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Characterisation of cytoplasm-filled processes in cells of the intervertebral disc

R. J. ERRINGTON[1]*, K. PUUSTJARVI[1]**, I. R. F. WHITE[1], S. ROBERTS[2], J. P. G. URBAN[1]

[1] *University Laboratory of Physiology, Oxford University, and* [2]*Centre for Spinal Studies, Orthopaedic Hospital, Oswestry, UK*

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ABSTRACT

We examined cells from the nucleus pulposus and annulus fibrosus of adult bovine intervertebral discs, using confocal laser scanning microscopy on living unfixed tissue. These cells were visualised using chloromethyl fluorescein diacetate, a membrane-impermeant fluorescent dye. The organisation of cells from the outer annulus was also determined using confocal microscopy after fixation and staining the actin-filaments with FITC-phalloidin. We found that cellular processes were a dominant feature of cells from all regions of the disc including the cells of the nucleus pulposus and inner annulus. These processes were also visible in histological sections of disc examined both at the light and electron microscope level, even though cells from the nucleus and inner annulus appeared chondrocyte-like, being rounded and enclosed in a capsule. The function of these processes is at present unknown. We suggest that they may serve to sense mechanical strain.

Key words: Chondrocytes; nucleus pulposus; annulus fibrosus; actin.

INTRODUCTION

The intervertebral disc is a remarkably acellular tissue. Cell density in the human disc is only around 6000 cells/mm^3 (Maroudas et al. 1975), i.e. the cells occupy less than 0.25% of tissue volume. Nevertheless, these are essential for disc function since throughout life they manufacture and turn over the macromolecules of the extracellular matrix which regulate the mechanical response of the disc to load (Johnstone & Bayliss, 1995).

The disc consists of 3 distinct morphological regions, the soft pulpy nucleus pulposus, the fibrocartilaginous inner annulus and the ligamentous-like outer annulus (Szirmai, 1970) (Fig. 1). There are marked differences in composition between these regions (Eyre, 1979; Bayliss et al. 1988; Oegema, 1993; Urban & Roberts, 1996) which are produced and maintained by apparently separate cell populations (Ichimura et al. 1991; Chelberg et al. 1995).

The different cell populations could arise from developmental differences between the regions (Peacock, 1952: Rufei et al. 1995). In the fetus, the nucleus is populated by notochordal cells (Walmsley, 1953). In some animals the notochordal cells of the nucleus persist throughout life, but in humans and in

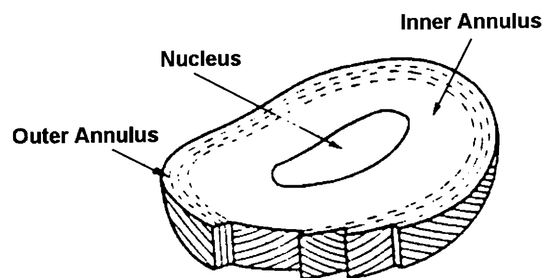

Fig. 1. Schematic view of the disc showing the nucleus, inner annulus and outer annulus with lamellae cut away to demonstrate the organisation of the outer annulus. (Adapted from Schultz, 1974).

Correspondence to Dr J. Urban, University Laboratory of Physiology, Oxford University, Tel: +441865 272509; fax: +441865 275469; e-mail: jpgu@physiol.ox.ac.uk
Present addresses: * Department of Cell Physiology, University of Nijmegen, The Netherlands; ** Department of Anatomy and Physical and Rehabilitation Medicine, University and University Hospital of Kuopio, Finland.

other species such as chondrodystrophoid dog breeds (Walmsley, 1953; Buckwalter, 1982; Trout et al. 1982; Butler, 1989), the notochordal cells disappear in infancy to be replaced by chondrocyte-like cells (Walmsley, 1953; Buckwalter, 1982). Whether this cell population is derived from dedifferentiation of notochordal cells, or whether the notochordal cells die and mesenchymal cells invade the nucleus space is not known (Walmsley, 1953; Taylor & Twomey, 1984). In contrast to the nucleus, the inner and outer annulus cells are both of mesenchymal origin; they separate into 2 distinct populations early in embryonic life. In humans, by 3.5 mo of fetal life, before any extracellular matrix is evident, the outer layer of cells, oriented with their long axes in gently curving columns, is distinct from the inners cells which appear rounded and chondrocyte-like (Walmsley, 1953). These differences in the appearance of the cells from the inner and outer annulus are maintained with growth and development.

In the adult human the cells of the inner region, the nucleus pulposus, are described mainly as chondrocytes or chondrocyte-like (Walmsley, 1953; Trout et al. 1982; Ichimura et al. 1991; Roberts et al. 1991; Banks et al. 1992; Maldonado & Oegema, 1992), being rounded with a spheroidal nucleus, and located within a capsule whose composition is distinct from that of the bulk matrix (Roberts et al. 1991). The cells occur singly or in clusters of 4 to 6 (Maroudas et al. 1975; Roberts et al. 1991). The chondrocyte-like appearance of the cells is in agreement with the finding that the major components of the nucleus are similar to those of articular cartilage, i.e. aggrecan and collagen II (Eyre & Muir, 1976; Eyre et al. 1991; Johnstone & Bayliss, 1995). The cells of the inner annulus are described both as chondrocyte-like and as fibrocartilaginous (Pritzker, 1977; Buckwalter, 1982; Roberts et al. 1991). These cells are also enclosed in a capsule and are rounded, but the matrix produced is described as fibrocartilaginous (Beadle, 1931; Buckwalter et al. 1993), being more collagenous than the nucleus though also containing high concentrations of aggrecan. Some collagen I fibrils are found in this region though the major collagen is still collagen II (Eyre et al. 1991). The cells of the outer annulus are elongated and fibroblast-like with no visible pericellular capsule (Buckwalter, 1982; Postacchini et al. 1984; Roberts et al. 1991) and are aligned with their long axes parallel to the collagenous bundles of the lamellae. Although this region contains a relatively high concentration of sulphated glycosaminoglycans (Urban & Maroudas, 1979), its composition and organisation is more like ligament or tendon than cartilage (Pritzker, 1977). The most noticeable features here are the visible concentric collagenous lamellae, consisting mainly of collagen I fibrils, running in oblique sheets between the vertebral bodies and anchoring the disc to the bone (Szirmai, 1970).

