Transforming and epidermal growth factors in degenerated intervertebral discs


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We studied the presence of anabolic growth factors in human herniated intervertebral discs (IVD) using a reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry. Messenger RNA (mRNA) was isolated from the nucleus pulposus using oligo (dT)₂₅ superparamagnetic beads and probing with gene-specific primers in RT-PCR.

mRNA coding for TGF-α (3/10), EGF (0/10), TGF-β₁ (0/10) and TGF-β₃ (2/10) or the EGF receptor (EGF-R; 0/10) and TGF-β type-II receptor (0/10) was found only occasionally. Beta-actin was always present and positive sample controls confirmed the validity of the RT-PCR assay. These RT-PCR findings were confirmed using immunohistochemical staining of EGF and TFG-β, whereas TGF-α protein was always found associated with discocytes.

We conclude that the nucleus pulposus of the herniated IVD is vulnerable to proteolytic degradation and depletion of proteoglycans due to the lack and/or low production of anabolic growth factors/receptors which could increase the local synthesis of the extracellular matrix.

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The functional unit of the lumbar spine consists of two vertebral bodies combined by a fibrocartilaginous intervertebral disc (IVD). This synchondrosis, or uncovertebral, joint allows movement in flexion and extension during which it functions as a shock absorber and as a dynamic cushion.² It is composed of a proteoglycan (PG) and water-rich nucleus (nucleus pulposus) surrounded by lamellae of collagen organised to a fibrous annulus.³,⁴ This arrangement is to a large extent responsible for the biomechanical properties of a healthy IVD.⁵ In a degenerated IVD this local homeostasis has not been maintained because of a decreased content of PG and water in the nucleus pulposus.⁶-⁸ The altered properties of the degenerating IVD increase the mechanical stress of the lumbar spine.⁹ Research has so far focused on the different catabolic factors and proteases which can degrade PG core proteins (proteoglycans).¹⁰-¹³ Less attention has been paid to the regenerative capacity of the nucleus.

An adult nucleus pulposus is an avascular tissue. Systemic regulatory factors and nutrients are delivered to the IVD cells by diffusion through the extracellular matrix, which also regulates the behaviour of discocytes by matrix-cell interactions.¹⁴,¹⁵ Another potential source of regulatory factors could be the local discocytes. The members of the transforming growth factor (TGF) super family have been shown to play an important role in the anabolic metabolism of the IVD. In mature canine IVDs, TGF-β and epidermal growth factor (EGF) elicited greater proliferative responses than other factors such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF).¹⁶ In addition, TGF can upregulate the expression of the tissue inhibitors of some metalloproteinases.¹⁷ Our aim was to assess the anabolic potential of degenerated/herniated IVDs by determining the local expression of TGF and EGF. The responsiveness of the discocytes to these soluble factors was assessed based on the demonstration of synthesis of their respective receptors.
Materials and Methods

Samples of nucleus pulposus from herniated IVDs were collected from ten patients, five men and five women with a mean age of 37.5 years (21 to 50). The clinical diagnosis had been confirmed before operation by MRI. The herniation was located between L4 and L5 in five and between L5 and S1 in five patients. The operation had been performed from the right or left depending on the side of the herniation. Fibrous tissue of the capsule of the hip, obtained at a total hip replacement performed for primary osteoarthritis, was used as a positive sample control. All the samples were frozen in dry ice precooled with isopentane immediately after removal.

For each mRNA extraction 80 to 90 tissue sections 15 µm thick were used. Aerosol-resistant pipette tips (Biohit, Helsinki, Finland) were used for pipetting hot DNA-containing solutions. The mRNA was extracted as previously described. Briefly, the procedure used oligo (dT)25 covalently attached to superparamagnetic polystyrene microbeads according to the manufacturer’s protocol (Dynal, Oslo, Norway). For each sample, 30 µl of beads (binding capacity 2 ng of poly(A)+ mRNA/µl beads) were used. They were washed with lysis/binding buffer (100 mM Tris-HCl, 2 M LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT, pH 8.0) using the magnetic particle collector supplied. The buffer (100 µl) was then added to the tissue sections and genomic DNA was sheared by passing the sample suspension 10 to 15 times through a 1 ml syringe with a 22G needle. The supernatant was incubated with the (dT)25 beads for 3 minutes at room temperature, washed twice with 200 µl of washing buffer with LiDS (10 mM Tris-HCl, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, pH 8.0), twice with washing buffer without LiDS and finally once with 0.5 RT buffer (45 mM KC1, 5 mM Tris-HCl, pH 8.3).

