An Investigation of the Biochemical and Histological Changes in the Collagen of the Kidney and Skeletal Muscle in Systemic Sclerosis

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Abstract

Changes in the distribution and quantity of different collagen types in some internal organs was determined in tissues from patients with systemic sclerosis. In addition, sera from patients with systemic sclerosis were assayed for circulating anti-collagen antibodies and were compared with normal sera. The results of a specific immunohistological survey of skeletal muscle and kidney showed a general increase in type I and III collagens in the fibrotic lesions when compared with age matched normal tissue. A thickening of basement membranes was also observed. Biochemical quantitation of the ratio of types I and III collagens in normal and affected kidney showed up to a three-fold increase in type III compared to type I collagen, consistent with the immunohistological data. Finally, quantitation, by the ELISA technique, of circulating anticollagen antibodies in sera from patients with systemic sclerosis demonstrated positive results for types I, III and IV collagens when compared with normal controls. These results are discussed in relation to the clinical progression and possible mechanisms of pathogenesis of the disease.

Key words: collagen antibodies, systemic sclerosis, fibrosis

Introduction

Systemic sclerosis is a disease characterized both by increased deposition of collagen in the skin and internal organs and by vascular changes involving the capillaries and small blood vessels of all affected tissues (Rodnan, 1979; Black, 1979).

The existence of at least five chemically and genetically distinct forms of collagen has now been established. Types I, II and III are fibrous. Skin tendon and bone contain mainly Type I, cartilage predominantly Type II, while Type III is
Tissue staining

6 µm sections of kidney and skeletal muscle sections were cut on a cryostat and air dried onto glass slides. The sections were either stained with the purified type specific anticollagen antibodies or normal rabbit serum as controls. Staining was generally carried out overnight. After exhaustive washing in PBS, the second antibody (anti-rabbit or anti-goat) conjugated with fluorescein was added and left for 30 min before further exhaustive washing. Sections were mounted in a gelatine-glycerine jelly.

Controls were performed by pre-incubation of the antibodies with purified antigen and by staining with non-immune sera.

Quantitation of Types I and III collagens in normal and systemic sclerosis kidneys

We found that the usual CNBr digestion method was not efficient enough for kidney and so samples were minced, briefly washed in water, extracted five times with 2% SDS (Laurent et al., 1981) and once with 0.5 M acetic acid and then digested with CNBr using two different methods. Firstly, in 70% (v/v) formic acid with an equal weight of cyanogen bromide at 25 °C for 4 h to give a final peptide concentration of 10 gm/ml (Method A). Secondly, samples (50 mg in 3 ml) were suspended in 30% (v/v) formic acid and heated at 56 °C for 5 min prior to homogenizing by hand in a ground glass Potter-Elvenhim homogenizer. The samples were heated for a further 5 mins at 56 °C and then were made up to 8 ml with 90% (v/v) formic acid (final concentration 67.5%). After cooling to 30 °C 50 mg of solid cyanogen bromide was added to each and incubation was carried out at 30 °C for 5 h (Method B). After digestion by either method, samples were freeze dried after a two-fold dilution with water. The use of the heat denaturation step increased solubilisation from around 65% to as much as 95%, consequently only samples solubilized in this way were used for quantitations.

The freeze dried samples were dissolved in gel buffer and run on 10% (w/v) polyacrylamide gels with a 5% (w/v) stacking gel (Laemmli, 1970). The relative proportions of Type I to Type III was assessed as previously described (Light, 1982) using the CNBr peptides α1(I)CB8 and α1(III)CB8.