In descriptions of disc cells there are intriguing mentions of cellular processes (Trout et al. 1982; Roberts et al. 1991) although these are not described specifically except in the rat annulus (Postacchini et al. 1984). Processes have however recently been described in tendon cells, visualised using confocal laser scanning microscopy (CLSM) (Ralphs et al. 1996) where it is thought they serve as mechanosensors. Here we report on our studies on adult bovine disc cells examined using CLSM, where we found these processes were a dominant feature of cells from all regions of the disc. These processes were also visible in histological sections of disc examined at the light and electron microscope (EM) level. The function of these processes is at present unknown. We suggest that, as in tendon, they may serve to sense mechanical strain.

METHODS

Source of tissue

Bovine tails from 18–24 mo steers were obtained from the slaughterhouse within 3 h of slaughter and the upper discs (CC1–CC2 or CC2–CC3) cut from the bone immediately before use; discs from 6 animals were used in this study. For confocal scanning microscopy, the disc was cut along the diameter, subsequently a strip (5 mm thick) was removed from each half. For light and electron microscopy, the discs were then divided into nucleus and inner and outer annulus in a humid chamber. Since the boundary between nucleus pulposus and inner annulus fibrosus was not clearly visible, only the central region of the disc was regarded as nucleus, and the boundary region was discarded. The outer annulus was considered to be the firm region of dense lamellae in the outer quarter of the disc; the junction between the outer and inner annulus was taken at the point where a colour change was visible by eye. The inner annulus was the remaining fibrocartilaginous region. The tissue from the 3 different regions was then divided for analysis and treated as described below.

Light microscopy

Disc slices from the different regions were fixed in 10% buffered formol saline and then processed

overnight through a series of alcohol, chloroform and xylene solutions before embedding in paraffin wax. Sections (10 μm) were stained with toluidine blue.

Transmission electron microscopy

Disc slices were fixed following the procedure of Hunziker et al. (1983). In brief, disc slices from the different regions were diced into 1 mm cubes in a bath of fixative and then fixation continued at room temperature for 4–6 h. The fixative contained 2.5% glutaraldehyde, 0.7% ruthenium hexamine trichloride and 1% $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4. The blocks were then washed in cold 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, rinsed in buffer and dehydrated in a series of ethanol solutions (70–100%) and propylene oxide. After dehydration, the disc blocks were embedded in epoxy resin and hardened at 60 °C in an oven. Sections (70 nm) were cut on an ultramicrotome and viewed under a 100CX JEOL electron microscope.

Confocal scanning laser microscopy

We examined living nucleus and inner annulus cells in fresh tissue slices using an intracellular fluorescent dye. We could not see cells in the outer annulus labelled in this manner because the dense collagen matrix was too opaque and had high reflective and scattering properties. In the outer annulus we therefore fixed the tissue and labelled the actin cytoskeleton of these cells with rhodamine phalloidin. This increased the visibility of the cells for 2 reasons. First, a higher concentration of fluorescent label could be used with fixed tissue than with the living disc. In addition the fixed annulus material could be mounted into a glycerol based mountant to reduce the refraction; the aberration was thus less than with live material.

Tissue preparation and dye loading (nucleus and inner annulus)

The tissue strip was enclosed in dialysis tubing and incubated at 37 °C for 15 min in HEPES buffered Dulbecco's modified Eagles' medium (standard DMEM) made to 400 mOsm by NaCl addition and containing 10% w/v polyethylene glycol 20000 (Fluka Chemicals, Glossop, UK) to prevent tissue swelling (Bayliss et al. 1986) and 4.5 μM chloromethyl fluorescein diacetate (CMFDA) (Molecular Probes, Oregon, USA). This nonfluorescent ester was readily taken up by the disc cells. Intracellular esterases cleaved the acetate form, leaving a membrane-impermeant fluorescent dye trapped inside the now positively labelled cells.

Actin labelling (outer annulus)

The method of actin staining was according to the protocol of Martin & Lewis (1992). In brief, blocks of tissue ($\sim 5 \times 5 \times 10$ mm) were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 1 h, rinsed in PBS for 3×30 min and labelled overnight with FITC-phalloidin (Sigma; 2.5 μg ml^{-1} in PBS). These specimens were washed as before and mounted in a glycerol-based mountant in a slide-coverslip sandwich.

Confocal microscopy and image visualisation

Once the dye was loaded, the tissue strip was placed in a sealed slide-coverslip chamber. This was mounted onto an MRC 600 CLSM (Bio-Rad Microscience, Hemel Hempstead, UK), attached to a Diaphot inverted microscope (Nikon UK, Surrey) set up for FITC imaging (488 nm excitation/515 nm emission). The tissue was always examined within 90 min of removal from the bone. All experiments were carried out using a $\times 25$, 0.8 NA Plan Neofluar objective lens. The CLSM imaging aperture was always fully closed, providing maximum optical sectioning.

Three-dimensional (3-D) fluorescence images were obtained through the region of interest. Typical images consisted of 768 pixels (0.5 μm sampling) by 250 lines by 60 sections, collected at 1 μm intervals. All data collection and manipulation was performed using the software packages COMOS and ThruView (BioRad Microscience) on a PC workstation. To visualise and hence present the large 3-D images the data were compressed into 2-D views using 1 of 2 different algorithms. The first was a simple maximum brightness projection. The second more complex reconstruction, presented both fluorescence and morphological information by introducing solidity into the view. Hence cells deeper into the original (transparent) tissue sample were realistically obscured by overlying cells (Errington et al. 1997). The actin staining of cells in the outer annulus was presented as a pixel shifted stereo pair.

