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An immunohistochemical study of the tissue bridging adult spondylolytic defects—the presence and significance of fibrocartilaginous entheses

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Abstract *Introduction* Spondylolytic spondylolisthesis is an osseous discontinuity of the vertebral arch that predominantly affects the fifth lumbar vertebra. Biomechanical factors are closely related to the condition. An immunohistochemical investigation of lysis-zone tissue obtained from patients with isthmic spondylolisthesis was performed to determine the molecular composition of the lysis-zone tissue and enable interpretation of the mechanical demands to which the tissue is subject. *Methods:* During surgery, the tissue filling the spondylytic defects was removed from 13 patients. Twelve spondylolistheses were at the L5/S1 level with slippage being less than Meyerding grade II. Samples were methanol fixed, decalcified and cryosectioned. Sections were labelled with a panel of monoclonal antibodies directed against collagens, glycosaminoglycans and proteoglycans. *Results:* The lysis-zone tissue had an ordered collagenous structure with distinct fibrocartilaginous entheses at both ends. Typically, these had zones of calcified and uncalcified fibrocartilage labelling strongly for type II collagen and aggrecan. Labelling was also detected around bony spurs that extended from the enthesis into the lysis-zone. The entheses also labelled for types I, III and VI collagens, chondroitin four and six sulfate, keratan and dermatan sulfate, link protein, versican and tenascin. *Conclusions:* Although the gap filled by the lysis tissue is a pathological feature, the tissue itself has hallmarks of a normal ligament—i.e. fibrocartilaginous entheses at either end of an ordered collagenous fibre structure. The fibrocartilage is believed to dissipate stress concentration at the hard/soft tissue boundary. The widespread occurrence of molecules typical of cartilage in the attachment of the lysis tissue, suggests that compressive and shear forces are present to which the enthesis is adapted, in addition to the expected tensile forces across the spondylolysis. Such a combination of tensile, shear and compressive forces must operate whenever there is any opening or closing of the spondylolytic gap.

Keywords Spondylolytic spondylolisthesis · Spondylolysis · Fibrocartilage · Aggrecan · Type II collagen

Introduction

Spondylolytic spondylolisthesis (SSL) is defined as an osseous discontinuity of the vertebral arch at the isthmus—the pars interarticularis—predominantly occurring in the fifth lumbar vertebra. Kilian [27] is credited with the first recognition of spondylolisthesis as anterior slippage of the last lumbar vertebra against the sacrum

[12, 40]. The first theoretical and subsequent biomechanical identification of a discontinuity of the pars interarticularis as a compulsory feature of spondylolisthesis, in addition to disruption of the intervertebral disc, was made by Robert [43]. Several epidemiological studies have since revealed the incidence in Caucasian populations to reach 4–6% [35, 52], which rise as high as 26% in secluded Eskimo populations [50]. The classical form of SSL does however not occur in non-ambulatory individuals [44], linking it not only to a genetic predisposition [18, 62, 58] but also to biomechanical factors associated with spinopelvic balance and repetitive loading [4, 15, 22, 25, 34, 59]. While several conventional histological investigations of the isthmic lesion have been performed in children [39, 49, 64] and adults [52, 58], the functional significance of the tissue bridging the bony defect (subsequently referred to as the 'lysis-zone tissue') is unclear. As this tissue forms a fibrous link between two regions of bone, it acts as a ligament. Normal ligaments have specialised bony attachment sites or 'entheses', which may be fibrous or fibrocartilaginous, depending on the mechanical loads to which they are subject [2, 3]. Although both types of entheses transfer tensile load to bone, fibrocartilaginous attachments are also subject to compression and shear [2, 3]. This is reflected by differences in the composition of the extracellular matrix (ECM) and in particular by the presence of aggrecan and type II collagen at fibrocartilaginous entheses [2, 3]. These molecules are typical of articular cartilage —which is a tissue noted for its ability to distribute compressive forces across synovial joints [55]. In any ligament where 'insertional angle' changes accompany joint movement, that ligament will inevitably be subject to compressive and shearing forces at its entheses. As the tissue bridging a spondylolytic defect can be viewed as a very short ligament, any relative movement of bone on either side of the spondylolysis should magnify insertional angle change. We have thus sought to determine the structure of the lysis-zone tissue through an immunohistochemical study of its ECM and, in particular, whether its entheses are fibrous or fibrocartilaginous, hereby-enabling interpretation of the mechanical demands to which the tissue is subject.

