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REGULAR ARTICLE

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Phenotypic characteristics of the nucleus pulposus: expression of hypoxia inducing factor-1, glucose transporter-1 and MMP-2

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Abstract Attempts to study the biology of the nucleus pulposus have been limited in scope due to the low rates of cell proliferation, difficulties in maintaining viable disc cells in culture and the absence of a clearly defined phenotype. The major objective of this communication is to construct a phenotypic signature for cells of the nucleus pulposus that is based on the hypothesis that in response to restriction on oxygen and nutrient flux, there is expression of HIF-1, GLUT-1 and MMP-2. Nucleus pulposus, as well as annulus fibrosus and cartilage of the vertebral end plates, was collected from rat spinal units. Western blot analysis and immunohistochemistry clearly showed that there was a significant level of expression of the HIF-1β isoform in the nucleus pulposus; HIF-1β was present at lower levels in cells of the annulus and the end plate. In contrast to HIF-1β, HIF-1α was expressed only in the nucleus pulposus. This isoform was absent from both the cartilage end plate and annulus. We detected HIF-1α immunohistochemically in the nucleus pulposus; however, the staining was light and diffuse. Cells of the nucleus pulposus expressed GLUT-1; in contrast, when probed by Western blot analysis the annulus and cartilage were negative for this protein. Western blot analysis also showed that in the nucleus pulposus the level of MMP-2 was high when compared to the adjacent tissues. We suggest that the differential expression of the two HIF isoforms, and GLUT-1 and MMP-2, provides a phenotypic signature that permits cells of the nucleus pulposus to be distinguished from neighboring tissues. Moreover, the presence of these isoforms provides evidence that cells of the disc respond to hypoxia and nutrient stress by upregulating stress-responsive genes.

Keywords Nucleus pulposus · Intervertebral disc · HIF · MMP-2 · GLUT-1| · Rat

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Introduction

Degenerative disc disease is one of the leading musculoskeletal disorders confronting the health system. It is of widespread prevalence, causing considerable pain and suffering, and if left untreated results in permanent dysfunction. The etiology of the disease process is not fully understood, although histopathological observations indicate that degenerative changes in the nucleus pulposus as well as the surrounding annulus fibrosus may occur early on in the disease process.

Attempts to study the biology of the nucleus pulposus cells have been limited in scope. This is partly due to the low rates of cell proliferation, difficulties in maintaining viable disc cells for long time periods and the absence of a clearly defined phenotype (Ishihara et al. 1997; Poiraudeau et al. 1999). Those studies that have been performed show that cells of the nucleus pulposus express a number of major collagen species (type I, II, III, V, VI, IX) (Nerlich et al. 1997); there is even a report to indicate that a highly specific cartilage collagen species (type X) (Boos et al. 1997) is present in nucleus pulposus cells. Other macromolecules synthesized by disc cells include aggrecan (Johnstone and Bayliss 1995; Sztrolovics et al. 1997), chondroitin sulfate or keratan sulfate (Roberts et al. 1994; Krajickova et al. 1995), hyaluronan and the hyaluronan binding receptor CD44 (Gan et al. 2000; Stevens et al. 2000). Treated with TGFβ, the cells express biglycan and some decorin

(Thompson et al. 1991; Johnstone et al. 1993; Scott et al. 1995; Gotz et al. 1997).

Together, the documented proteins and proteoglycans of the disc provide an indistinct phenotype profile (Chelberg et al. 1995). Many of the collagens are present in other connective tissues and the proteoglycans are ubiquitous. More importantly, cells of the annulus fibrosus and the cartilage end plates (tissues that surround the nucleus pulposus) express similar macromolecular profiles (Nerlich et al. 1997; Sztrolovics et al. 1997; Roberts et al. 1994; Scott et al. 1994). This finding has led some authorities to label the cells as chondrogenic, thereby assuming that specific phenotypic markers do not exist.