RESULTS

Light microscopy

Nucleus cells. Sections through the nucleus of the bovine disc showed rounded cells separated from each other by extensive loose extracellular matrix. Fig. 2*a*, a typical section, shows only 4 rounded cells, well separated from each other in an area 0.05 mm², demonstrating the low cell density of the tissue. In this section darkly stained protrusions were visible from all the cells. It is possible that these protrusions delineated disturbances in the organisation of the pericellular matrix produced by cellular processes. There was no evidence of the presence of notochordal cells in any section examined.

Outer annulus cells. In the annulus, the cells were typically long and thin, lying along the direction of the collagen lamellae, as described by others. Long protrusions were visible extending from the cell body parallel to the direction of the collagen fibrils (Fig. 2*b*).

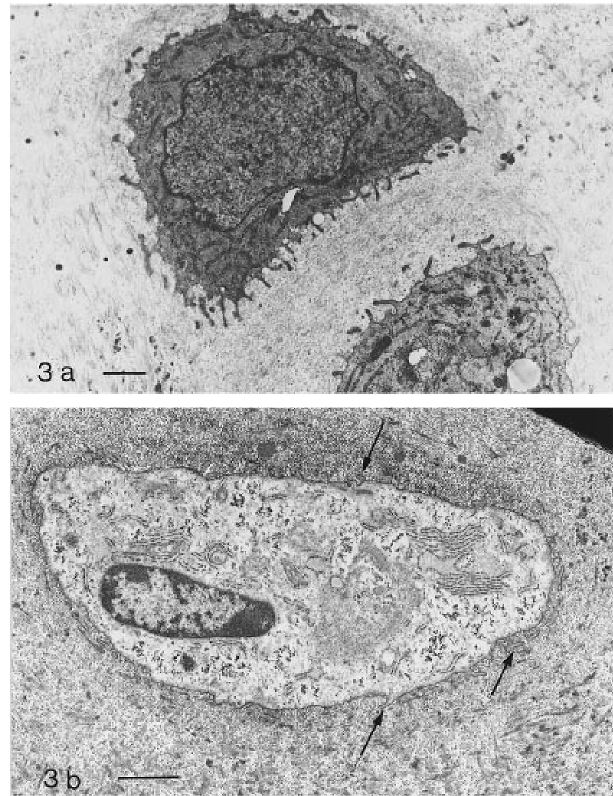

Fig. 3. Transmission electron micrographs of cells from the nucleus pulposus. (*a*) Pair of nucleus pulposus cells showing multiple processes; (*b*) Nucleus pulposus cell: processes indicated by arrows. Bars, 1 μm.

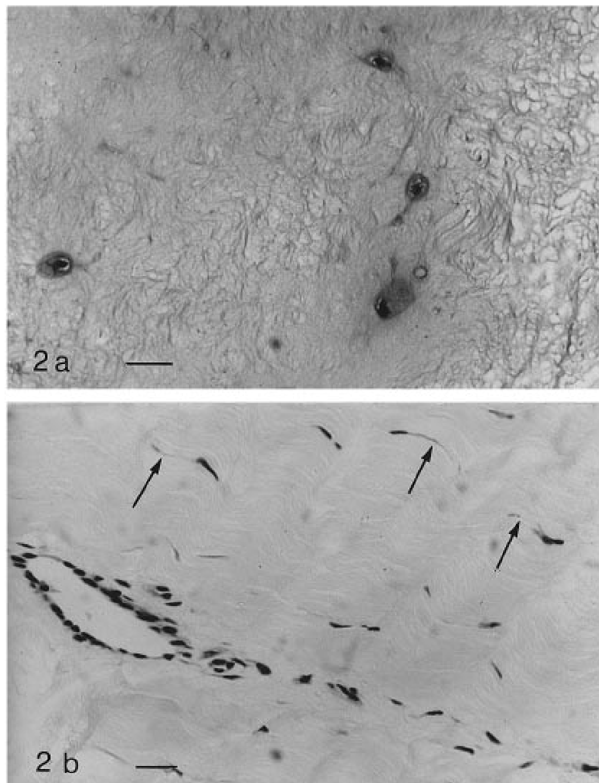

Fig. 2. (*a*) Light microscopic view of nucleus cells, wax-embedded and toluidine blue stained. Bar, 10 μm. (*b*) Light microscopic view of outer annulus cells; small blood vessels are also visible; wax-embedded and H & E stained. Arrows point towards possible processes. Bar 10 μm.

Transmission electron microscopy

Nucleus cells. Cells from the nucleus viewed using transmission EM had a chondrocyte-like appearance and were surrounded by extracellular matrix. The pericellular matrix was denser and appeared more organised than the matrix further from the cell (Fig. 3*a*, *b*). These cells thus appeared surrounded by a capsule as described previously at the light microscope level (Roberts et al. 1991). Although the cells had a chondrocyte-like appearance, numerous processes were visible projecting from the plasma membrane in the plane of the section (Fig. 3*a*). These processes do not appear to be an artifact arising from cell shrinkage as extracellular matrix appears to adhere closely to the cell boundary. Processes were not always so easily seen. Figure 3*b* shows another cell from the nucleus where processes were not evident unless the section was carefully examined. Short processes can be seen, and also sections of processes detached from the membrane around the cell periphery.

Outer annulus cells. Cells from the outer annulus were mainly elongated, running in the direction of the collagen fibres (Fig. 4*a*). The cells were heavily vacuolated and processes were readily seen extending

