

Biochemical changes of intervertebral discs in patients with spondylolisthesis or with tears of the posterior annulus fibrosus

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SUMMARY Surgically removed discs from patients with either spondylolisthesis or tears of the posterior annulus fibrosus were analysed for water, proteoglycan, and collagen content and compared with post-mortem control material. Discs from patients with spondylolisthesis had a reduced proteoglycan content in all sections sampled and less collagen in the outer annular layers. In contrast discs containing tears in the posterior annulus were unaltered biochemically, although extended studies on 2 patients indicated that there may be localised biochemical changes in the region of the tear itself. Collagen types I, II, and III and proteoglycan distributions were studied qualitatively by immunofluorescence. Collagen types I and III appeared to be reduced in discs from patients with spondylolisthesis, but again little change was found in patients with tears in the posterior annulus fibrosus.

Spondylolisthesis describes the forward displacement of a vertebral body, and is commonly seen at the lumbosacral joint. True spondylolisthesis occurs in approximately 6% of the population,¹ and in the vast majority of cases the displacement is slight. There is, however, a distinct group, usually seen in adolescents, where gross displacement occurs with the body of L5 coming to lie almost in front of the first sacral body.¹

Traumatic injuries of the lumbar spine frequently result in tears of the annulus fibrosus which can be detected by discography.² Such tears commonly occur at the lumbosacral joint, are located posteriorly, and are clearly visible during subsequent surgery for disc removal by the anterior approach. These groups of patients, both those with spondylolisthesis, either mild or severe, and those with tears in the posterior annulus represent clinical conditions in which the intervertebral discs have been subjected to abnormal stress or trauma. There is evidence from x-rays and discograms for disc degeneration in all these patients, but no biochemical or morphological studies have been done on either group.

The aim of this study has been to investigate biochemical changes in sections of anterior annulus fibrosus and nucleus from the appropriate intervertebral discs removed at surgery. Collagen,

proteoglycan, and water profiles were obtained, together with qualitative distribution patterns of collagen types I, II, and III and compared with age-matched post-mortem controls.

Materials and methods

COLLECTION OF MATERIAL

Specimens of intervertebral disc were taken from patients undergoing anterior disc excision and spinal fusion. Diagnosis was made on clinical and radiological evidence. Three patients underwent fusion for mild spondylolisthesis, while 6 severe cases had both reduction and fusion.³ Ten patients had tears of the posterior annulus fibrosus.² One patient case (4) had tears in 2 discs, another (case 9) had a posterior tear in one disc and a mild degree of spondylolisthesis in another. The age range for spondylolisthesis patients was 9 to 39 years, the 6 severe cases being under 21. All affected discs were lumbosacral. Patients with tears of the posterior annulus ranged in age from 24 to 40 years; 8 were lumbosacral and 3 were lumbar 4–5 in position.

At surgery the sagittal anterior section of disc was removed. It contained outer and inner annulus, usually some nuclear material, and in 2 cases of annular tears (Cases 1 and 8) included tissue adjacent to the tear. Immediately on removal the tissue was

placed in a sterilised chamber kept at 100% humidity to prevent evaporation.⁴ For long-term storage the specimen was frozen in N-hexane (boiling range 67–70°C, BDH Chemicals Ltd) at 90°C and kept in liquid nitrogen.

Control material from post-mortem spines obtained within 24 hours of death was treated in a similar manner. The same area was removed from corresponding intervertebral discs of age-matched and where possible sex-matched subjects.

WATER CONTENT

Disc material was removed from storage, divided radially into approximately 3 mm sections, and labelled from A, the outer anterior annulus, across the disc as far as the tissue allowed. The average extended to E but in the case of 2 controls, where there were complete sections of intervertebral disc, as far as J at the outer posterior annulus. This was to correspond with the 2 annular tear patients in whom there was extensive excised material, including some of the tear region. The wet weight of each section was obtained and the dry weights measured after drying to a constant weight over phosphorous pentoxide. Water content was calculated as % water/wet weight.