Materials and methods

The lysis-zone tissue filling the spondylytic defects of 13 individuals (age range 36–60 years, mean 45 years, both sexes) undergoing spondylodesis for low-grade isthmic spondylolisthesis was removed during surgery. All patients had bilateral spondylolysis and all of the spondylolisthesis were at the L5/S1 level except one, which was at L4/5. In no case was slippage greater than Meyerding grade II (i.e. greater than 50% [61]). Moderate to advanced disc degeneration, with obvious loss of disc height or loss of hydration signal on magnetic resonance imaging, was present in all cases. Persistent or exacerbated back pain had been present for at least 3 months in all patients and most reported a history of recurring low back pain for several years along with mild symptoms of L5 nerve root compression. Informed consent was obtained from all patients for the entire procedure and the Declaration of Helsinki (http://www.fda.gov/oc/health/helsinki89.html) was strictly followed. The spondylolytic region was identified during surgery and the lysis-zone tissue and a small part of the adjacent bone was removed as completely as possible without endangering the neighbouring nerve root. In six patients, the tissue could be collected from both sides and in seven, from one side only. Thus a total of 19 specimens were examined. In 12 of these, both entheses were included, but only one enthesis was available in the others. Tissue samples were fixed for 24 h in 90% methanol at 4°C. The tissue was decalcified in 5% EDTA, infiltrated with a 5% sucrose solution in PBS for 12 h and cryosectioned at 12 μm on a HMV500 Microm cryostat. Sections were stained with toluidine blue and labelled with a panel of monoclonal antibodies directed against collagens (types I, II, III and VI), glycosaminoglycans (chondroitin four and six sulfates, keratan and dermatan sulfates) and proteoglycans (aggrecan, link protein, versican and tenascin). Details of the antibodies are given in Table 1, together with pre-treatment procedures. The activity of endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol and any non-specific binding of the secondary antibody was reduced by incubating the sections with horse serum. Antibody binding was detected with a Vectastain ABC 'Elite' avidin/biotin/peroxidase kit (Vector Laboratories, Burlingame, CA, USA) and control sections were made by omitting the primary antibody. All control sections were unlabelled

Results

The position of the lysis-zone tissue in a typical spondylolytic defect at L5/S1 is shown in Fig. 1a. In all specimens, the tissue was highly organised with structurally ordered collagen bundles and distinct fibrocartilaginous entheses at both its pedicle and vertebral arch ends (Figs. 1b, 2a). Typically, these entheses had zones of calcified and uncalcified fibrocartilage that were separated from each other by a tidemark (Fig. 2a). The cells in the uncalcified fibrocartilage were typically arranged in longitudinal rows (Fig. 2a).