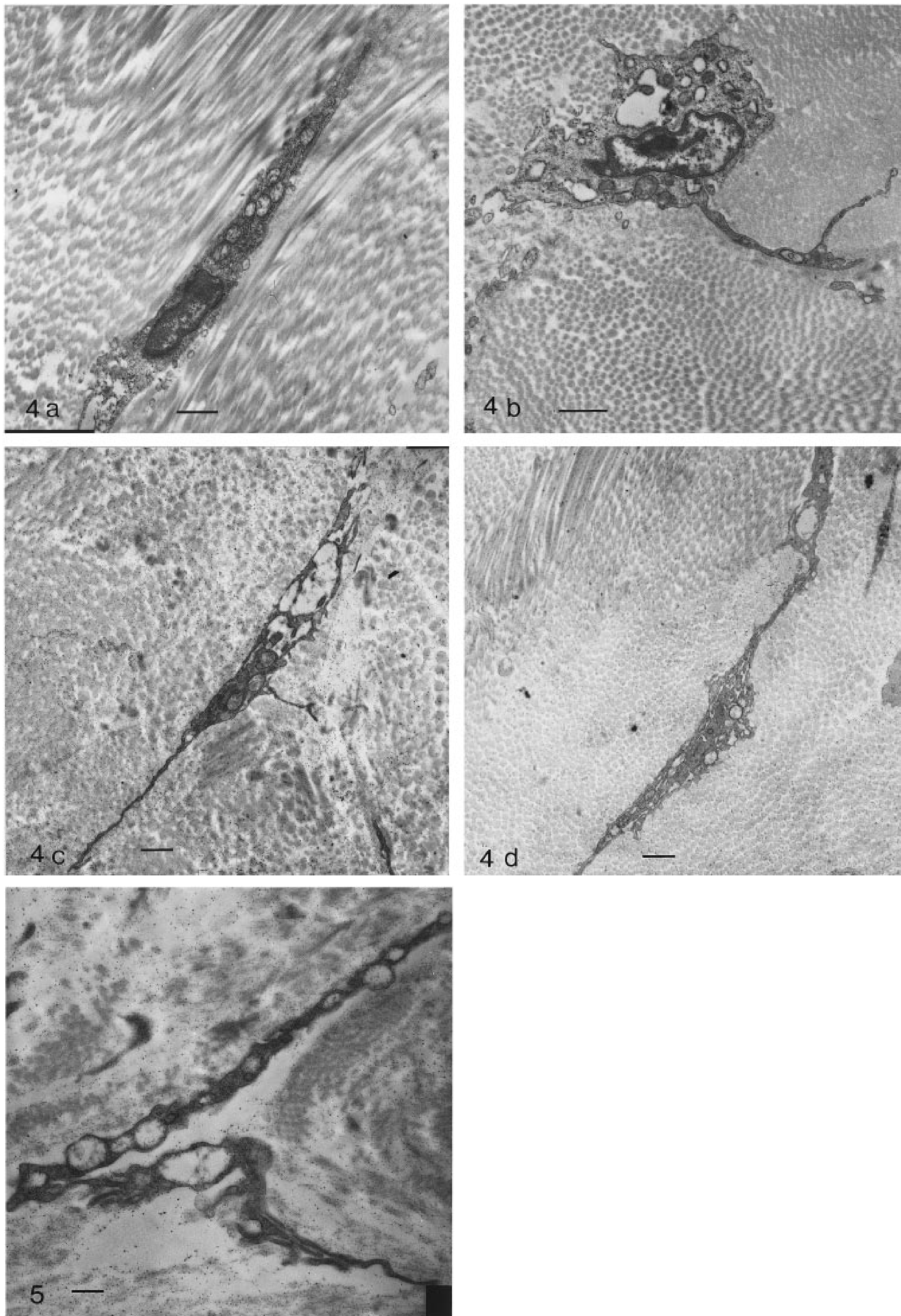

Fig. 4. Transmission electron micrographs of cells from the outer annulus. (*a*) Cross section through an elongated annulus cell stretched along collagen fibrils. (*b*) Cross section through a rounded annulus cell showing thick processes extending into the matrix. (*c*) Elongated cell from the outer annulus showing processes running both along the long axis of the cell and also a process at right angles to it. (*d*) Pair of outer annulus cells showing a possible junction. Bars, 1 μm.

Fig. 5. Transmission electron micrograph showing details of processes. Bar, 0.1 μm.

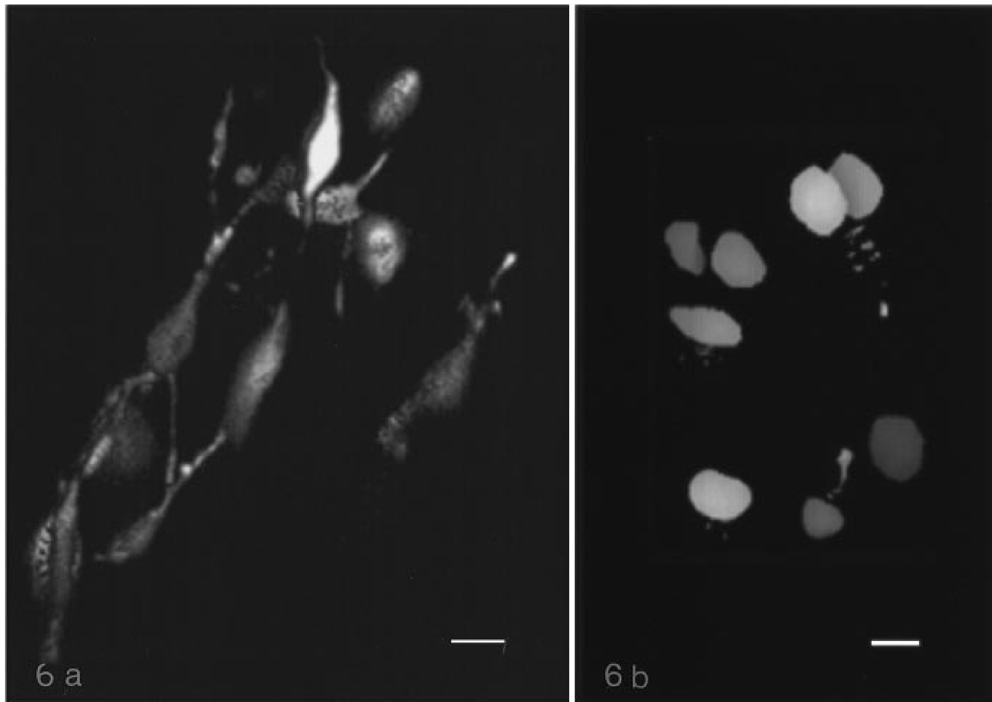

Fig. 6. Confocal images of cells from the nucleus pulposus. (*a*) Low magnification of elongated living nucleus cells. (*b*) Low magnification of chondrocyte-like living nucleus cells. Bars, 10 µm.

along the long axis between cells. In addition, a second type of annulus cell, much rarer than the elongated type, was seen (Fig. 4*b*). This cell type was also heavily vacuolated, but was more rounded, with thick processes; these also contained vacuoles. The diameter of these processes was large in relation to the diameter of the adjacent collagen fibrils. From the elongated cells, processes extended both in the direction of the cell body and into the matrix as shown in Figure 4*c*, where a process extends perpendicular to the long axis of the cell. Figure 4*d* shows 2 cells with processes extending towards each other and possibly forming a cellular junction. At higher magnification, the extensive vacuolation of the processes was clearly visible. Figure 5 shows such processes running between collagen bundles. One of the processes appears to end in a splayed-out foot.