COLLAGEN CONTENT

Dried sections of disc (approximately 5 mg) were accurately weighed and hydrolysed in 6 M HCl for 16–24 hours at 100°C. After cooling, the solutions were neutralised to pH 7.0 with 5 M NaOH, with BDH 678 used as indicator. Approximately 0.5 g of a mixture of charcoal and Dowex 1-X8 (1:2 w/w) was added to remove charred carbohydrate and decolourise the solution. Samples were filtered and made up to 10 ml with distilled water. Dilutions of 80 µl in 2 ml distilled water were made, and the hydroxyproline content was estimated colourimetrically according to Woessner.⁵ The percentage collagen/wet weight for each section was calculated from mean hydroxyproline values (of at least 6 readings, mean SD 6.9) with a multiplication factor of 7.55, based on 13.2% w/w hydroxyproline for type I collagen, which is the major collagen found in the anterior annulus.⁶

PROTEOGLYCAN CONTENT

Accurately weighed dried sections (approximately 5 mg) of disc were hydrolysed with 9 units of papain (type IV; Sigma) in 1 ml of 0.01 M cysteine-HCl/0.005 M EDTA/0.2 N Na acetate, pH 6.0, at 60°C for 3–6 hours until all the tissue went into solution. Hydrolysed samples were then made up to 10 ml volume with saturated benzoic acid solution and aliquots analysed for uronic acid.⁷ The proteoglycan content was calculated from mean uronic acid values

(of at least 4 readings, mean SD 7.4) with a multiplication factor of 7.00.⁸

Significance between means was calculated according to Student's *t* test for a small sample number and where the 2 means are not assumed to have the same variance.

IMMUNOCHEMICAL LOCALISATION OF COLLAGEN TYPES AND PROTEOGLYCAN

The preparation and characterisation of antibodies to collagen types I and II and proteoglycan has been described previously.⁹ Antisera to collagen types I and II were specific for native collagen molecules. Antisera to proteoglycan showed greatest reaction with the hyaluronate binding region of cartilage and disc proteoglycan, together with weak reaction to the keratan sulphate and chondroitin sulphate rich-regions. Type III collagen was prepared from human placenta and used to raise antisera as described previously.¹⁰

Nine discs from patients with annular tears and 6 with severe spondylolisthesis were investigated by immunofluorescence and compared with 6 and 5 controls, respectively. Disc tissue, stored under liquid nitrogen, was sectioned on a Bright's rotary cryostat. Sections (7 µm) were stained for collagen by indirect immunofluorescence as previously described⁹ after being treated with 0.1 mg ml⁻¹ trypsin (type III, 11 700 units mg⁻¹ protein, Sigma) for 15 min at 37°C. This enhanced the staining of collagen, possibly by removing other proteins¹¹ or proteoglycan,¹² which were covering the fibres. It did not appear to release any type III molecules from the tissue, since less than 0.2% of the tissue hydroxyproline was lost after protease incubation. Sections stained with antiproteoglycan or haematoxylin and eosin were not pretreated with trypsin. All sections were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline, pH 7.4, for 10 minutes at room temperature before being treated with antisera. Fluorescence was viewed on a Leitz Dialux 20 microscope fitted with incident illumination and Phloempak 2.4 filter unit.

Results

WATER CONTENT

Percentage of water content was measured in 96 sections of patient material and 74 controls. The mean values for both patient groups were compared with those for the corresponding control group at each site across the intervertebral disc. In all cases the percentage water/wet weight increased from outer to inner annulus, as has been shown in canine discs.¹³ No significant differences were found when comparing patient and control groups (Figs. 1a, 1b).