In all specimens, the ECM of the enthesis fibrocartilage labelled strongly for type II collagen and aggrecan (Fig. 2b, c). Labelling was also detected around bony spurs that extended from the enthesis into the lysis-zone in some specimens (Fig. 2d) and in regions of chondroid bone (Fig. 2e). At the latter sites, the fi-
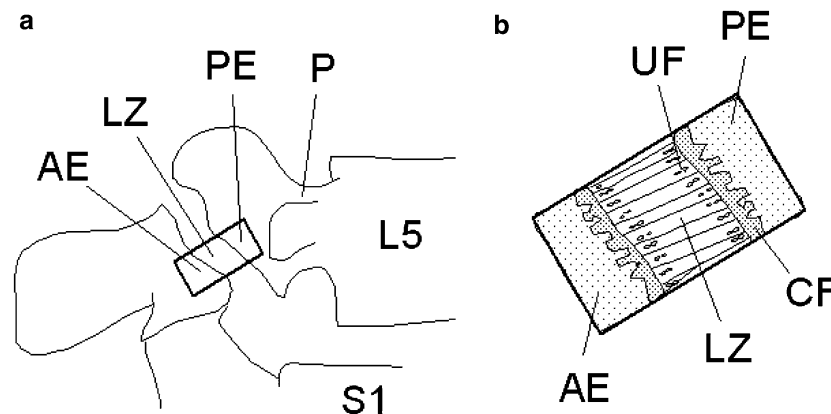
Table 1 List of monoclonal antibodies used, together with their dilutions, pretreatments and sources

Antigen(s) recognized:	Antibody	Dilution	Enzyme pretreatment	Source	References
Collagen I	Col 1	1:2000	Hyal (1.5 IU/ml) and ChABC (0.25 IU/ml)	Sigma	None
Collagen II	CIICI	1:5	Hyal (1.5 IU/ml) and ChABC (0.25 IU/ml)	DSHB	21
Collagen III	III-53 (4H12)	1:500	Hyal (1.5 IU/ml) and ChABC (0.25 IU/ml)	ICN	None
Collagen VI	5C6	1:6	None	DSHB	20
Chondroitin-4-sulfate	2B6	1:1500	ChAC (0.25 IU/ml)	B. Caterson	10
Chondroitin 4 and dermatan sulfates	2B6	1:1500	ChABC (0.25 IU/ml)	B. Caterson	10
Chondroitin-6-sulfate	3B3	1:80	ChABC (0.25 IU/ml)	B. Caterson	10
Keratan sulfate	5D4	1:1500	None	B. Caterson	9
Aggrecan	1C6	1:10	ChAC (0.25 IU/ml) after reduction and alkylation	DSHB	8
Link Protein	8A4	1:10	ChAC (0.25 IU/ml) after reduction alkylation	DSHB	8
Versican	12C5	1:10	ChAC (0.25 IU/ml)	DSHB	None
Tenascin	T2H5	1:100	ChAC (0.25 IU/ml)	Novocastra	56

All antibodies are mouse monoclonals, except for 3B3, which is a rat monoclonal. *DSHB* developmental studies hybridoma bank, *ChAC* Chondroitinase ACII (Sigma), *ChABC* Chondroitinase ABC (Sigma), *Hyal* Hyaluronidase (Sigma)