The major objective of this communication is to construct a phenotypic signature for cells of the nucleus pulposus that is based on the predicted physiological status of the tissue in vivo. We argue that for survival and growth in the avascular confines of the disc, in response to severe restriction on oxygen and nutrient flux, the nucleus pulposus cells must express a unique set of genes. Using Western blot and immunohistochemical analysis, we show that cells of the nucleus pulposus express hypoxia inducing factor-1 (HIF-1), glucose transporter-1 (GLUT-1) and matrix metalloproteinase-2 (MMP-2). The raised level of expression of these proteins provides a molecular profile that can be used to characterize disc cells and distinguish them from cells of the surrounding tissues.

Materials and methods

Design of the study

The major hypothesis to be tested is that in response to the low vascularity of the disc, cells of the nucleus pulposus preferentially express HIF-1, GLUT-1 and MMP-2. Nucleus pulposus, as well as annulus fibrosus and cartilage of the vertebral end plates, were collected from rat spinal units and proteins extracted. Since the cartilaginous and ligamentous tissues are dense, we used a Triton extraction procedure to efficiently solubilize cellular proteins. All of the three tissues were probed by Western blot analysis for both HIF-1α and HIF-1β, GLUT-1 and MMP-2; we used aggrecan, which is expressed in large amounts by nucleus pulposus cells as a positive control. In addition, HIF-1 and MMP-2 expression was examined immunohistochemically in order to localize the expression of the antigens. All the experiments were repeated at least 3 times.

Isolation of nucleus pulposus and surrounding tissues

Adult rats approximately 8–10 weeks of age weighing between 180 and 200 g were used for this study. Immediately after sacrifice, the spine was isolated and ribs and other adherent structures were removed with rongeurs. Disc units (the intervertebral disc and adjacent vertebrae) from the mid-thoracic to the lumbar region of the spine were obtained under aseptic conditions. Adherent ligamentous tissue from the annulus and the vertebral bone fragments from the cartilage end plate were removed from the complete intervertebral discs. For immunohistochemistry, disc units were fixed in 4% formalin in phosphate-buffered saline (PBS) for 3–4 days.

Disc cell collection

The disc unit was immersed in calcium- and magnesium-free Hanks' buffered salt solution (HBSS), pH 7.4, supplemented with 80 mM NaCl (Ishihara et al. 1997). A transverse cut, parallel to the disc axis, was made through the superior surface of the annulus with a scalpel blade (no. 15) and the two halves of the disc were held open with fine forceps. This procedure facilitated release of contents of the disc into the high osmolality medium. This extract contained both the nucleus pulposus and the transitional zone. The cells were centrifuged at 2,500 rpm for 10 min. The supernatant was removed and the cell pellet collected. To isolate the annulus and the cartilage end plates, the discs were then transferred to a second dish containing HBSS. The adhering annulus tissue and the cartilage end plate were then removed. In practice, only about two-thirds of the middle portion of the annulus was isolated. Likewise, small pieces of tissue from the central translucent region of the end plate were harvested. The end plate tissue fragments, the annulus, and the nucleus pulposus were suspended in 0.1% Triton-X 100 in PBS (v/v) using the method described by Teixeira et al. (1995). To prevent denaturation, the extraction medium was supplemented with phenylmethylsulfonyl fluoride (0.5 µM), leupeptin (1 µg/ml), pepstatin (1 µg/ml) and aprotinin (1 µg/ml). The extracts were polytron homogenized and then sonicated. Samples were stored at –80°C until analyzed.

Western blotting

Extracts of the nucleus pulposus, annulus and the cartilage end plates were isolated from the disc units as described above. Equal amounts of protein were electrophoresed on sodium dodecyl sulfate (SDS) polyacrylamide gels (6% for aggrecan, 10% for GLUT-1, and 12% for HIF-1 subunits and MMP-2). For aggrecan, samples were incubated with 0.1 unit chondroitinase ABC (Sigma Chemical Co., St. Louis, MO), in 50 mM TRIS acetate, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6, for 1 h at 37°C (Lark et al. 1995). To ensure equal amounts of loading and transfer, gels were stained with Ponceau red. Actin was also used as internal loading control to normalize the expression. The protein bands were transferred to a nitrocellulose membrane, blocked and treated with primary antibodies to aggrecan (1:2,500), actin (1:1,000), HIF-1α (1:100) and HIF-1β (1:200) (Novus Biologicals, Littleton, CO) and MMP-2 (1:200) (Chemicon Internationals Inc., Temecula, CA) and GLUT1 (1:200) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blots were incubated with the peroxidase-labeled secondary antibody, and the protein bands were detected using the light emitting ECL Western blotting detection systems (Amersham Pharmacia Biotech, Piscatway, NJ). Protein was measured using the DC protein assay (Biorad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Immunohistochemistry