Results

Tissue staining

Kidney

The distribution of the collagens I, III, IV and V in normal human kidneys (Figs. 1a–d) were shown to be similar to those described by Roll et al., 1980. Types I and III were present in fibres around the glomeruli and tubules, in the interstitium and around the large blood vessels. Type IV collagen was distributed in the basement membrane of the tubules, glomeruli and Bowman's capsule, whereas Type V appeared to be present primarily in the interstitium and mesangium with little basement membrane staining in agreement with Gay et al. (1981) In the diseased kidney, immunofluorescence staining with type specific anticollagen antibodies revealed a marked increase in Type III throughout the tissue especially in the interstitium. A similar but less dramatic increase was observed for Type I
Table 1. Clinical details of patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Sex</th>
<th>Duration of Disease</th>
<th>Clinical Organ Involvement</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>69</td>
<td>M</td>
<td>12 years</td>
<td>Skin, joints, muscles, <em>heart</em>, lung, <em>kidney</em> (chronic), oesophagus</td>
<td>Heart failure</td>
</tr>
<tr>
<td>MF</td>
<td>62</td>
<td>M</td>
<td>11 weeks</td>
<td>Skin, <em>kidney</em> (acute)</td>
<td>Respiratory infection</td>
</tr>
<tr>
<td>FW</td>
<td>58</td>
<td>M</td>
<td>8 years</td>
<td>Skin (total), muscle, oesophagus, small and large bowel, lungs</td>
<td>Acute renal failure</td>
</tr>
<tr>
<td>SK</td>
<td>44</td>
<td>F</td>
<td>10 years</td>
<td><em>Kidney</em> (acute), hypertension skin, oesophagus, lung</td>
<td>Hypertensive encephalopathy. Restrictive airways disease</td>
</tr>
<tr>
<td>GD</td>
<td>41</td>
<td>F</td>
<td>15 months</td>
<td>Skin, muscle joints, oesophagus, lungs, <em>heart</em></td>
<td>Haematemesis Congestive cardiac failure</td>
</tr>
<tr>
<td>MM</td>
<td>33</td>
<td>F</td>
<td>7 years</td>
<td>Skin, oesophagus, <em>heart</em> <em>Kidney</em>, lung, heart, joints, bowel and skin</td>
<td>Cardiac arrest Renal failure</td>
</tr>
<tr>
<td>AS</td>
<td>57</td>
<td>F</td>
<td>2 years</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagen in systemic sclerosis

The tubular and glomerular basement membranes also appeared thickened and showed an increased intensity of staining with anti-type IV collagen (Fig. 1e and f). The tubular and glomerular basement membranes also appeared thickened and showed an increased intensity of staining with anti-type IV collagen (Fig. 1e and f). Anti-type V antibodies showed an increase in this collagen type in the interstitium as well as the glomerular mesangial matrix (Fig. 1h). These changes were observed even in systemic sclerosis patients who in life had no clinical evidence of renal disease.

Skeletal muscle

The distribution of the polymorphic forms of collagen Types I, III, IV and V in normal human muscle was similar to that previously reported (Duance et al., 1980a, 1980b). Antibodies to Type I collagen minimally stained the endomysium and the perimysium whereas anti-type III collagen antibodies strongly stained the perimysium and to a lesser extent the endomysium. The endomysium also stained intensely with antibodies to both Type IV and V collagens.

Staining of the diseased skeletal muscle with the type-specific anticollagen antibodies showed that Type III was substantially increased in the perimysium particularly around blood vessels and endomysium (Fig. 2a and c). Type I increased in the same locations but was less marked. Both the basement membranes of the endomysium and the small blood vessels were more intensely stained with anti-type IV collagen antibodies as well as appearing to be thickened when compared with normal tissue (Fig. 2b and d).
Fig. 1. Immunofluorescence staining of normal (a–d) and systemic sclerosis kidney (e–h) stained with antibodies to type I (a and e), type III (b and f), type IV (c and g) and type V (d and h) collagen × 100.

An increase in staining is evident for all types of collagen but is particularly marked for type III.
Quantitation of collagen Types I and III

The usual CNBr digestion technique was found to be relatively ineffective in solubilising diseased kidney (Method A). We therefore used a modified method (Method B) which involved a preliminary heat denaturation step. This procedure allowed up to 95% of the tissue to be solubilized by the cyanogen bromide (Fig. 3). Coomassie Blue stained gels were scanned and quantitated (Fig. 4). We were thus able to establish the ratio of Type III to Type I collagen in normal and diseased kidneys (Table 2) but due to technical difficulties discussed elsewhere (Light, 1982) it was not possible to reliably quantitate specific collagen type increases in skeletal muscle.

Normal kidney contained 16.4% ± 2.1% Type III relative to Type I (from determinations made from five normal subjects) but the kidneys of all five cases of systemic sclerosis investigated contained significantly elevated proportions of Type III (Table 2). This was highlighted in the cases in which death was caused by renal failure when the ratio of Type III to Type I collagen had increased by more than two times from 16% to 40% (Fig. 3e). At present it is not possible to quantitate the changes in the proportions of Types IV and V collagens by the CNBr peptide method as little is yet known of the characteristics of the peptides of these less abundant collagens.
Fig. 3. Biochemical quantitation of the ratio of type I and type III collagen in normal and fibrotic kidney samples.