brocartilage cells were densely, but irregularly, arranged on the soft tissue side of the bony interface (Fig. 2f). The entheses consistently also labelled for types I, III and VI collagens, several glycosaminoglycans (chondroitin four and six sulfate, keratan sulfate and dermatan sulfate), link protein, versican and tenascin. However, labelling for type I collagen (7 of 19) and versican (2 of 19) was locally absent in some specimens close to the tidemark (Fig. 2g). In some specimens, the subchondral bone at the enthesis contained conspicuous fragments of calcified cartilage that were readily identifiable by their positive labelling for type II collagen and by the absence of labelling for type I collagen (Fig. 2h).
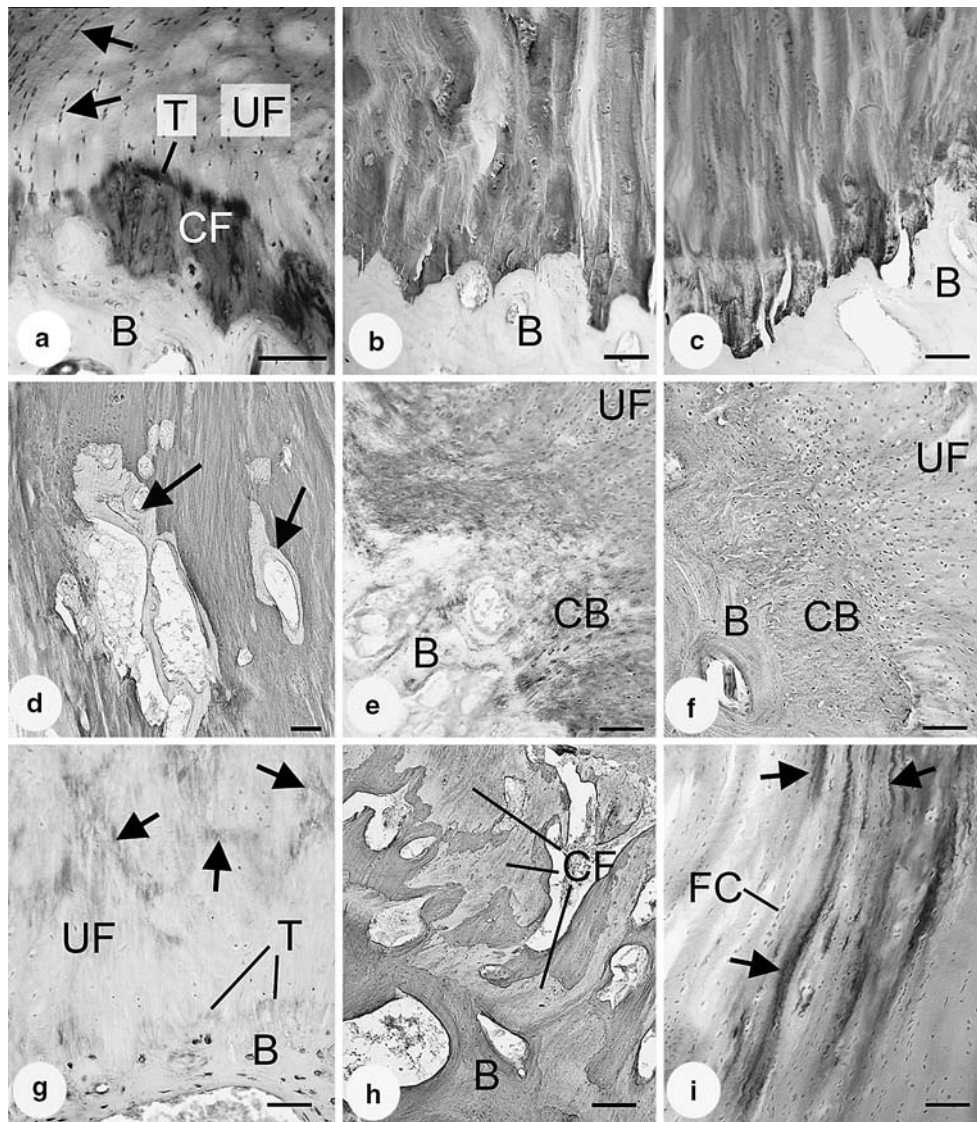
Evidence of fibrocartilage differentiation was also seen in the mid-substance of the lysis-zone tissue along with occasional islands of bone. The fibrocartilaginous ECM labelled for aggrecan and link protein in all specimens and for type II collagen in 14 out of 19 cases (Fig. 2i).

Discussion

Ossification of the isthmus region begins bilaterally from the medial circumference of the vertebral arch at the prenatal gestation age of 8 weeks [53], appearing first at L1 and last at L5 [1]. Bone first starts to appear as a perichondral sheath and progresses to almost complete replacement of cartilage by bone by the time of birth [53]. Correspondingly, spondylolytic lesions of the isthmus have rarely, if ever, been observed in the fetus or new-born [57,63]. Nevertheless, the developmental process of the vertebral arch remains active through bone remodelling up to 7-9 years of age [42]. Interestingly, spondylolytic defects of the lumbosacral spine are most commonly recognised between 4-6 years of age [31] and