Following tissue fixation, disc units were embedded in paraffin. Transverse and coronal sections 8–10 µm in thickness were then prepared. The sections were deparaffinized in xylene, rehydrated through graded ethanols. For aggrecan, samples were incubated with the primary antibody in a 1% bovine serum albumin in PBS at a dilution of 1:100 at 4°C overnight. The antibody was raised in rabbit against the peptide sequence from residues 24–151 of the mouse aggrecan core protein precursor (Walcz et al. 1994). After thoroughly washing the sections, the bound primary antibody was incubated with horseradish-peroxidase-conjugated anti-rabbit goat secondary antibody, at a dilution of 1:500 (Boehringer Mannheim Indianapolis, IN) for 1 h at room temperature. For HIF-1 and MMP-2, the sections were first treated with 1 unit hyaluronidase for 1 h at 37°C. The sections were washed with PBS and then were incubated with primary antibodies of HIF-1α (1:10) and β (1:20) (Novus Biologicals, Littleton, CO) and MMP-2 (1:50) (Chemicon Internationals Inc., Temecula, CA) overnight at 4°C in

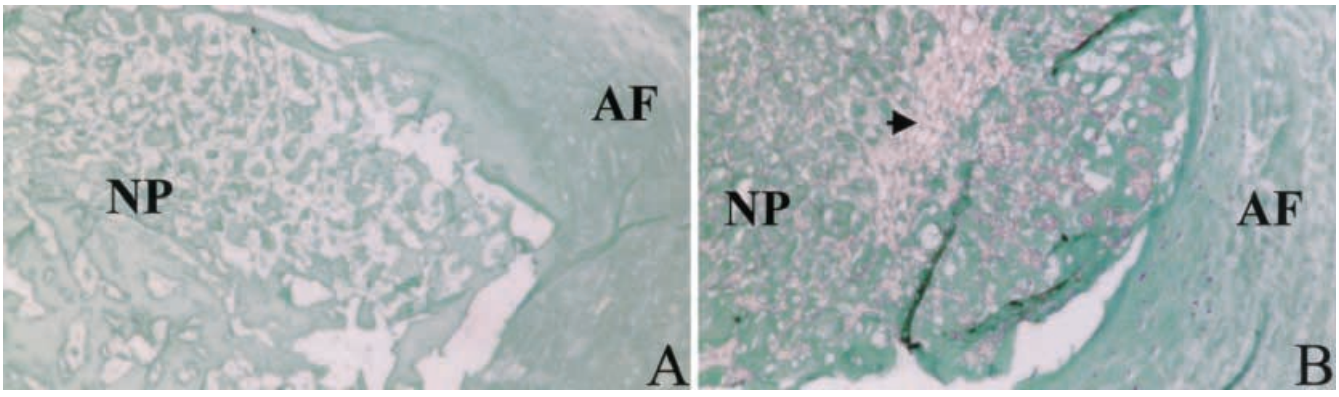

Fig. 1A, B Distribution of aggrecan in the intervertebral disc. A transverse tissue section (8–10 μm) of a rat lumbar intervertebral disc was treated with an antibody to aggrecan core protein and detected with horseradish peroxidase staining. The section was counterstained with Alcian blue. **A** Control (no primary antibody); **B** aggrecan. The central region of the disc is occupied by the nucleus pulposus (*NP*), which is surrounded by the ligamentous annulus fibrosus (*AF*). In this plane, the end plates are not visible. Note aggrecan is present in cells as well as the extracellular matrix (*arrow*) of the nucleus pulposus. In the inner region of the annulus fibrosus, cells and extracellular matrix are weakly stained (*arrow*). ×20

PBS containing 1% bovine serum albumin as a blocking agent. The samples with the bound primary antibodies were washed and then treated with peroxidase labeled secondary antibodies at a dilution of 1:100 for 2 h at room temperature. Color development was achieved using diaminobenzidine-H_2O_2. Tissues were counterstained with Alcian blue, mounted in Permount and viewed by light microscopy.

