IMMUNOFLOUORESCENT STAINING FOR COLLAGEN AND PROTEOGLYCAN IN NORMAL AND SCOLIOTIC INTERVERTEBRAL DISCS

HELEN K. BEARD, SALLY ROBERTS, JOHN P. O'BRIEN

From the Charles Salt Research Centre and the Department for Spinal Disorders, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry

Specific antisera to collagen Types I, II and III and proteoglycan were used to investigate the distributions of these molecules in normal human intervertebral discs. Immunofluorescent staining indicated the presence of small amounts of Type III collagen located pericellularly in normal adult intervertebral discs. This finding had not been demonstrated previously by other methods. Similar specimens of intervertebral discs from 17 patients with scoliosis of varying aetiologies were examined, but no evidence was obtained for primary connective tissue defects. Secondary changes, especially marked vascularisation of the inner annulus, were apparent in a number of scoliotic discs, and some of these showed enhanced staining for collagen Type I and proteoglycan, and intercellular matrix staining for Type III collagen.

Scoliosis frequently occurs alone—idiopathic scoliosis—or in association with other symptoms—paralytic scoliosis and inherited disorders (McKusick 1972), such as Marfan's syndrome, Ehlers–Danlos Type VI, or homocystinuria. The causes of scoliosis are not clear, although primary changes in the connective tissue have been suggested as one possibility (Ponseti et al. 1976). Specific metabolic errors have been identified in a number of inherited disorders which commonly involve scoliosis (Pinnell et al. 1972; Seigel 1975; Kreig and Muller 1977; Sykes, Francis and Smith 1977). In Ehlers–Danlos Type VI, there is a deficiency in the enzyme lysine hydroxylase which leads to a defect in the cross-linking of collagen (Pinnell et al. 1972), and in osteogenesis imperfecta the ratio of Type I to Type III collagen in the skin is less than normal (Sykes et al. 1977).

Changes in the connective tissue have also been identified in idiopathic scoliosis, but these are less well defined. The stability of polymeric skin collagen to alkali was reduced in adolescent patients with idiopathic scoliosis as compared with controls of a similar age (Francis, Sanderson and Smith 1976), and the pepsin extractability of collagen from intervertebral discs in a similar group was found to be abnormal (Bushell et al. 1979). An increase in the content of collagen, particularly in the nucleus pulposus, in scoliosis has also been demonstrated (Pedrini, Ponseti and Dohrman 1973; Bushell, Ghosh and Taylor 1978). These changes, together with an increased capacity to aggregate platelets by collagen from the fascia of patients with both idiopathic and congenital scoliosis (Udén, Nilsson and Willner 1980), suggested that in these patients there may be primary changes in collagen in several tissues. In addition, both quantitative and qualitative changes in proteoglycan have been observed in idiopathic scoliosis (Pedrini et al. 1973).

The intervertebral discs in scoliotic patients are often grossly deformed (Schmorl and Junghanns 1971; O'Brien, Dwyer and Hodgson 1975), although it is not known whether these changes are primary or secondary. Recent biochemical studies have increased the understanding of the normal structure of these tissues (Eyre 1979). Collagen and proteoglycan together account for 80 per cent of its dry-weight (Beard and Stevens 1980), and whereas collagen predominates in the annulus fibrosus, proteoglycan is the major component of the nucleus pulposus (Adams, Eyre and Muir 1977). Studies on porcine and human intervertebral discs indicated that the major collagen types in the disc, Types I and II, were inversely distributed from the outer annulus to the nucleus (Eyre and Muir 1976; Adams et al. 1977). Investigations using specific antisera showed similar distributions and indicated that, in the annulus fibrosus of the pig, postnatal developmental changes in distribution of the collagen types occurred (Beard et al. 1980).

In this study specific antisera were used to investigate the distribution of collagen Types I, II and
III and proteoglycans in normal human intervertebral discs. The possibility that these tissue components may be abnormally distributed in scoliotic spines was investigated.