Fig. 1 Diagrammatic representation of the lysis-zone tissue filling a spondylolytic defect between L5 and S1. The low power view on the left (**a**) shows the position of the lysis-zone tissue (*LZ*) and the distinction between its two entheses—the 'vertebral arch' enthesis (*AE*) and the 'pedicle' enthesis (*PE*). The drawing on the right (**b**) summarises diagrammatically the ligamentous organisation of the lysis-zone tissue. There are fibrocartilaginous entheses at both the pedicle and vertebral arch ends of the tissue and a distinction can be made between zones of uncalcified (*UF*) and calcified (*CF*) fibrocartilage. *P* pedicle

several well documented cases of de novo formation of the defect exist in the literature [57]. Histologically, the defect is found to begin at the lateral and anterior circumference of the isthmus with regions of bone resorption and remodelling, which vary from a regionally weakened isthmus to complete osseous disruption [64]. These findings correspond to a certain degree with finite element analyses that reveal the anterior isthmus to be most vulnerable to mechanical stress in various loading conditions [24]. Progressive or high grade vertebral slippage can occur in the vertebral growth plate [23,26,28,46], altering the loading situation of the paediatric isthumus and potentially leading to formation of the spondylolytic defeat. When it occurs, the paediatric defect is highly vascularised with disordered collagenous and cartilaginous tissue spanning the defect [52,64], whereas the adult defect is characterised by ordered

tissue [52,58]. In juveniles, osseous reunion may occur [31,49,55,60], however, if there is non-union, the lysis-zone tissue will conform to a specific mechanical environment that persists into adulthood.

Despite the fact that the gap in which the lysis tissue forms is a pathological feature, the tissue obtained in our study has an ordered structure and is always attached to bone by fibrocartilaginous entheses, that are typical of a normal ligament [17,45]. Mechanical stresses are known to be important in regulating tissue differentiation both during normal development and in regeneration or repair. There are clear parallels between the formation of fibrocartilaginous entheses in the lysis-zone tissue, the re-establishment of such entheses after the surgical re-attachment of a ligament or tendon [11,16,54,60] and in the formation of fibrocartilage in pseudoarthroses [30,19]. Fibrocartilage at a bony interface is widely be-

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Fig. 2 Immunohistochemical labelling of extracellular matrix molecules at the entheses of the lytic tissue in spondylolytic defects. **a** Toluidine blue staining of an enthesis to show the presence of distinct zones of calcified (*CF*) and uncalcified fibrocartilage (*UF*) that are separated from each other by a tidemark (*T*). Note the longitudinal rows of fibrocartilage cells (*arrows*). *B* bone. *Scale bar* 100 μm. **b** Type II collagen labelling on the soft tissue side of the enthesis. *B* bone. *Scale bar* 100 μm. **c** Aggrecan labelling on the soft tissue side of the enthesis. *B* bone. *Scale bar* 100 μm. **d** Small bony spurs surrounded by type II collagen staining matrix. *Scale bar* 200 μm. **e** Aggrecan labelling of chondroid bone (*CB*). Note the adjacent labelling in the uncalcified fibrocartilage (*UF*). *Scale bar* 100 μm. **f** Type I collagen labelling in lamellar bone (*B*), chondroid bone (*CB*) and the zone of uncalcified fibrocartilage (*UF*). *Scale bar* 100 μm. **g** Patchy labelling (*arrows*) for versican. Note the local absence of labelling near the tidemark (*T*). *B* bone, *UF* uncalcified fibrocartilage. *Scale bar* 100 μm. **h** Isolated fragments of calcified fibrocartilage (*CF*) within the bone beneath the enthesis. The fragments can be readily identified because they do not label with type I collagen—unlike the surrounding bone (*B*). *Scale bar* 200 μm. **i** Streaky labelling for type II collagen (*arrows*) in the mid substance of the spondylolytic ligament in association with the presence of fibrocartilage cells (*FC*). *Scale bar* 100 μm