Results

Figure 1 shows a transverse section of the rat lumbar disc counterstained with Alcian blue. The nucleus pulposus occupies the center of the section and is surrounded by concentric lamellae of the annulus fibrosus (in this orientation, the end plate is not visible). Since aggrecan is known to be present in the nucleus pulposus and the surrounding tissues of the intervertebral disc, we immunostained this section of the disc with an antibody to this proteoglycan. Since both cells and matrix of the nucleus pulposus react positively for aggrecan, the cell outlines appear to be somewhat indistinct. There is also some weak staining of the matrix of the annulus fibrosus; most of the stain is localized to the fibroblasts and extracellular matrix of the inner annulus.

Sagittal sections through discs stained with antibodies to HIF-1β and MMP-2 are shown in Fig. 2. Nucleus pulposus cells react strongly with the HIF-1β antibody (Fig. 2B), although the staining is diffuse. In comparison, there is a low level of staining in the annulus fibrosus; of those cells stained most are in the inner one-third of the tissue. In the end plate, only the hypertrophic chondrocytes were HIF-1β positive (faintly visible). We also evaluated the presence of HIF-1α staining in disc cells. The cells displayed very low levels of stain in all the regions of the intervertebral disc (not shown). Similarly, MMP-2 staining is limited to the nucleus pulposus (Fig. 2C). Very little staining is detectable either in the annulus or in the end plate cartilage.

We performed Western blot analyses of protein extracted from the nucleus pulposus, the annulus and the end plate cartilage. We first examined the extracts for the presence of aggrecan. Figure 3 shows that a band of about 230 kDa is present in the nucleus pulposus extracts. In contrast, very low levels of aggrecan are seen in the extracts of the annulus and end plate cells. Western blot analysis of HIF-1α and -1β is indicated in Fig. 4. A band corresponding to HIF-1α (about 110 kDa) is present in the nucleus pulposus. Neither the annulus fibrosus nor the end plate cells express this isoform (Fig. 4A). In contrast, all the tissues express HIF-1β (92 kDa, Fig. 4). HIF-1β is significantly higher in extracts of the nucleus pulposus compared to annulus and end plate cartilage (Fig. 4C). A small band possibly corresponding to pre-HIF-1β is evident (142 kDa), together with some lower molecular weight fragments (75–95 kDa).

We also examined the expression of the glucose transporter GLUT-1 isoform in the cells of the rat intervertebral disc (Fig. 5). It is apparent that only the cells of the nucleus pulposus express this 37-kDa protein; the diffuse nature of the band probably reflects low levels of the protein and the lability of the molecule. The cells of the annulus and the end plate contain very low levels of the transporter. Finally, we evaluated MMP-2 expression. Figure 6 shows a Western blot of MMP-2 in extracts of the nucleus pulposus, annulus fibrosus and cartilage end plate. It is clear that MMP-2 is strongly expressed by cells of the nucleus pulposus. A number of bands at 60, 64 and 70 kDa probably represent breakdown products of the protein. In contrast to the nucleus pulposus, low levels of the enzyme are present in annulus and the end plate cells.