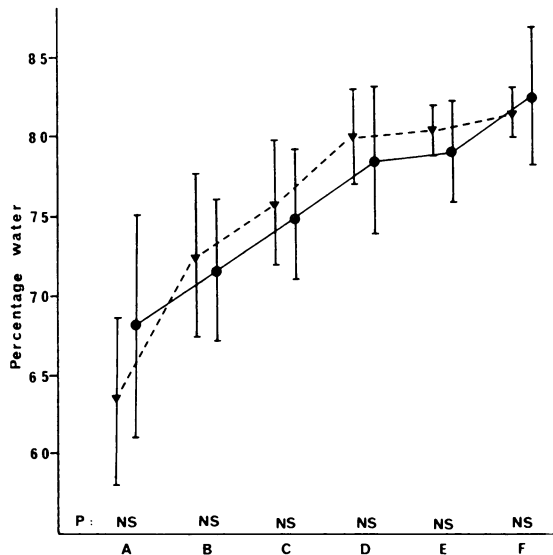


Figure 1a

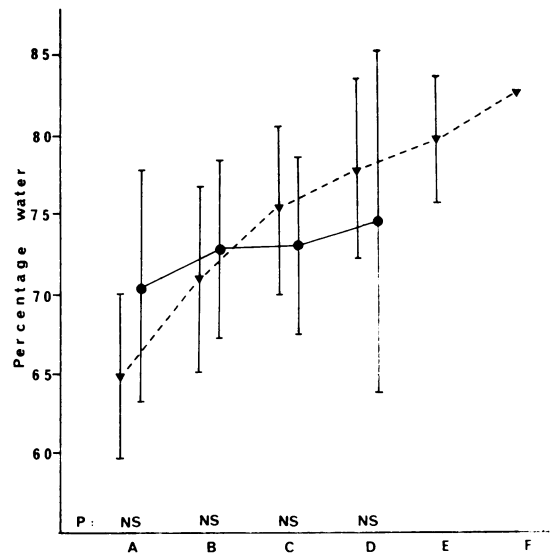


Figure 1b

Fig. 1 Variation of water content across disc. (a) Posterior annular tear patients. (b) Spondylolisthesis patients. ●—● Patients. ▼---▼ Controls. Vertical bars show standard deviation.

COLLAGEN CONTENT

The collagen content for all groups was found to decline from outer to inner annulus, and again this is consistent with previous work.¹⁴ The annular tear patients gave values for percentage collagen/wet

weight closely resembling those obtained for the control group (Fig. 2a). Spondylolisthesis patients were also similar to their controls, with the exception of section A, which had significantly less collagen than the controls (Fig. 2b).

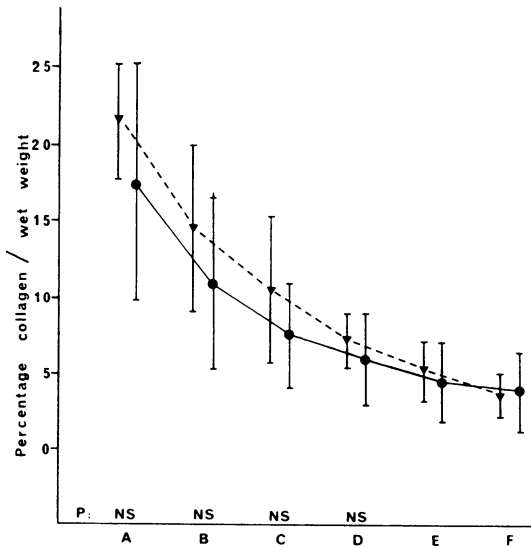


Figure 2a

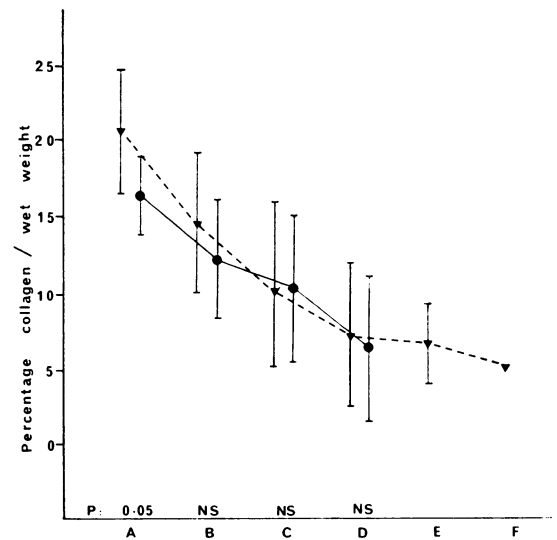


Figure 2b

Fig. 2 Variation of percentage collagen/wet weight across disc. (a) Posterior annular tear patients. (b) Spondylolisthesis patients. ●—● Patients. ▼---▼ Controls. Vertical bars show standard deviation.