MATERIALS AND METHODS

Collection of material. Intervertebral discs were excised from scoliotic patients at operation and anterior spinal fusion usually made by Dwyer’s technique. The anterior region of the disc containing the annulus was removed and in approximately half the specimens a sample of the nucleus was also removed. The tissue was immediately placed in a 100 per cent humidity chamber, and was processed within several hours of operation. The fragment was sketched, measured, and divided into sections including the annulus and nucleus, and rapidly frozen by dropping into hexane kept at minus 80 degrees Celsius in a bath of solid carbon dioxide and acetone.

Control material was taken from cadaveric spines within 24 hours of death. No evidence of autolysis affecting the immunofluorescence assay was found. Specimens were removed from the same region of the intervertebral disc, and treated in the same way as the surgical specimens.

Preparations of antisera. Antisera to collagen Types I and II and to proteoglycan were prepared and characterised as previously described (Beard et al. 1980). Antisera to Type III collagen were raised in guinea-pigs and goats after immunisation with Type III collagen prepared from human placenta (Beard, Lea and Ryvar 1979). Specificity of these antisera was tested by haemagglutination and immunoprecipitation using human collagen Types I, II, III, and IV, proteoglycan, human serum and purified fibronectin from the rat (kindly donated by Dr M. J. Marshall). Guinea-pig antisera were specific to Type III collagen whereas goat antisera also reacted with Type IV collagen from the human placenta. The goat serum was therefore purified by using immunoadsorbent columns for Type I, III and IV collagen. Guinea-pig antisera and the purified fraction from goat antisera gave similar staining patterns.

Immunofluorescent staining. The tissue of the disc was stored under liquid nitrogen and sectioned on a Bright’s rotary cryostat. Sections (seven micrometres thick) were stained for collagen by indirect immunofluorescence as previously described (Beard et al. 1980) except that the sections from human discs were pretreated with 0.1 milligrams per millilitre trypsin (11700 units per milligram of protein) for 15 minutes at 37 degrees Celsius. This was found to enhance the staining of the collagen and may have resulted from removal of the proteins sheathing the collagen fibres (Buckwalter, Maynard and Cooper 1978). Although trypsin is known to cleave Type III collagen at a single site (Miller et al. 1976), it is unlikely to release collagen molecules from the tissue; indeed hydroxyproline analysis indicated that less than 0.2 per cent of the tissue collagen was lost during treatment with trypsin.

RESULTS

Staining patterns obtained with normal human intervertebral discs. Staining for Type I collagen was negative, or very weak, in the nucleus pulposus and in the inner

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Staining for Type III collagen in human intervertebral discs. Figure 1—Inner annulus stained with antibodies to Type III collagen (× 40). Figure 2—Nucleus stained with antibodies to Type III collagen counterstained with ethidium bromide. Arrow denotes cell nucleus (× 40). Figure 3—As in Figure 2 (×134). Figure 4—As in Figure 2 (×134). Cell cluster can be seen here.
annulus of human intervertebral discs. The outer regions of annulus, however, showed clear staining and exhibited a striated pattern in the intercellular matrix, similar to that seen in discs from pigs (Beard et al. 1980).

Antisera to Type II collagen stained the nucleus, and the whole of the annulus except for the outer lamellae. This outermost zone was clearly negative for Type II collagen in some adult human discs, and as found in porcine discs was approximately one millimetre thick (Beard et al. 1980). However, in other adult specimens this outer region was not clearly demarcated. No striated pattern was detected with Type II antisera and the staining was more diffuse.

Antisera to Type III collagen showed characteristic pericellular staining which was largely restricted to the nucleus and inner annulus (Figs 1 and 2). Single cells, which could be readily identified by counterstaining the deoxyribonucleic acid in the nucleus with ethidium bromide, showed a clear halo of fluorescence with anti-Type III antisera (Figs 2 and 3). In some sections of the nucleus groups of cells could be seen which were surrounded by Type III collagen, although the matrix beyond was negative (Fig. 4). The cells of the cartilage end-plate adjacent to the disc did not stain and the intercellular matrix throughout the disc was stained only very rarely (Table I).