Discussion

Previous studies of cells of the nucleus pulposus have indicated that the tissue is chondrogenic. In other words, its phenotypic profile is similar to that of cartilage (Boos et al. 1997; Nerlich et al. 1997). For this reason, efforts have been directed at defining the tissue, using selected

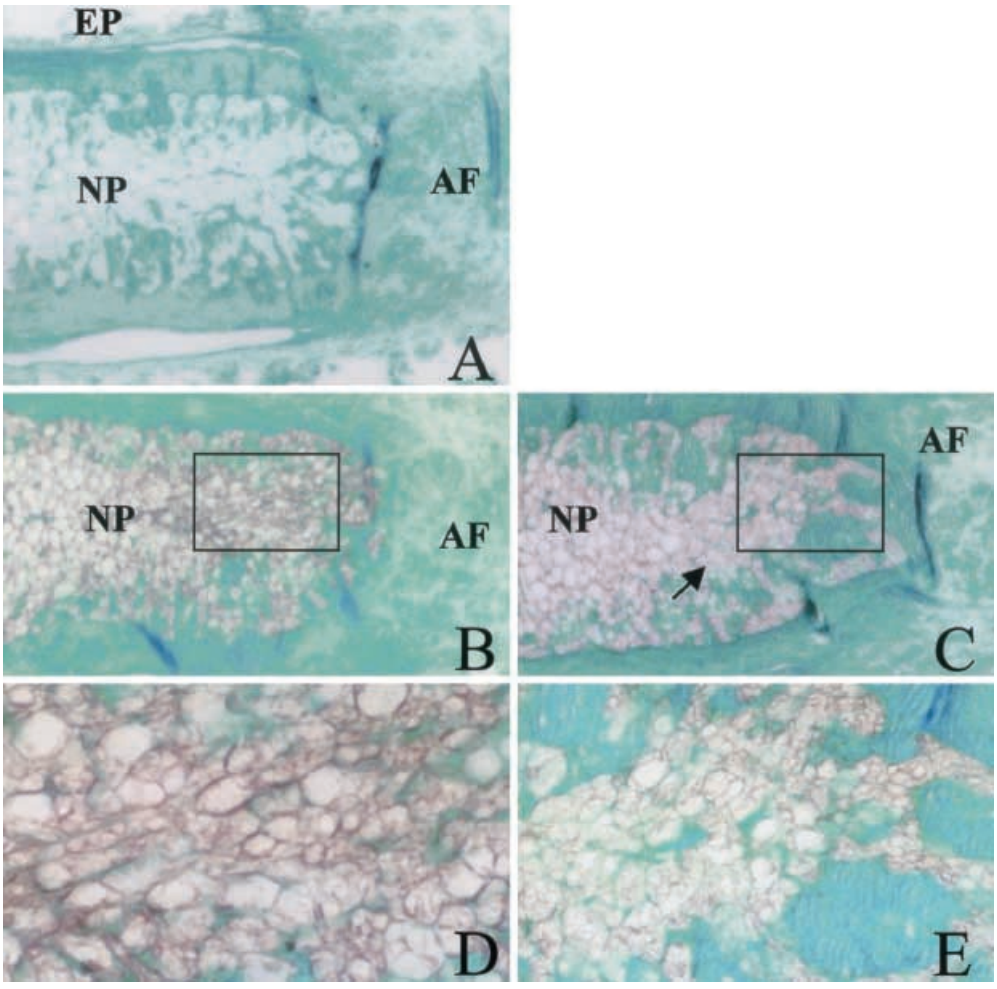

Fig. 2A–E Immunohistochemical localization of HIF-1 and MMP-2 in the rat intervertebral disc. A sagittal tissue section of rat thoracic intervertebral disc was treated with hyaluronidase prior to being stained with antibodies to HIF-1β and MMP-2. Sections were counterstained with Alcian blue. **A** Control; **B** HIF-1β; **C** MMP-2; **D** and **E** are high-power views of the *box* regions shown in **B** and **C** respectively. Note cells in the nucleus pulposus (*NP*) are lightly and diffusely stained by both the antibodies (*arrow*). Very little staining is visible in the annulus fibrosus (*AF*) or the cartilage end plate. ×20 **(A–C)**, ×200 **(D, E)**