Confocal laser scanning microscopy

Nucleus cells. Low magnification views of sections of the unfixed, living nucleus, incubated in CMFDA showed cells located in clumps within the extracellular matrix (Fig. 6*a*, *b*); large regions of the matrix were acellular. In general, the cells could be classified into 2 types. The first (Fig. 6*a*) consisted of cells with long and short multiple cytoplasm-containing processes; these cells had a slightly elongated cellular body. The second type (Fig. 6*b*) were cells of more chondrocyte-like appearance usually with 1, but with no more than 2, very short (20% length) stumpy processes. The short processes were either directed towards another cell or randomly into the matrix.

Figure 7 shows a typical assortment of cells from the nucleus at higher magnification. Figure 7*a*, *b* shows cells with multiple thin extensions. Figure 7*c*–*e* shows elongated cells with short globular extensions. Figure 7*f* shows cells of chondrocyte-like appearance, displaying short extensions.

Inner annulus cells. Cells of the inner annulus, visualised using CMFDA, mostly had rounded cell bodies and were chondrocyte-like except that they invariably had cytoplasm-filled processes. These, in general, were shorter and fatter than those seen in the nucleus cells (Fig. 8*a*, *b*).

Outer annulus cells. The outer annulus cells, fixed and stained with rhodamine phalloidin and examined by confocal laser scanning microscopy, were mostly elongated in the direction of the collagen fibrils with processes extending both along the major cell axis and normal to it (Fig. 9). Figure 10*a* shows a stereo pair of a typical elongated cell with several processes extending in different planes from the cell body. The punctate nature of the actin filament staining is evident. The more rounded cell type visible at EM level was also seen. Figure 10*b* shows a more rounded cell with much longer processes extending in several directions.

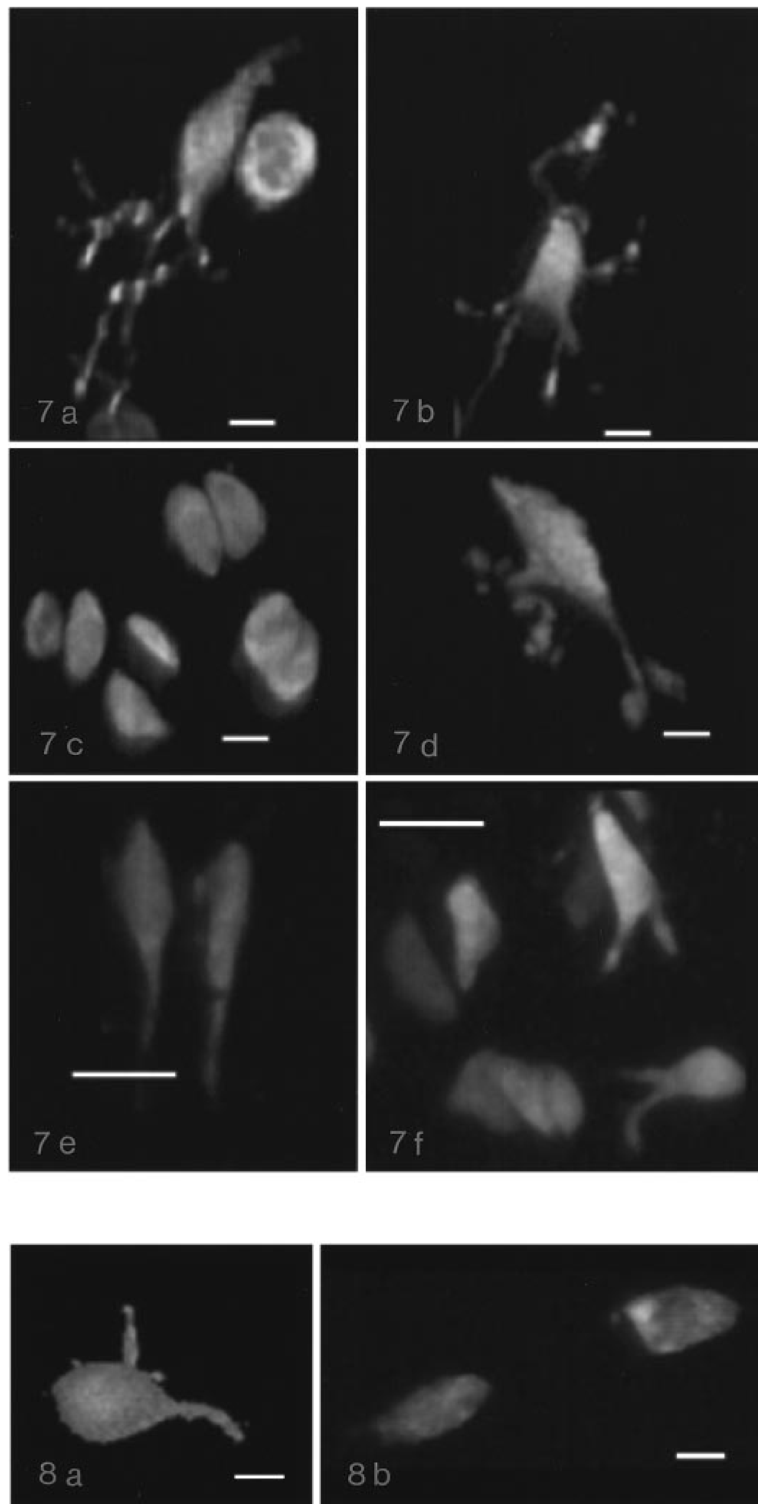

Fig. 7. Higher magnification confocal images of living nucleus cells showing the different shapes visible. (*a*, *b*) Cells with multiple thin extensions. (*c*, *d*, *e*) Elongated cells with short globular extensions; (*f*) Chondrocyte-like cells, some in doublets with short fine extensions. Bars, 5 μm.