SDS-polyacrylamide gel photograph of four normal kidney samples (a–d) and two fibrotic kidneys (e–f). All samples were extracted × 5 with 2.5% SDS and × 1 with 0.5 M acetic acid prior to CNBr digestion.

Tracks a–d represent ages 20, 35, 37 and 58 respectively. Track (e) was processed from a kidney obtained at post-mortem from a systemic sclerosis patient who died from renal failure (Patient AS) whereas track (f) was from a systemic sclerosis patient who showed renal fibrosis but died from other causes (Patient AC). The two bands used for quantitation are labelled.

Collagen antibodies

We investigated the naturally occurring antibodies to collagen Types I, II, III, IV and V by means of the sensitive ELISA technique. The results showed a significant increase in the titers to Types I, III and IV collagens (Table 3).
Collagen in systemic sclerosis

Fig. 4. Quantitation of type I and type III collagen in normal and systemic sclerosis kidney. Scans of SDS-polyacrylamide gel CNBr peptide maps from (a) kidney collagen from a 57 year old systemic sclerosis patient who died from renal failure (Patient AS) and (b) kidney collagen from a 58 year old normal control. The two peaks used for quantitation by methods previously described (Light, 1982) are labelled.

Table 2. Quantitation of the percentage type III collagen with respect of type I collagen in normal and systemic sclerosis kidneys

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Percent Type III collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>14 months</td>
<td>13.2</td>
</tr>
<tr>
<td>Normal</td>
<td>20 years</td>
<td>16.2</td>
</tr>
<tr>
<td>Normal</td>
<td>35 years</td>
<td>15.5</td>
</tr>
<tr>
<td>Normal</td>
<td>37 years</td>
<td>19.0</td>
</tr>
<tr>
<td>Normal</td>
<td>58 years</td>
<td>14.6</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>33 years (MM)</td>
<td>34.4</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>58 years (FW)</td>
<td>32.1</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>62 years (MF)</td>
<td>40.0</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>57 years (AS)</td>
<td>38.7</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>69 years (AC)</td>
<td>33.7</td>
</tr>
</tbody>
</table>

1 Means of calculations made from two separate tracks on the same gel.
Table 3. Systemic sclerosis/collagen antibody study

<table>
<thead>
<tr>
<th>Collagen antibody</th>
<th>Control subjects n = 43</th>
<th>Systemic sclerosis patients n = 106</th>
<th>Patients vs controls</th>
<th>P – value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>277 ± 27</td>
<td>426 ± 20</td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Type II</td>
<td>132 ± 14</td>
<td>142 ± 8</td>
<td></td>
<td>Not sig.</td>
</tr>
<tr>
<td>Type III</td>
<td>286 ± 31</td>
<td>661 ± 29</td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Type IV</td>
<td>140 ± 19</td>
<td>212 ± 12</td>
<td></td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Type V</td>
<td>96 ± 14</td>
<td>84 ± 8</td>
<td></td>
<td>Not sig.</td>
</tr>
</tbody>
</table>

Discussion

Most of the information concerning the metabolism of collagen in systemic sclerosis has come from work with dermal tissue (Uitto et al., 1969; Perlish et al., 1976; Lovell et al., 1978; Fleischmajer et al., 1978). A study of collagen in the internal organs in systemic sclerosis has been more difficult to achieve and yet it is the internal organs, especially the heart, lung and kidney, which determine morbidity and mortality. To date the chemical nature, distribution and proportion of both the fibrous and basement membrane collagens in the internal organs in systemic sclerosis has never been fully investigated and the only report of the fibrous collagens in the internal organs is that of Seyer et al. (1981). In their study on the lung, the relative content of Type I versus Type III collagen was unchanged. In contrast to their finding we have shown by immunofluorescence studies that there is an excess deposition of both the fibrous and basement membrane collagens in the kidney and skeletal muscle. Using the type-specific antibodies this increase was shown to be predominantly Type III collagen, which persists long after the early phase of the disorder, and does not revert to Type I. We have confirmed the observed increase in the relative proportion of Type III collagen in the kidney by direct chemical analysis of the CNBr peptide patterns.