### Table I. Immunofluorescent staining of the three collagen types and of proteoglycan in normal human discs

<table>
<thead>
<tr>
<th>Age of patient</th>
<th>Sex</th>
<th>Anulus</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Newborn</td>
<td>M</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>3 months</td>
<td>F</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4 years</td>
<td>M</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td>9 years</td>
<td>M</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>12 years</td>
<td>F</td>
<td>+ +</td>
<td>++</td>
</tr>
<tr>
<td>15 years</td>
<td>M</td>
<td>+ +</td>
<td>++</td>
</tr>
<tr>
<td>28 years</td>
<td>M</td>
<td>+ +</td>
<td>+++</td>
</tr>
<tr>
<td>46 years</td>
<td>F</td>
<td>+ +</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Staining appeared in the inner annulus only.

Table II. Analysis of intervertebral discs taken from scoliotic patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age at onset</th>
<th>Age at operation</th>
<th>Discs</th>
<th>Curve (apex)</th>
<th>Clinical features</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>17</td>
<td>L3-4</td>
<td>Thoracolumbar (T10, L4)</td>
<td>Wedged L4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Birth</td>
<td>10</td>
<td>L2-3</td>
<td>Lumbar (L1)</td>
<td>Hemivertebra L1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>13</td>
<td>T7-8 to T11-12</td>
<td>Thoracic (T10)</td>
<td>Fused T9-10</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>34</td>
<td>T11-12 to L2-3</td>
<td>Thoracolumbar (T6, L1)</td>
<td>Abnormal facet joints</td>
<td>L2-3 vascularised. All discs*</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>11</td>
<td>T8-9 to L4-5</td>
<td>Thoracolumbar kyphoscoliosis (T9)</td>
<td>Multiple deformities</td>
<td>T11-12*</td>
</tr>
<tr>
<td>6</td>
<td>Birth</td>
<td>20</td>
<td>L2-3</td>
<td>Lumbar (L2)</td>
<td>Hip dislocation</td>
<td>L2-3*</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>38</td>
<td>L2-3, L3-4</td>
<td>Lumbar (L3)</td>
<td>Poliomyelitis</td>
<td>L2-3*</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>16</td>
<td>T11-12 to L4-5</td>
<td>Lumbar (L3)</td>
<td>Poliomyelitis</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>28</td>
<td>T12-L1 to L5-S1</td>
<td>Lumbar (L2)</td>
<td>Poliomyelitis</td>
<td>L3-4* vascularised</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>T7-8</td>
<td>Thoracic kyphoscoliosis (T8)</td>
<td>Pott's disease</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>44</td>
<td>T10-11 to L2-3</td>
<td>Thoracic (T11)</td>
<td>Idiopathic</td>
<td>L2-3 vascularised, T10-11, L2-3*</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>34</td>
<td>T7-8 to T12-L1</td>
<td>Thoracic (T8)</td>
<td>Idiopathic</td>
<td>T7-8, T12-L4*</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>41</td>
<td>T10-11 to L2-3</td>
<td>Thoracic (T12)</td>
<td>Idiopathic</td>
<td>T11-12*, L1-2* vascularised</td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>38</td>
<td>L1-2 to L3-4</td>
<td>Thoracic kyphoscoliosis (T11)</td>
<td>Idiopathic</td>
<td>L3-4*</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>40</td>
<td>L3-4, L4-5</td>
<td>Lumbar (L2)</td>
<td>Idiopathic</td>
<td>L4-5 degenerate</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>32</td>
<td>T10-11</td>
<td>Thoracic (T12)</td>
<td>Idiopathic</td>
<td>L1-2* vascularised</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>7</td>
<td>T8-9 to T12-L1</td>
<td>Thoracic (T11)</td>
<td>Idiopathic</td>
<td>T11-12*</td>
</tr>
</tbody>
</table>

* Matrix of the discs shows staining with antisera to Type III collagen.

† Staining with antisera to Type III collagen in the end-plate lesion.