Fig. 8. (*a*, *b*) Confocal images of living inner annulus cells. Bars, 5 μm.

DISCUSSION

The results presented here show that cytoplasm-filled processes are a feature of cells from all regions of the bovine intervertebral disc. They were clearly visible in the nucleus and inner annulus using CLSM of living cells. Here a 3-D image of the whole cell was reconstructed from 1 μm sections. It was, however, not possible to image living cells from the outer annulus using CLSM because of the opacity of the

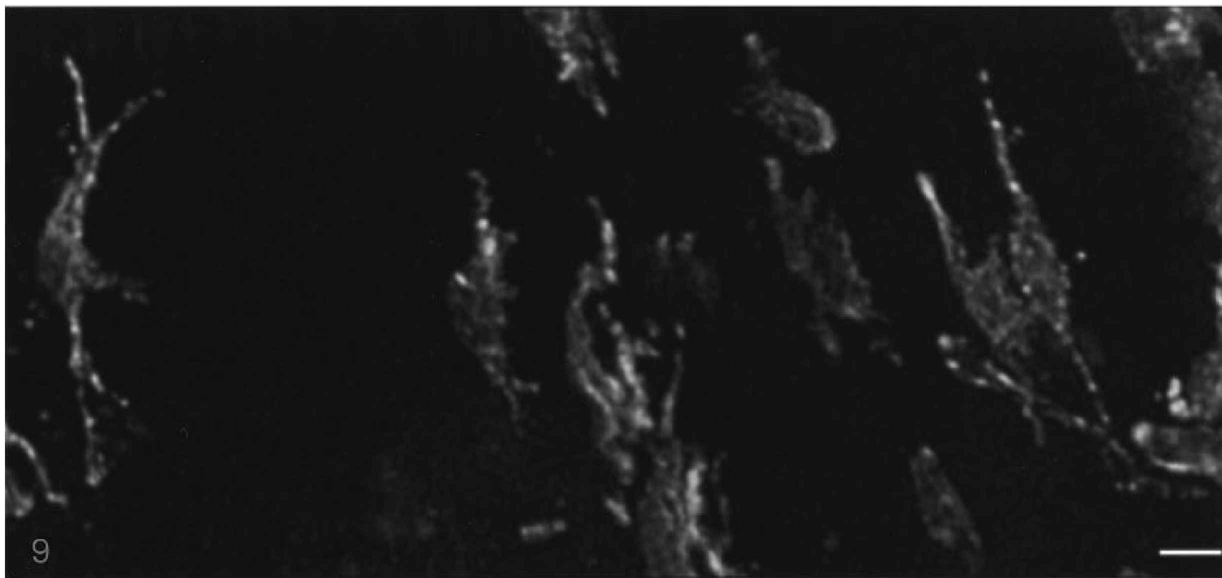

Fig. 9. Confocal images of outer annulus cells, fixed and stained with rhodamine-phalloidin, showing their orientation. Bar, 10 μm.

dense collagenous matrix. In this region of the disc, we used CLSM with rhodamine-phalloidin to visualise the 3-D actin network. This technique indicated that the outer-annulus cells also had extensive processes.

In addition, processes were visible in all regions using light and electron microscopy (Figs 2–4). However with these conventional techniques they were not readily apparent in many sections of the nucleus (Fig. 3*b*) or annulus (Fig. 2*b*) and were only easily visible when the section bisected the long axis of the process (Figs 2*a*, 4*c*).

The organisation of these processes was distinct in cells from the different regions of the disc. The nucleus and inner annulus cells were rounded and separated by an extensive extracellular matrix and appeared chondrocyte-like, not notochordal. Processes were visible on all cells, penetrating some distance into the matrix especially in the nucleus. The processes appeared to extend towards neighbouring cells in the nucleus (Figs 2*a*, 3*a*, 6*a*, *b*) and annulus (Fig. 4*d*). We have not yet investigated whether gap junctions were present by use of specific antibodies. The processes on the inner annulus were in general shorter and thinner than those of the nucleus or outer annulus (Fig. 8*a*, *b*). In the outer annulus most cells were elongated, running in the direction of the collagen fibres. Processes were seen extending from and in the direction of the long axis of the cell. These processes possibly form junctions with neighbouring cells (Fig. 4*d*) as described in tendon (McNeilly et al. 1996). Processes were also seen extending into the matrix, perpendicular to the long axis of the cell (Fig. 4*c*). Rounded and heavily vacuolated cells were seen in the outer annulus; these cells had vacuolated processes extending into the matrix (Fig. 4*b*).

There have been some earlier reports indicating the presence of cell processes on the outer and inner annulus cells but these have not been discussed in most studies of disc cells. Postacchini et al. (1984) have described long cytoplasmic projections in the inner and outer annulus cells of new-born, young and old rats. Projections have also been seen in young human nucleus cells by Trout et al. (1982), while Roberts et al. (1991) reported projections from adult human nucleus cells but thought these were an artefact of processing. With traditional microscopic imaging, the processes are not always easily seen (e.g. Figs 3*b*, 4*a*). For this reason it is possible that these features of the bovine disc cells which appear so prominent when viewed in 3-D reconstruction using CLSM, have almost been ignored until now and may be a general feature of disc cells.