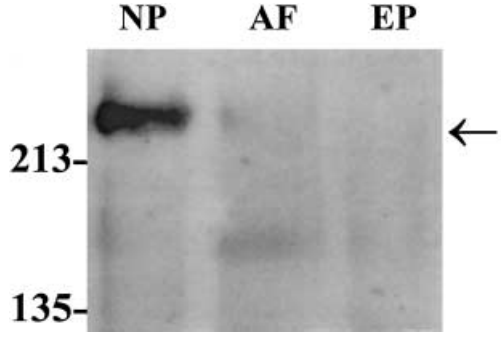

Fig. 3 Western blot analysis for aggrecan. Extracts of freshly isolated nucleus pulposus, annulus fibrosus and cartilage end plate were analyzed for aggrecan. Equal amounts of protein (20 µg), treated with chondroitinase ABC for 1 h at 37°C, were loaded onto 6% SDS-PAGE. The protein bands were transferred to nitrocellulose, incubated with an aggrecan antibody and detected by chemiluminescence. Note the presence of a protein band with a molecular weight of about 230 kDa. This probably corresponds to the partially deglycosylated core protein. Low levels of the protein were detected in the annulus fibrosus and the endplate (*NP* nucleus pulposus, *AF* annulus fibrosus, *EP* cartilage end plate)

cartilage transcripts, rather than determining if nucleus pulposus cells expressed a unique phenotypic profile. Instead of focusing on cartilage, we considered the physiology of the tissue, especially its relationship to the vascular supply. Western blot analysis of the nucleus pulposus clearly showed that there was a significant level of expression of the HIF-1β isoform in the nucleus pulposus; HIF-1β was present at lower levels in cells of the annulus and the end plate. In contrast to HIF-1β, HIF-1α was expressed only in the nucleus pulposus. This isoform was absent from both the cartilage end plate and annulus. We suggest that the differential expression of the two HIF isoforms (together with use of the other markers discussed below) provides a phenotypic signature that permits cells of the nucleus pulposus to be distinguished from neighboring tissues.

In retrospect, the observation that HIF-1α was expressed in the nucleus pulposus was not surprising as one characteristic feature of this tissue is its avascular structure (Amonoo-Koufi 1991). The low number of blood vessels would be expected to impose a hypoxic state on cells of the nucleus pulposus, and that state is enhanced by the low vascularity of the surrounding annulus fibrosus and the superficial layers of the end plate cartilage (Holm et al. 1981). Bartels et al. (1998) report-