This predominance of Type III collagen was observed by immunofluorescence studies in all the internal organs examined. The collagen was deposited mainly in the perimysium of skeletal muscle and in the interstitium in the kidney. There was also excessive deposition around the larger blood vessels in both the kidney and skeletal muscle. Preliminary results show a similar increase in Type III collagen in cardiac muscle (data not shown).

The endomysial basement membrane of skeletal muscle and the basement membrane of the glomeruli, tubules and blood vessels in the kidney (which are clearly defined when stained with antibodies to Types IV collagen) are apparently thickened in the disease tissues. Although it is possible that this may be an artifact produced by tissue shrinkage, this is an unlikely interpretation of the results, since the effect was consistently observed in such structurally diverse tissues. There have been reports of capillary and glomerular basement membrane thickening in the internal organs in systemic sclerosis (Lapenas et al., 1978) and we have observed in our own studies that the small blood vessels are more apparent in the diseased muscle and kidney when stained with antibodies to Types IV and V collagens.

In addition, in our patients an excess of Type III collagen can be seen surrounding the larger arteries. The deposition of collagen in the kidney was most marked
Collagen in systemic sclerosis

We sought to confirm the results of our immunohistological studies by chemical quantitation of the collagens in systemic sclerosis kidney and skeletal muscle. Preliminary work showed that accurate quantitation was not possible in tissue such as muscle where collagen is normally only a minor component as extractions of non-collagenous constituents proved extremely difficult (Light, 1982). However, we were able to obtain reproducible assays of the ratio of Type I and III collagen in normal and systemic sclerosis kidneys. It should be noted that we observed anomalous results when the tissue was initially digested with pepsin as noted by other workers (Laurent et al., 1981; Light, 1982). We found that a heat denaturation step prior to CNBr digestion of the whole tissue increased the solubilization of the kidney from 60% to as much as 95%. Analysis of material so treated confirmed the immunohistological results and indicated more than a two-fold increase in Type III collagen relative to Type I above normal in the most severe cases studied.

Interference with such quantitations by Type IV collagen CNBr peptides was adjudged to be minimal. A minor Type V collagen CNBr peptide comigrates with α1(I)CB8 and elevated quantities of this component may have been present. However, as, in general, Type V to Type I ratios are very small, this interference would not significantly alter the results. If there was any such interference it would tend to artificially reduce Type III to Type I ratios thus our results may be taken as underestimates.

Measurement of circulating antibodies to collagen showed an increased titre of antibodies to collagens Type I, III and IV correlating well with the observed systemic increases in these particular collagens. These antibodies could be present as a result of altered collagen metabolism, however, whether they have a role in the pathogenesis of the disease is unknown. Immunofluorescence studies have shown a deposition of immunoglobulins and complement along the basement membranes, particularly in the kidney, and these lesions could be due to the formation of an antibody to a normal or altered collagen in the vessel wall (Steffen, 1965). It is also possible that these antibodies play a role in perpetuating the disease.

The pathological and clinical implications of change in both the fibrous and basement membrane collagens in systemic sclerosis is partly speculative. The excessive fibrous collagen deposited in both skeletal and cardiac muscle must certainly interfere with function, and be partly responsible for the contractures, atrophy and myocardial dysfunction. The same may also be said of the fibrous collagen deposited in the interstitium and around the larger blood vessels in the kidney where it exaggerates the deteriorating renal function. It has been suggested that the earliest changes in systemic sclerosis occur in the blood vessels via a circulating factor, and the initial response may be a thickening of capillary basement membrane with a restriction of nutrients to the organ. This restriction could cause an increase in the proportion of tissue fibroblasts and thus an increase in the rate of collagen synthesis (Lipton, 1977). This excess collagen would then restrict nutrient supply further resulting in accelerated organ dysfunction. The
observed circulating anti-type IV antibodies may arise in response to metabolic abnormalities in one or more relatively minor primary basement membrane lesions and could be instrumental in vessel wall injury, leading to the secondary more general fibrosis.

Further studies on the involvement of collagen in the pathogenesis of systemic sclerosis will be beneficial for our complete understanding and treatment of this disease.

Acknowledgement

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References


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