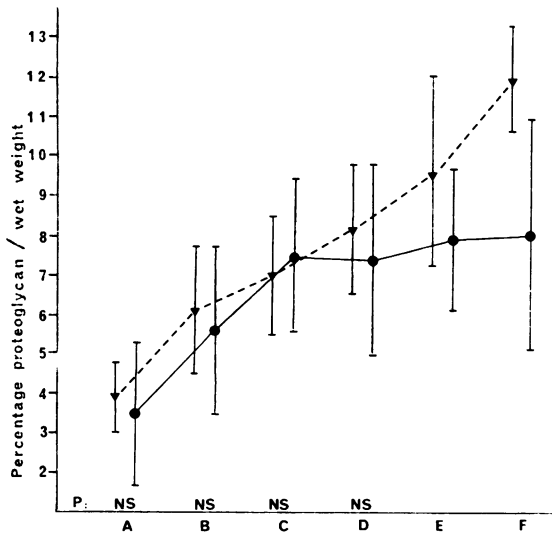


Figure 3a

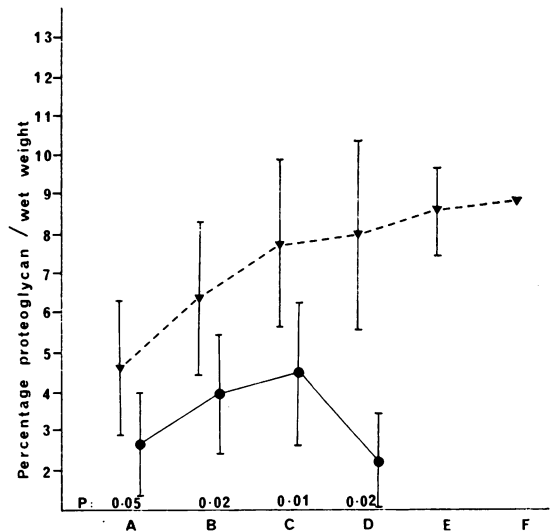


Figure 3b

Fig. 3 Variation of percentage proteoglycan/wet weight across disc. (a) Posterior annular tear patients. (b) Spondylolisthesis patients. ●—● Patients. ▼---▼ Controls. Vertical bars show standard deviation.

PROTEOGLYCAN CONTENT

The proteoglycan content increased from outer to inner annulus. Again there was no significant difference between corresponding sections from discs with annular tear and controls, though there was an apparent lowering of percentage proteoglycan/wet weight in patient material in the nucleus and therefore towards the region of the tear (Fig. 3a). The spondylolisthesis group, however, gave significantly lower values in all sections analysed (Fig. 3b). The percentage loss compared with that in controls is shown in Table 1. In one patient (Case 9), who had one disc with an annular tear and another with spondylolisthesis, the percentage of loss of proteoglycan was calculated for each (Table 2). As expected the disc with a spondylolisthesis showed a greater loss in proteoglycan in all but the outer section of annulus.

Complete profiles were done on 2 patients (Cases 1 and 8) in which posterior tears of the annulus were clearly visible at operation. Results for percentage water, collagen, and proteoglycan content/wet

Table 2 Comparison of percentage of proteoglycan loss in patient (Case 9) between disc with spondylolisthesis (1) and disc with posterior annular tear (2)

Section of disc	% loss in 1	% Loss in 2
A	68.3	70.5
B	58.9	42.3
C	73.5	44.3
D	87.9	56.7

weight were compared with their respective age-matched controls (Figs. 4a, 4b). These results suggested that in one patient (Case 1) there may have been changes, notably loss of collagen and increase of proteoglycan and water, which occurred around the tear and were not apparent in the remainder of the disc. The other patient resembled the controls. There was some difficulty in matching the positions of patient and control exactly, because the complete disc was removed in the case of the post-mortem material while in the patient it was obviously limited by the danger of damage to the dura. The tear region was estimated to correspond with section I of the controls (Fig. 4).