The function of these processes is unknown. It is possible to speculate that they may act as mechano-transducers since a network of cytoplasm-filled processes appears to exist, similar in some respects but by no means as extensive as that produced by osteocytes in bone. The processes of the osteocytes are thought to play a major role in sensing changes in mechanically induced strain (Klein-Nulend et al. 1995). Could the processes of the disc cells act in a similar fashion? It is known that the metabolism of disc cells alters in response to applied mechanical stresses (Puustjarvi et al. 1993; Ohshima et al. 1995; Ishihara et al. 1996). In life, the disc is constantly

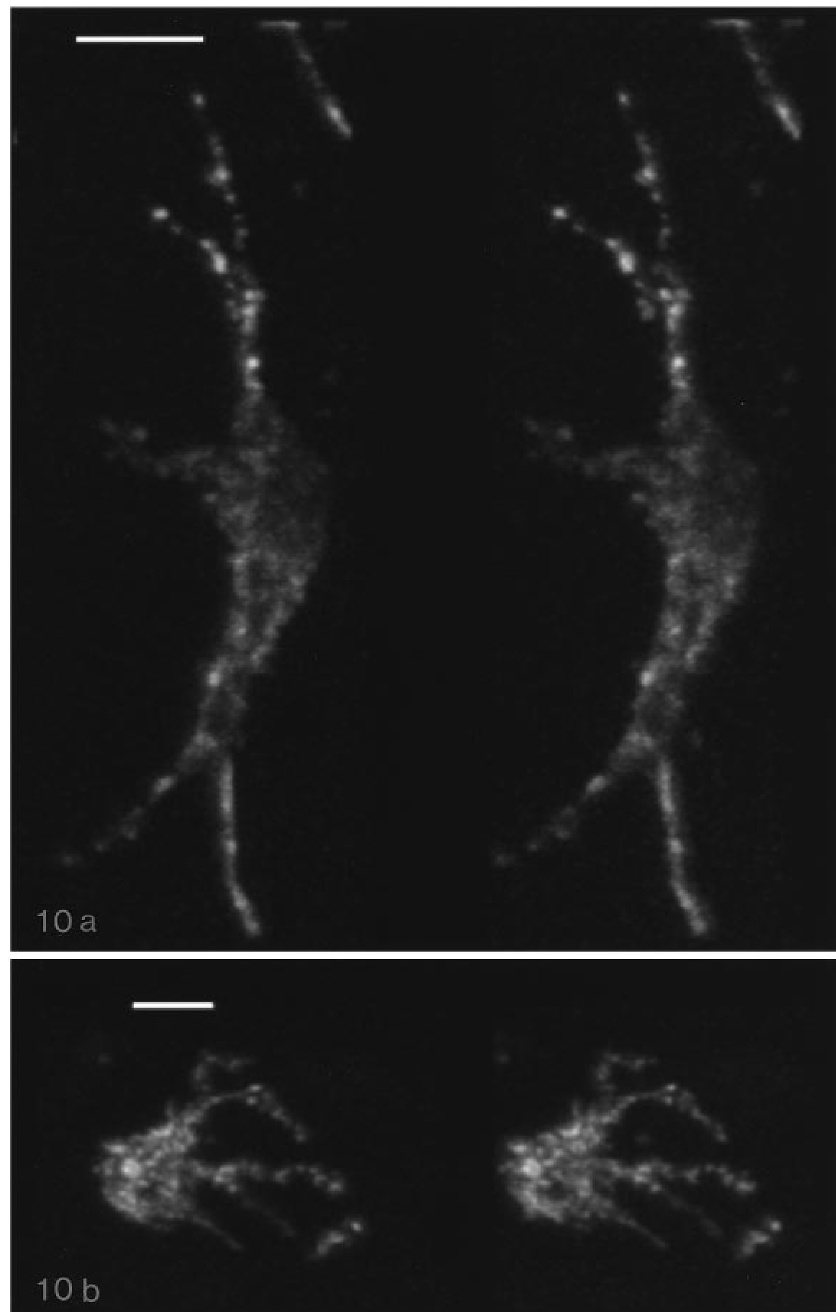

Fig. 10. Confocal images of outer annulus cells, fixed and stained with rhodamine-phalloidin. High magnification stereo-pairs of (*a*) a thin, extended, outer annulus cell type, (*b*) a rounded outer annulus cell type. Bars, 10 µm.

under mechanical loads which alter with change in posture and activity (Urban & Roberts, 1995). For adequate functioning of the tissue, the cells must be able to respond to the customary stresses by laying down and maintaining an extracellular matrix which is of appropriate composition and organisation. The complicated pattern of deformation, pressurisation and flow (Urban & Roberts, 1995) arising in the disc as the result of load changes might better be sensed by slender processes than rounded cells; their role in this response should now be investigated.

Disc cells are often described as 'chondrocyte-like' and it is assumed that their responses to environmental stimuli are similar to those of articular chondrocytes. However processes such as those seen in the disc cells are never seen in bovine, porcine, rabbit or human articular chondrocytes using CLSM (R. Errington, unpublished observations). It thus appears that, morphologically at least, and perhaps also functionally in their response to the external environment, disc cells are distinctly different from articular chondrocytes, and their behaviour cannot necessarily be directly extrapolated from that of articular cartilage chondrocytes.

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REFERENCES

Banks GM, Chelberg MK, Oegema TR (1992) Identification of heterogeneous cell populations in normal intervertebral disc. *Transactions of the American Orthopaedic Research Society* **17**, 681.

Bayliss MT, Urban JPG, Johnstone B, Holm S (1986) In vitro method for measuring synthesis rates in the intervertebral disc. *Journal of Orthopaedic Research* **4**, 10–17.

Bayliss MT, Johnstone B, O'Brien JP (1988) Proteoglycan synthesis in the intervertebral disc: variation with age, region and pathology. *Spine* **13**, 972–981.

Beadle OA (1931) *The Intervertebral Discs. Observations on their Normal and Morbid Anatomy in relation to Certain Spinal Deformities*, London: His Majesty's Stationery Office.

Buckwalter JA (1982) The fine structure of the intervertebral disc. In *Idiopathic Low Back Pain* (ed. White AA, Gordon SL), pp. 108–143. St Louis: C. V. Mosby

Buckwalter JA, Woo SL-Y, Goldberg V, Hadley EC, Booth F, Oegema TR (1983) Current concepts review: soft tissue aging and musculoskeletal function. *Journal of Bone and Joint Surgery* **75A**, 1533–1548.