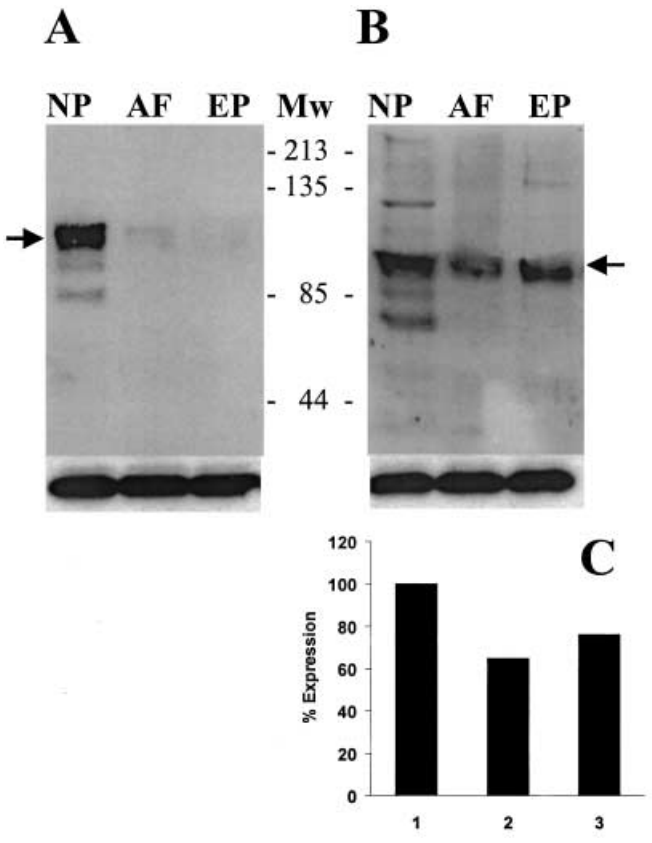

Fig. 4 Western blot analysis of **A** HIF-1α and **B** HIF-1β subunits in the intervertebral disc. Equal amounts of protein (20 μg) were electrophoresed on 10% SDS-PAGE. The protein bands were transferred to a nitrocellulose membrane and reacted with the primary antibody and detected by chemiluminescence. The molecular size of HIF-1α subunit is close to 110 kDa, and the molecular weight of HIF-1β subunit is 92 kDa. Note both HIF-1α and -1β are present in the nucleus pulposus only. In contrast, HIF-1β is present in all three tissues. Western blot for actin, which was used as a loading control, is shown below **A** and **B**. **C** Comparison of HIF-1β levels in NP, AF and EP. The level of HIF-1β in NP (lane 1) is greater than AF (lane 2) and NP (lane 3) when normalized against β-actin (*NP* nucleus pulposus, *AF* annulus fibrosus, *EP* cartilage end plate, *Mw* molecular weight markers)

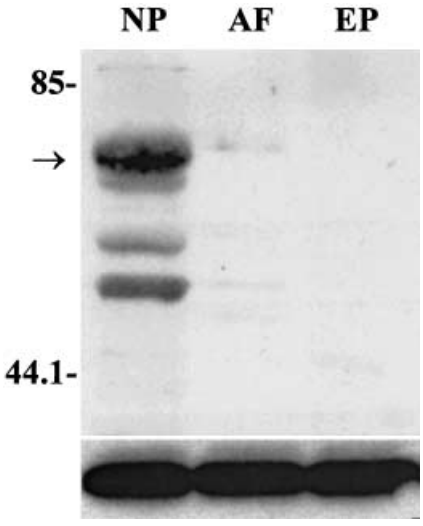

Fig. 5 Western blot analysis for the glucose transporter GLUT1 in tissues of the rat intervertebral disc. Tissue extracts of the nucleus pulposus (*NP*), annulus fibrosus (*AF*) and cartilage end plate (*EP*) were analyzed for GLUT1 on 10% SDS-PAGE with actin as a loading control. Note only cells of the nucleus pulposus express the GLUT-1 protein (37 kDa)

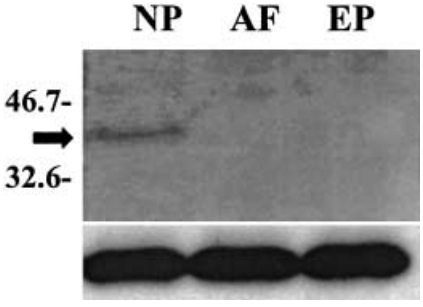

Fig. 6 Western blot analysis for MMP-2 in rat intervertebral disc. Tissue extracts were analyzed for MMP-2 on a 10% SDS-PAGE with actin as loading control. A strongly staining band is seen in the nucleus pulposus (*NP*) at 72 kDa, corresponding to MMP-2. The lower molecular weight bands at 60, 64 and 70 kDa are probably degraded MMP-2 protein (*AF* annulus fibrosus, *EP* cartilage end plate)

ed that a pO_2 gradient exists in the disc, the highest pO_2 values being seen at the periphery of the annulus and close to the end plate, and lowest in the center of the disc. In other tissues, when the O_2 concentration is low, and there is reliance on the glycolytic pathway to generate metabolic energy, there is an increased synthesis of glycolytic enzymes and an accumulation of the end products of anaerobic metabolism (Bunn and Poyton 1996; Wenger and Gassman 1997). Since the increase in glycolytic activity is mediated by HIF-1, a transcription factor that serves to transactivate hypoxia sensitive genes (Semenza et al. 1994, 1996), it was not surprising to find that there was expression of both isoforms. The HIF-1α subunit is a very short lived protein which is rapidly degraded under normoxic conditions; however, for tissues that are hypoxic, there is accumulation of the protein due to the formation of a stable heterodimer with the HIF-1β subunit (Kallio et al. 1999). When this occurs, the protein level of the HIF-1α is generally 2–5 times greater relative to the HIF-1β subunit (Jiang et al. 1996; Kallio et al. 1997). In the nucleus pulposus, the HIF-1α isoform was present, although it was not expressed by either the annulus or the resting cartilage cells. This observation was unexpected and as such it provides a novel and unique marker of the nucleus pulposus.