lieved to provide a two-tier system of protection against stress concentration [2,3,48]. The zone of calcified fibrocartilage anchors the ligament to the bone and enables it to withstand shearing forces occurring during changes of insertional angle, while the uncalcified fibrocartilage is believed to dissipate bending forces away from the hard/soft tissue boundary. Both of these zones are present in the lysis tissue and this suggests that such tissue is subject to comparable types of mechanical loading which direct the differentiation of mesenchymal progenitor cells within it. The widespread occurrence of molecules typical of cartilage in the lysis tissue – notably type II collagen, aggrecan, link protein and chondroitin 6 sulfate, strongly suggests that compressive and shear forces are acting within the tissue. While tensile forces are expected to be present across the spondylolytic defect through axial loading of the lumbar spine, shear and compressive forces must operate whenever there is any motion within the gap. Gap dimensions inevitably change in bilateral defects, when movements occur between the spine and the pelvis. They occur because there is a shift in the instantaneous axis of rotation in flexion and extension from the disc and lower adjacent vertebral body to the spondylolytic vertebra [47]. The bony ring in which the defect lies, is hereby subject to mechanical loading which deforms it and thus alters the gap size. An analogous mechanical situation occurs in the normal acetabulum in connection with its transverse ligament [29,32]. Here, the size of the acetabular notch across which the ligament spans, changes with the loading on the hip joint.

The presence of fibrocartilage in the midsubstance of the ligament is comparable to a similar finding in the transverse ligament of the atlas, in the region where the ligament is compressed against the dens [37]. In the spondylolytic gap, compressive forces that trigger such a differentiation could be a consequence of torsional movements of the spine. The exact reason why the 'mid-substance fibrocartilage' composition did not include collagen type II in all specimens (14 of 19) is unclear, but may reflect inter-individual variations in the torsional forces. While our findings reveal clear structural parallels between the spondylolytic tissue and normal ligaments, the origin is not embryological but develops from a paediatric bone defect [31, 57, 63]. From this perspective, the spondylolysis may be likened to a pseudoarthrotic bone defect, in which the connective tissue bridging the defect has undergone specific structural adaptation. The structure of the fibrocartilage is probably analogous to that reported by Heggeness et al. [19] in lumbar pseudoarthroses. Correspondingly, chondroid bone (a form of woven bone), which has been reported here at the spondylolytic tissue entheses, is also a feature of the newly formed tissue boundary in surgical reconstructions. According to Oguma et al. [41], it develops prior to the re-appearance of fibrocartilage. The presence of woven bone in association with the lysis-zone tissue and the identification of islands of calcified fibrocartilage isolated within the bone just beneath the entheses, suggests an attempt at osseous healing that occurs in some, but not all, patients. Woven bone is typical of new bone formation and is well-documented both in fracture healing and during normal development [14]. Burr et al. [7] have suggested that woven bone production can be a normal response to abnormal strains. Calcified fibrocartilage fragments persisting within the bone that underlies an attachment site probably suggests recent episodes of endochondral ossification—which is again indicative of bone turnover in some patients. We support the view of Cullinane et al. [13] that there is a direct relationship between mechanical loading conditions, gene expression and tissue differentiation within a healing bone defect. The molecular architecture of lysis-zone entheses suggests that mechanical influences on tissue repair can recapitulate some aspects of the early enthesis development within attempts at healing in a pseudoarthrotic adult bone defect.

In summary, despite the genetic predisposition for SSL that has been reported by other authors [18, 62], our finding that the lysis-zone tissue associated with spondylolytic defects, has fibrocartilaginous entheses, suggests that specific mechanical forces act at its attachment sites. As fibrocartilaginous entheses are typical of normal ligaments in which compressive, tensile and shear forces act [5, 6, 33, 36–38], we suggest that compression and shear at the entheses of the lysis-zone tissue may be a consequence of insertional angle changes that are inevitably magnified in short ligaments. While we cannot ascertain the structural strength of the lysis-zone tissue, the ECM composition clearly indicates a functional adaptation to the described combination of tensile and shearing forces.

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