Butler WF (1989) Comparative anatomy and development of the mammalian disc. In *The Biology of the Intervertebral Disc*, (ed. Ghosh P), pp. 84–108. Boca Raton: CRC Press.

Chelberg, MK, Banks GM, Oegema TR (1995) Identification of heterogeneous cell populations in normal human intervertebral disc. *Journal of Anatomy* **186**, 43–53.

Errington RJ, Fricker MD, Wood JL, Hall AC, White NS (1997) Four-dimensional imaging of living chondrocytes in cartilage using confocal microscopy: a pragmatic approach. *American Journal of Physiology* **272**, C1040–51.

Eyre DR (1979) Biochemistry of the intervertebral disc. *International Review of Connective Tissue Research* **8**, 227–290.

Eyre DR, Caterson B, Benya P, Heinegard D, Oegema TR, Pearce RH et al. (1991) The intervertebral disc. In *New Perspectives on Low Back Pain* (ed Gordon S, Frymoyer J), pp. 147–209. Philadelphia: American Institute of Orthopaedic Surgery.

Eyre DR, Muir H (1976) Types I and II collagens in intervertebral disc. Interchanging radial distributions in annulus fibrosus. *Biochemical Journal* **157**, 267–270.

Hunziker EB, Herrmann W, Schenk RK (1983) Ruthenium Hexammine Trichloride (RHT)-mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chondrocytic plasma membranes in situ during cartilage fixation. *Journal of Histochemistry and Cytochemistry* **31**, 717–727.

Ichimura K, Tsuji H, Matsui H, Makiyama N (1991) Cell culture of the intervertebral disc of rats: factors influencing culture, proteoglycan, collagen and deoxyribonucleic acid synthesis. *Journal of Spinal Disorders* **4**, 428–436.

Ishihara, H, McNally DS, Urban JPG, Hall AC (1996) Effects of hydrostatic pressure on matrix synthesis in different regions of the intervertebral disk. *Journal of Applied Physiology* **80**, 839–846.

Johnstone B, Bayliss MT (1995) The large proteoglycans of the human intervertebral disc: changes in their biosynthesis and structure with age, topography and pathology. *Spine* **20**, 674–684.

Klein-Nulend J, Van Der Plaas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ et al. (1995) Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB Journal* **9**, 441–445.

Maldonado BA, Oegema TR (1992) Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. *Journal of Orthopaedic Research* **10**, 677–690.

Maroudas A, Nachemson A, Stockwell R, Urban J (1975) Some factors involved in the nutrition of the intervertebral disc. *Journal of Anatomy* **120**, 113–130.

Martin P, Lewis J (1992) Actin cables and epidermal movement in embryonic wound healing. *Nature* **360**, 179–183.

McNeily CM, Banes AJ, Benjamin M, Ralphs JR (1996) Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions. *Journal of Anatomy* **189**, 593–600.

Oegema TR (1993) Biochemistry of the intervertebral disc. *Clinics in Sports Medicine* **12**, 419–439.

Ohshima H, Urban JPG, Bergel DH (1995) The effect of static load on matrix synthesis rates in the intervertebral disc measured in vitro by a new perfusion technique. *Journal of Orthopaedic Research* **13**, 22–29.

Peacock A (1952) Observations on the postnatal structure of the intervertebral disc in man. *Journal of Anatomy* **86**, 162–179.

Postacchini F, Bellocci MA, Massobrio M (1984) Morphologic changes in annulus fibrosus during aging. An ultrastructural study in rats. *Spine* **9**, 596–603.

Pritzker HP (1977) Aging and degeneration in the human intervertebral disc. *Orthopaedic Clinics of North America* **8**, 65–77.

Puustjarvi K, Lammi M, Kiviranta I, Helminen HJ, Tammi M (1993) Proteoglycan synthesis in canine intervertebral discs after long distance running training. *Journal of Orthopaedic Research* **11**, 738–746.

Ralphs JR, McNeilly CM, Hayes AJ, Banes AJ, Benjamin M (1996) 3D modelling of tendon cell shape in vivo by confocal microscopy. Cell-cell communication and the role of gap junctions. *Transactions of the American Orthopaedic Research Society* **21**, 1.

Roberts S, Menage J, Duance V, Wotton SF, Ayad S (1991) Collagen types around the cells of the intervertebral disc and cartilage endplate: an immunolocalization study. *Spine* **16**, 1030–1038.

Rufei A, Benjamin M, Ralphs J (1995) The development of fibrocartilage in the rat intervertebral disc. *Anatomy and Embryology* **192**, 53–62.

Schultz AB (1974) Force-deformation properties of human costosternal and costo-vertebral articulations. *Journal of Biomechanics* **7**, 311–318.

Szirmai JA (1970) Structure of the intervertebral disc. In *Chemistry and Molecular Biology of the Intercellular Matrix* (ed. Balazs EA), pp. 1279–1308. New York: Academic Press.

Taylor JR, Twomey LT (1984) The role of the notochord and blood vessels in vertebral column development and in the aetiology of Schmorl's nodes. In *Modern Manual Therapy* (ed. Grieve J), pp. 21–29. Edinburgh: Churchill Livingstone.

Trout JJ, Buckwalter JA, Moore KC (1982) Ultrastructure of the human intervertebral disc 1. Cells of the nucleus pulposus. *Anatomical Record* **204**, 307–314.

Urban J, Maroudas A (1979) Measurement of fixed charge density and partition coefficients in the intervertebral disc. *Biochimica Biophysica Acta* **586**, 202–223.

Urban JPG, Roberts S (1995) Development and degeneration of the intervertebral discs. *Molecular Medicine Today* **1**, 329–335.

Urban JPG, Roberts S (1996) The Intervertebral Disc. In *Structure and Function of the Extracellular Matrix* (ed. Comper W), pp. 203–233. Reading, UK: Gordon and Breach.

Walmsley, R. (1953) The development and growth of the intervertebral disc. *Edinburgh Medical Journal* **60**, 341–364.