Although the Western blot analyses provided unambiguous evidence that HIF-1α and -1β were present in the nucleus pulposus, the results of the immunohistochemical studies were not clear cut. We detected HIF-1α in the nucleus pulposus; however, the staining was light and diffuse. The most plausible explanation for the low signal level was the lability of the protein and inaccessibility of the antigenic epitopes of the HIF-1α protein.

Another possibility is that in the intact tissue the osmolarity is very high and the cells are under hydrostatic pressure. Thus, the fixative would enter the tissue slowly and release of pulposus pressure would serve to enhance cell damage and reduce nuclear staining.

We also evaluated a second phenotypic marker, the glucose transporter protein GLUT-1. We chose to evaluate this isoform as it is sensitive to hypoxia and its activity is elevated when there is an increase in HIF expression (Sun et al. 1994; Brosius et al. 1997). We noted that cells of the nucleus pulposus expressed this protein; in contrast, the annulus and cartilage failed to evidence this protein when probed by Western blot analysis. These results suggest that the nucleus pulposus responds to hypoxia by increasing glucose uptake into the tissue, thereby enhancing glycolysis and preventing ischemia-induced injury. Aside from providing additional support for the view that cells in the nucleus pulposus are committed to the glycolytic pathway, probably in response to the low O_2 tension, the finding that the cells express this transporter provides yet another useful phenotypic marker that along with the HIF-1 can be utilized to follow and characterize the cells of the rat spine.

While HIF-1 and GLUT relate to the unique metabolism of the nucleus pulposus, expression of these proteins reflects normal cell metabolism and it is likely that their activities are minimally altered in disease. For this reason, we decided to extend the investigation to include MMP-2, a protein that is known to be synthesized by nucleus pulposus cells and with MMP-9 has also been linked to hypoxia and disc disease (Krtolica and Ludlow 1996; Sedowofia et al. 1982). Crean et al. (1997) reported that both the pro- and active forms of MMP-2 and -9 were increased in disc degeneration. It was noted that there was a positive correlation between the activities of the MMPs and the severity of the disease. Again, Western blot analysis showed that the level of MMP-2 in the nucleus pulposus was high when compared to the other adjacent tissues. The relatively low level of MMP-2 in the annulus was somewhat surprising, although it is likely that activity levels may rise when the tissue is stressed. Nevertheless, with respect to normal cells, the fact that there is a substantive difference in the level of the MMP-2 protein between the nucleus pulposus and the surrounding tissues provides a third useful phenotypic marker. The results also suggest that it may be valuable to assess other MMPs in the disc and surrounding tissues especially in relationship to remodeling of the extracellular matrix during normal development and disease.

In summary, based on the studies reported here we have delineated three novel markers of the nucleus pulposus phenotype. While these results are not in conflict with the notion that the cells of the disc are chondrogenic, they do indicate a specialized and separate phenotype from that expressed by the ligamentous cells of the annulus and the resting cells of the superficial layer of the end plate. We recognize that our results pertain to the adult rat; of course with age the possibility exists that an increasing number of cells in the annulus fibrosus and the transitional zone also may be exposed to hypoxic conditions. If this does occur, these cells may express HIF and other stress proteins; indeed the possibility exists that hypoxia may even trigger cells in the transitional zone to express the nucleus pulposus phenotype. Experiments are ongoing to learn if this type of environmental challenge causes cells of the annulus fibrosus to express these three phenotypic markers when maintained in long-term culture. Outcomes from all of these studies should facilitate and extend understandings of the growth and development of the spinal unit and possibly provide a new approach to engineering disc tissue.

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