wet weight of rat ventricles (Table 1). In contrast to type VI collagen, no change was observed in this protein as a consequence of hypertension; in the diabetic heart, although the mean value for type IV was increased over normal, its variability was too great to be significant.

The possibility that diabetes might result in increased amounts of type I collagen, which would remain insoluble at the conclusion of the sequential extraction protocol, was evaluated on the cyanogen bromide solubilized residues both by immunoblotting as well as by amino acid analysis. The electrophoretic patterns and amounts of anti-type I collagen reactivity in normal and diabetic hearts were indistinguishable and no difference was found between normal ($n = 8$) or diabetic ($n = 10$) hydroxyproline and hydroxylysine values (hydroxyproline: $36.2 \pm 6 \mu\text{g/g}$ for normal and $32.9 \pm 4 \mu\text{g/g}$ for diabetic hearts). The collagen remaining insoluble in SDS-mercaptoethanol, based on a typical hydroxyproline content of 100 residues/1000 for type I collagen [13], would be approximately 0.36 mg per g ventricle (wet weight).

Measurement of laminin in the sequential heart extracts indicated that 90% was present in the guanidine extract; its levels were substantially less than either type VI or type IV collagen (Table 1) and were unaffected by diabetes. Analyses which were carried out for fibronectin indicated that the perfused rat heart (from which serum fibronectin was removed) had very small amounts of this

protein ($93 \pm 10 \mu\text{g/g}$, ($n = 5$)) and no alteration was observed in diabetes for which the value was 89.3 ± 4.6 ($n = 6$).

Effect of diabetes and hypertension on myosin content and ventricle weight of rat hearts. Immunochemical determination of myosin, which represents the principal and characteristic protein of ventricular myocytes, indicated a slight but not significant decrease of this protein in the diabetic heart (Table 1). Hypertension in itself did not alter the ventricular myosin content per g but when hypertension and diabetes were combined the level of this protein was significantly increased compared to the content present in diabetes alone ($p < 0.025$).

The ventricle weight expressed as g per 100 g body weight was 0.31 ± 0.01 for normal rats; 0.28 ± 0.02 for diabetic rats; 0.36 ± 0.01 for hypertensive animals and 0.34 ± 0.03 for hypertensive-diabetic rats. Only the hypertensive rat ventricle weight was significantly different from normal ($p < 0.01$).

Discussion

To evaluate the effect of diabetes on the heart, two distinct targets must be considered, the myocytes and the cells of the small blood vessels. In the early stages of the disease in either genetically diabetic mice [22] or streptozotocin-diabetic rats [23], myocyte damage has been observed which involved mitochondria, myofilaments and sarcoplasmic reticulum; moreover, alterations have been found in myosin isotypes [24] as well as in a variety of cellular functions such as myosin-ATPase activity, calcium uptake, catecholamine sensitivity and $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ [24–26].

In the chronic diabetic state, the microvascular disease affecting many tissues may have an important impact on cardiac performance. Evidence for small blood vessel involvement has been indicated by increased PAS staining detected in light microscopic studies of myocardium from human diabetic patients [1–4], as well as from the capillary basement membrane thickening observed by electron microscopy in biopsied myocardial tissue from diabetic subjects taken at the time of cardiac bypass operation [27]. Similar changes have been reported for the hearts from streptozotocin-diabetic rats which had substantially more myocardial connective tissue [28] and statistically significant increases in the width of myocardial capillary basal lamina [29]. Although in dogs with a mild alloxan diabetes, hydroxyproline values indicated increased amounts of collagen in the myocardium [30], similar measurements in streptozotocin-diabetic rat ventricles showed levels which were significantly increased only at 6 weeks of diabetes [31]; since in the present study only type VI collagen was found to be increased, the unchanged hydroxyproline values of the other collagens would mask this change.

Substantially elevated fibronectin mRNA levels have been reported for streptozotocin-diabetic hearts, even after restoration of blood glucose levels by insulin treatment [32]. Since the present investigation found no alteration in myocardial fibronectin content in alloxan

diabetes, it is likely that if an increased synthesis of this protein occurs, its degradation must also be enhanced.

The data in the present report reconcile the findings of increases in both collagen and PAS staining by showing that type VI collagen, a strongly PAS-reactive molecule [5], is significantly elevated in hearts of rats with alloxan diabetes. While it has long been recognized that myocardium contains types I and III collagen [33–35] and the presence of type IV in myocyte basement membranes and capillary walls has also been shown [35, 36], it is only recently that type VI collagen has been demonstrated in this tissue [5]. The present data which quantitate the amounts of several matrix components indicate that for rat heart as has been found for cornea [37] and several other tissues [38], type VI collagen may be considered a major extracellular constituent.

While type I collagen was found not to be increased in the diabetic rats, it is possible that in the hypertensive hearts the amount of this protein may have been elevated together with type VI. Indeed, hypertension induced by banding of the aorta has been reported to result in substantial cardiac hypertrophy and fibrosis, together with increased levels of both hydroxyproline and type I collagen [36].

Our studies have demonstrated that the effect of diabetes on extracellular matrix constituents is dependent upon the tissue being studied. The elevation of type VI collagen in the myocardium appears to be similar to changes which may occur in the mesangial component of the glomerular extracellular matrix in diabetes. This is in contrast to the alterations in the basement membrane itself where increases in type IV collagen predominate, probably reflecting the contribution of the epithelial cells [6–8]. An investigation of the biosynthetic potential of the individual cell types present at sites in which the characteristic diabetic complications occur will hopefully provide clues to the molecular basis of the microvascular disease.

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