Table 1 Percentage proteoglycan lost in spondylolisthesis discs

Section of disc	% Loss
A	43.5
B	39.1
C	43.6
D	67.5

IMMUNOCHEMICAL LOCALISATION OF COLLAGEN TYPES AND PROTEOGLYCAN

The staining patterns in all the control material showed a distribution pattern comparable to that in pig and normal human intervertebral discs.¹⁰ The

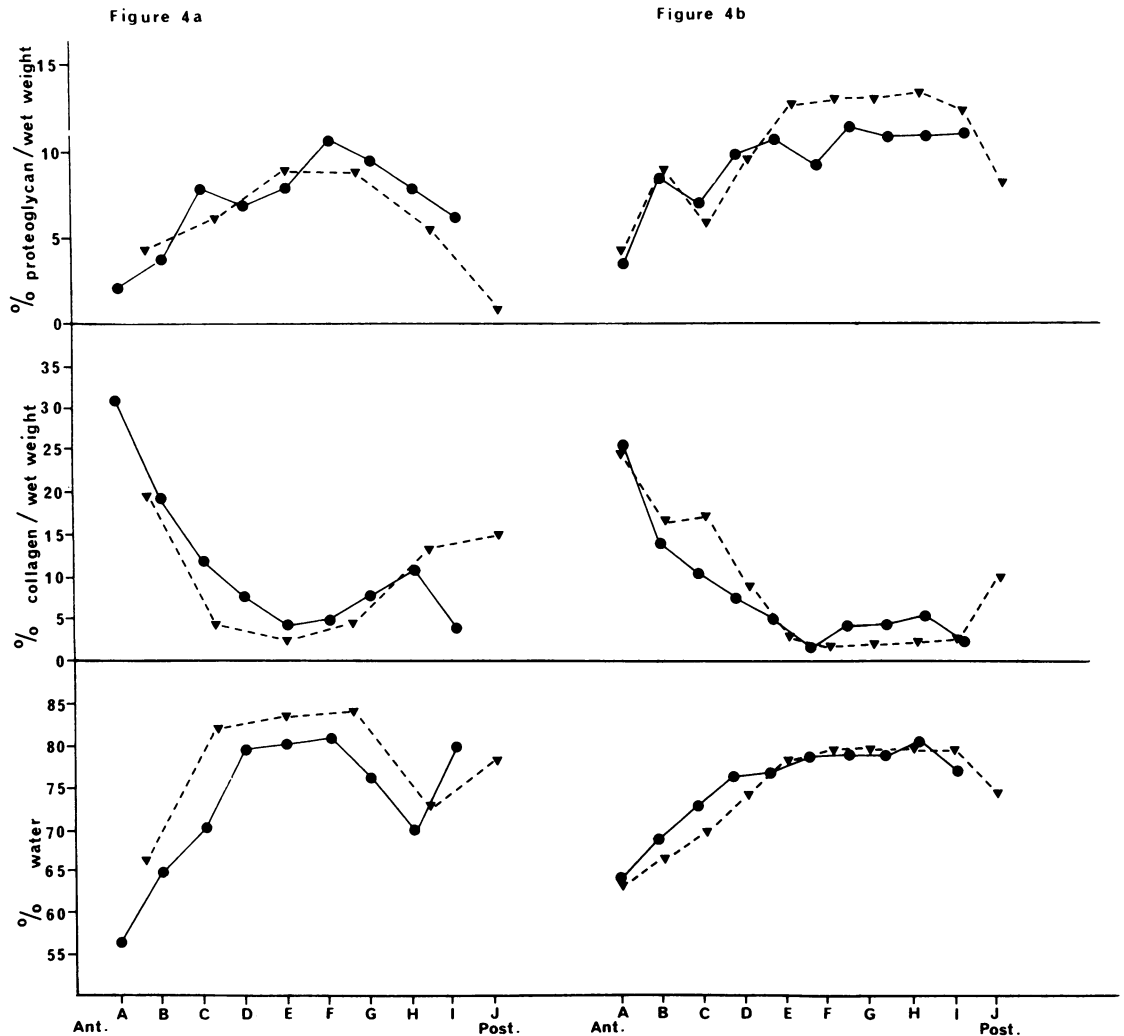


Fig. 4 Variation in water, collagen, and proteoglycan content across the discs in 2 posterior annular tear patients. I is the tear region. (a) ●—● Case 1. ▼---▼ Control. (b) ●—● Case 8. ▼---▼ Control.