VOL. 63-B, No. 4, 1981
Antisera to proteoglycan showed clear staining in the nucleus and inner annulus, but the outer annulus was largely negative. Staining in this latter region was restricted to areas of fissuring or degeneration. This lack of staining in the outer annulus differs from the findings in adult pig intervertebral discs where there was a mesh-like staining pattern with anti-proteoglycan antisera (Beard et al. 1980). In both cases the distribution patterns were distorted by loss of proteoglycan (estimated at 30 per cent) during processing.

**Effect of location in the spine.** Lumbar, thoracic and cervical discs from three different cadaveric spines were compared by immunofluorescence. These discs taken from a newborn boy, a girl aged three months and a man aged 40 years showed no change in staining pattern with antisera to collagen Types I, II and III or proteoglycan according to their location in the spine.

**Effect of age.** Lumbar discs from eight cadaveric spines at different ages were tested by immunofluorescence. As seen in Table I, the distributions observed for Type I collagen and proteoglycan were similar in specimens from neonates and people up to 46 years of age. However, the distributions of Type II collagen varied: Type II was restricted to the nucleus in discs from the

---

**Fig. 5**
Section to show vascularisation of the inner annulus in a scoliotic disc.
(Haematoxylin and eosin, ×33.)

**Fig. 6**
Section showing lesion site in the cartilage end-plate (Patient 17) and T11–12 stained with antibodies to Type I collagen (×40). Figure 7—Adjacent area stained with antibodies to Type III collagen (×40). Figure 8—Inner annulus of the L1–2 disc from Patient 13, showing enhanced staining for Type I collagen in an otherwise weakly stained area (×40). Figure 9—Inner annulus of the L1–2 disc from Patient 16, showing Type III staining in the intercellular matrix close to a capillary blood vessel (×134).
newborn and children aged three months, but occurred in both annulus and nucleus in older specimens. The intervertebral discs from the two youngest spines were also negative with antisera to Type III collagen, whereas all the older specimens showed characteristic pericellular staining in the nucleus and inner annulus. 

Staining patterns obtained with intervertebral discs from scoliotic patients. Specimens of intervertebral disc were collected from 17 different scoliotic patients undergoing anterior fusion operations. Five of these patients had congenital scoliosis (Table II). One patient had a history of congenital displacement of the hip and four patients had scoliosis after poliomyelitis. One further patient who developed a severe kyphosis after Pott’s disease was also included. The remaining seven were classified as having idiopathic scoliosis (adolescent six patients, infantile one).

Frozen sections from all discs were stained with antibodies to collagen Types I, II and III or proteoglycan by indirect immunofluorescence, and additional sections were stained using haematoxylin and eosin. The distribution patterns obtained for collagen Types I and II, and for proteoglycan were generally similar to that found in the cadaveric specimens. Some scoliotic discs showed areas of vascularisation (Fig. 5) and these areas frequently showed enhanced staining with antisera to Type I collagen or proteoglycan (see Fig. 8). Antisera to Type III collagen showed more variable staining. One patient (Patient 17) gave no staining for Type III in any of the six discs sampled, although in one of these specimens (T11–12) a fragment of cartilage end-plate was included, which showed positive staining with antisera to Type III collagen around a lesion close to the border of the disc. This site also showed strong fluorescence with antibodies to Type I collagen (Figs 6 and 7). All the other discs tested showed characteristic pericellular staining with antisera to Type III collagen, but nine discs showed additional staining in the intercellular matrix.

Parallel sections stained with haematoxylin and eosin showed discrete areas of degeneration in one disc (L4–5) from Patient 15 which was probably associated with osteoarthritic changes in this region detected by radiology. Five discs showed extensive vascularisation in the inner annulus and in all patients these areas showed matrix staining with antisera to Type III collagen (Figs 8 and 9).

DISCUSSION

The possibility that scoliosis may result from a primary defect in collagen or proteoglycan metabolism has not been eliminated. The genetic background in almost half of the patients with idiopathic scoliosis (Riseborough and Wynne-Davies 1973), and the obvious abnormalities of connective tissue in some forms of scoliosis demand that these two areas be further explored. The qualitative changes in collagen noted in idiopathic scoliosis, reduced pepsin extractability in the nucleus (Bushell et al. 1978), lowered alkali stability (Francis et al. 1976) and enhanced capacity to aggregate platelets (Udén et al. 1980), could arise from defective collagen assembly, cross-linking, or an alteration in collagen types.