results are summarised in Table 3. Staining with type I antisera was generally strongest in the outer annulus, while that for type II was stronger in the inner annulus and nucleus. Antiproteoglycan gave a similar pattern with the nucleus staining strongly, but no staining in the outer annulus. Antisera to type III, however, gave a discrete pericellular pattern, occurring mainly in the nucleus and inner annulus. Chondrocytes and fibroblasts both gave pericellular staining. Discs from patients with annular fissures showed little deviation from this pattern except in 2 patients, Cases 1 and 4 (L5-S1), which showed relatively weak and infrequent pericellular staining with antisera to type III.

In the group of spondylolisthesis patients there appeared to be a greater difference from the control material. Some discs showed weaker staining to anti-type I collagen, while most showed fewer cells and weaker staining with anti-type III. One patient (Case 17) showed particularly weak staining with antisera to all 3 types. When the method was repeated for this patient after pretreating the frozen sections with chondroitinase ABC (Miles Biochemicals; 5 units ml⁻¹) for 30 min at 37°C, there was markedly enhanced staining for all 3 types. This did not occur with post-mortem disc material and suggests that proteoglycan may have masked the collagen fibres in this specimen.

Table 3 Fluorescent staining intensities with antisera to collagen types I, II, and III and proteoglycan

Patient	Sex	Disc	Age	Age of onset/trauma	I	II	III	Pg
<i>Posterior annular tears</i>								
1	F	L5-S1	24	12	+	±	+Few	++
2	M	L5-S1	24	23	+	++	+++	++++
3	F	L5-S1	26	22	+	+	+++	++++
4	M	L4-5	32		+	+	+++	++++
		L5-S1			+	+	+Few M	++
5	F	L5-S1	38	30	+	+	+++	NT
6	M	L5-S1	38	30	+	+	+++	++++
7	M	L5-S1	38	31	NT	+	+++	++
8	F	L4-5	40	33	+	+	+++M	++++
9	M	L4-5	31	26	NT	NT	NT	NT
10	M	L5-S1	37	31	NT	NT	NT	NT
<i>Controls</i>								
11	F	L5-S1	23		±	++	+++	++++
12	M	L5-S1	23		+	+	+++	++++
13	M	L4-5	28		++	++	+++	++
		L5-S1			+	++	+++	++
14	M	L5-S1	40		+	+	+++	++
15	F	L5-S1	42		NT	+	+++	++++
16	M	L5-S1	38		NT	NT	NT	NT
<i>Spondylolisthesis</i>								
17	F	L5-S1	9	7	±	±	±M	++
18	F	L5-S1	13	10	±	±	+Few	+
19	M	L5-S1	14	13	+	+	+Few	++
20	M	L5-S1	16	15	±	±	++	+++
21	M	L5-S1	17	15	++	++	+Few M	++
22	M	L5-S1	21	17	+	+	+Few	++
23	M	L5-S1	16	13	NT	NT	NT	NT
9	M	L5-S1	31	26	NT	NT	NT	NT
24	M	L5-S1	39	38	NT	NT	NT	NT
<i>Controls</i>								
25	M	L5-S1	9		+	+	+++Few	++
26	F	L4-5	12		+	+	+++	++
		L5-S1	12		++	+	+++	+++
27	M	L5-S1	15		++	±	++	++
28	M	L5-S1	21		+	+	+++	++

M = matrix staining. Pg = proteoglycan. Few = fewer cells staining. NT = not tested.

Discussion

The annular tear patients gave no significant changes from normal in the biochemical studies, but since only the anterior annulus and sometimes the nucleus were sampled in all but 2 cases this was not surprising, for the pathology appears to be in the posterior part of the disc. The finding that the anterior regions of the disc were biochemically unchanged fits with clinical evidence and observations at surgery. In 1 of the 2 patients (case 1) from whom more material was obtained there was a suggestion that biochemical changes might be taking place in the posterior annulus. The increased water and proteoglycan observed together with reduced collagen were not in accordance with findings in other disc pathology.¹⁵⁻¹⁷ However, studies in animals suggested that the biochemical changes following trauma may be highly variable. Lipson and Muir¹⁸ induced a rabbit model of disc degeneration which showed that the water

content initially fell and then rose rapidly again, before showing a gradual loss over an extended period. Proteoglycan levels followed this closely. If there is a similar trend after trauma in annular tear patients the water content in relation to normal would depend on time of sampling, and could explain the apparent discrepancies of water and proteoglycan levels.

There was a significant loss of proteoglycan in the group of spondylolisthesis patients, whether mild or severe and in all sections sampled. These changes were similar to those found in osteoarthritic articular cartilage, which had lower proteoglycan,¹⁹ accompanied by increased water^{20 21} and an increase in collagen synthesis and chondrocyte activity.²² In this situation it appeared that the chondrocytes themselves were affected and either had a decreased ability to synthesise proteoglycan¹⁹ or synthesised proteoglycans which were more readily extracted,²³ possibly owing to shorter chondroitin sulphate chains.^{23 25} The mechanical stress experienced in the disc in spondylolisthesis and in articular cartilage in osteoarthritis may be similar in inducing biochemical changes in the proteoglycans in these tissues.

Collagen levels in degenerative joint disease showed less dramatic changes; indeed in many studies they appeared not to be altered at all.^{21 26} In this study there was a significant change only in the outer annular section of the spondylolisthesis group, which was lower than the controls. Venn and Maroudas²⁰ found a similar loss in osteoarthritic cartilage. There is some evidence for degraded collagen fibrils arranged in a halo round chondrocytes of prolapsed discs,²⁷ and of 'reaction products' round chondrocytes in normal and more intensely in chondromalacic cartilage.²⁸ In other studies both collagen synthesis and degradation were increased in osteoarthritic cartilage,²² so that an overall balance was maintained.

The apparent decrease in type I and type III collagen in spondylolisthesis discs was interesting. No corresponding rise in type II collagen was seen, but the distribution of other collagen types (for examples IV and V) was not investigated. Since the technique used was entirely qualitative and dependent on the tissue architecture, the changes observed may have resulted from the redistribution of other tissue components such as proteoglycan rather than a quantitative change in collagen type ratio. However, changes in type of collagen have been demonstrated in osteoarthritic cartilage, where type I was synthesised as well as type II,^{29 30} but this was refuted by Floman *et al.*³¹ and Lippiello *et al.*²² Adam *et al.*³² have also shown that collagen of osteoarthritic cartilage was more soluble and less resistant to

proteolytic enzymes, again indicating some change in structure and possibly type of collagen. Disturbances in collagen types have been found in a number of inherited pathological conditions.³³

In the majority of patients studied here the initiation of symptoms can be explained by previous trauma or a defect in the pars interarticularis, though it is possible that the annular tear patients have been predisposed owing to an inherent defect in the biochemical structure of the disc. It is clear from this study that the affected disc in spondylolisthesis showed a loss in proteoglycan, with the possibility of an accompanying change in collagen types indicated by immunofluorescence. In the annular tear group the intervertebral disc was largely unchanged biochemically, though extended study of 2 patients suggested that localised changes may occur in the region of the posterior fissure.

The mechanisms underlying these secondary biochemical changes remain obscure. There is considerable evidence that chondrocytes can respond to stress and can make some attempt at repair in damaged articular cartilage in rabbits.^{34 35} McDevitt and Muir²³ suggested that chondrocytes synthesised proteoglycans resembling immature cartilage in response to altered mechanical stress, while Caterson and Lowther³⁶ have shown a decrease in hexuronic acid content of non-loadbearing cartilage in sheep, together with a decrease in molecular weight of the proteoglycan present. Although in aging and degeneration proteoglycan shows the most obvious quantitative change, there is still speculation as to which, if either, proteoglycan³⁷ or collagen³⁸ is the first to be affected. Discs from spondylolisthesis patients provide a suitable sample group for biochemical studies, and investigations may show some interesting relationships between biochemical and biomechanical changes.

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