In this study, immunochemical methods were used to look for changes in the types of collagen and proteoglycan in intervertebral discs from scoliotic patients. The distribution patterns obtained with antisera to collagen Types I and II and to proteoglycan in normal human discs were similar to previous findings in discs from pigs (Beard et al. 1980) and agreed well with earlier biochemical data (Eyre and Muir 1976). As in the pig, there appeared to be a postnatal developmental change in the annulus in humans, and in the limited number of specimens tested it seemed that in humans this change occurred between birth and four years of age. Previous immunofluorescence studies on human intervertebral discs did not report such changes in the annulus (Wick et al. 1976), but these workers used antisera to bovine collagens and obtained weak reactions for both Types I and II using adult tissues.

Antisera to Type III collagen showed a very discrete pericellular staining pattern which was restricted to the nucleus and inner annulus. This pattern also appeared to be age dependent: it occurred around a few cells in the four-year-old normal specimen, and in all older specimens, but was absent in those under four years of age. Since Type III collagen has not been isolated from intervertebral discs, and previous biochemical studies suggest that Types I and II account for approximately 90 per cent of collagen in the disc (Eyre and Muir 1977), these findings must be treated with caution. Although the specificity of the antisera was carefully checked against other collagen types, serum proteins and the cell surface associated protein fibronectin, it is difficult to eliminate the presence of other contaminating antibodies directed against, for instance, uncharacterised tissue glycoproteins. Nevertheless, minor collagen types are known to occur in other tissues (Butler, Finch and Miller 1977) and the distribution patterns obtained with Type III antisera in normal human discs would be compatible with an overall content of less than 10 per cent.

Our investigations on scoliotic discs showed no gross abnormalities in the distributions of collagen Types I and II and proteoglycan. It is interesting to note that all patients had Type II collagen in the annulus, including the six patients who developed scoliosis before the age of four years. Clearly in this small group of patients there were none who showed either primary or developmental changes in the distribution of collagen Types I and II or proteoglycan. This does not, of course, exclude the possibility of quantitative changes in the Type I to Type II collagen ratio. Distribution patterns obtained with antisera to Type III collagen showed
particularly interesting results with Patient 17 who showed no staining in any of the intervertebral discs sampled, although positive staining was apparent in an end-plate lesion. The interpretation of these low levels of Type III collagen is difficult for various reasons. The techniques used were qualitative and the poor staining observed may have resulted from poor availability of this antigen in the tissue rather than from a lack of Type III collagen. Secondly, the age of this patient is close to that at which positive staining for Type I first appeared in normal discs, and her clinical problem and subsequent treatment since the age of one may have resulted in retarded development.

A number of discs from several patients showed evidence of vascularisation which was frequently associated with enhanced staining for Type I collagen and proteoglycan and with intercellular staining using antiserum to Type III collagen: the latter was also observed in one normal disc. Increased Type III collagen has been demonstrated in healing wounds in many different situations (Gay et al. 1978; Weber, Meigel and Spier 1978; Rojkind, Giambrone and Biimpica 1979) and it seems possible that these staining patterns may reflect tissue repair occurring in the discs secondary to scoliotic deformity. In the small group described here there appeared to be no clear link between these apparent secondary changes and the location of the disc with respect to the apex of the scoliotic curve.

In conclusion, within the limitations of the techniques and the sample material used, we were unable to demonstrate any clear primary changes in proteoglycan or in collagen types in scoliotic intervertebral discs. However, our findings suggested that there may be low levels of Type III collagen present in normal intervertebral discs and that secondary changes associated with changes in collagens Types I and III and proteoglycan may occur in scoliotic discs. Such changes could explain the increase in nuclear collagen (Pedrini et al. 1973) and its reduced peptic extractability (Bushell et al. 1978) in scoliotic discs and merit further investigation.

REFERENCES


Seigel RC. The connective tissue defect in homocystinuria. Clin Res 1975;23:263A.


THE JOURNAL OF BONE AND JOINT SURGERY