The rTth reverse transcription (Perkin Elmer, Branchburg, New Jersey) reaction was performed using 5 U of the enzyme in a total volume of 20 µl (90 mM KC1, 10 mM Tris-HCl, pH 8.3, 1 mM MnCl2, 200M of dATP, dCTP, dGTP and dTTP) in thin-walled PCR tubes (Plastic Trade, Helsinki, Finland) topped with 50 µl of mineral oil (Sigma, St Louis, Missouri) using a thermal cycler (Pharmacia, Sollentuna, Sweden). The reaction was run for 1 minute at +95°C to melt the cDNA:RNA hybrid. The beads containing the first strand of cDNA were immediately transferred to a new PCR tube in which were performed 35 (for β-actin) or 45 cycles (TGF-β1, TGF-β3, TGF-βII-R, TFG-α, EGF and EGF receptor (EGF-R)) of PCR reactions consisting of 1 minute of denaturation at +95°C, 1 minute of annealing at a primer-specific temperature of +60°C for TGF-β1, TGF-β3, EGF and EGF-R, +61°C for β-actin, +63°C for TGF-βII-R and TFG-α and 1 minute of extension at +72°C. For the last cycle 5 minutes of extension at +72°C were used. The beads containing the first strand of cDNA of each sample were used for all of the genes studied. Amplified DNA was run on a 1.5% modified NuSieve agarose gel (FMC BioProducts, Rockland, Maine) for verification of size. PCR fragments were extracted from the gel using silica-gel-membrane-based QIAquick columns according to the manufacturer’s protocol (Qiagen Inc, Chatsworth, California) which were quantified, sequenced (20 to 75 ng/PCR fragment) using fluorescein-labelled dye terminator kits (PE Applied Biosystems, Foster City, California) and analysed on an automatic sequencer 373 A (PE Applied Biosystems).

Frozen tissue samples were cut into sections 8 µm thick, mounted on gelatin-coated glass slides and fixed in acetone for 5 minutes at +4°C. Intrinsic peroxidase activity was abolished by pretreating the tissue sections in 0.1% H2O2 in methanol for 30 minutes. Sections were rinsed in 0.3% Triton-X in 0.1M PBS before staining using the avidin-
The biotin-peroxidase complex (ABC) method\(^{10}\) as described elsewhere.\(^{20,21}\) Briefly, serial sections were sequentially incubated with:

1) normal rabbit or horse serum (1:20; Vector Laboratory, Burlingame, California) depending on the primary antibody used for 20 minutes at +22°C;

2) primary antibodies consisting of a) polyclonal goat anti-human EGF IgG 10 µg/ml (R&D Systems, Abingdon, UK) (no cross-reactivity detected by Western blot or direct ELISA for rhTGF-α, rhIL-α, rhIL-1β, rhIL-2, rhIL-3, rhIL-4, hPDGF); b) monoclonal mouse anti-human TGF-α antibodies, diluted 1:400 (Chemicon, Temecula, Canada) (no cross-reactivity detected with EGF or TGF-β) or c) monoclonal mouse anti-human TGF-β\(_1\), β\(_2\), β\(_3\), IgG\(_1\), 20 µg/ml (Genzyme Diagnostics, Cambridge, Massachusetts) (primary antibodies were incubated overnight in a moist chamber at +4°C);

3) biotinylated rabbit anti-goat IgG or biotinylated horse anti-mouse IgG, respectively, for 1 hour at +22°C;

4) ABC complexes in 0.1M PBS (1:100; Vector Laboratory) for 1 hour at +22°C; and

5) 0.06% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemicals) and 0.006% H\(_2\)O\(_2\).

All sections were dehydrated in a graded ethanol series, cleared in xylene and mounted in Diatex. The specificity of the reactions was tested by the omission of the primary antibody, which was replaced with the corresponding normal goat serum or monoclonal mouse IgG control antibody.
of the same subtype and concentration, but with irrelevant specificity (Dakopatts a/s, Glostrup, Denmark).

Results

All samples of the herniated lumbar IVD and of the positive control fibrous capsule contained beta-actin demonstrating the successful extraction of mRNA (Figs 1 and 2). The fibrous capsule control contained TGF-α, EGF, TGF-β1, TGF-β3, TGF-α, EGF-R and TGF-β type II receptor showing that the processing of the sample, probing and amplification protocol worked to full satisfaction. The identity of the PCR amplification products was confirmed, not only by verification of size, but also by nucleotide sequencing. All the experiments were run twice and gave consistent results. Histological examination of the semi-serial sections confirmed that the samples contained chondrocyte-like discocytes embedded in amorphous extracellular matrix, whereas spindle-shaped, fibroblast-like cells embedded in fibrillar collagenous matrix were practically absent from all samples.

Regarding the herniated IVD samples, TGF-α was found in 3 out of 10 samples (Fig. 2, panel B). In addition, TGF-β3 was found in 2 out of 10 samples (Fig. 1, panel C) which, however, were not the same as those containing TGF-α mRNA (Fig. 1, panel C, Fig. 2, panel B). EGF (Fig. 2, panel C), TGF-β1 (Fig. 1, panel B), EGF-R (a receptor shared by EGF and TGF-α; Fig. 2, panel D) and TGF-β type-II receptor (Fig. 1, panel D) were not found in any of the IVD samples studied.

Immunohistochemical ABC staining of TGF-α, TGF-β (β1, β2, β3) and EGF (Fig. 3) in the positive sample control, i.e., fibrous capsule of the hip, gave positive staining results with moderate to strong staining of the fibroblast-like cells of the capsule (Figs 3C and 3D). TGF-α was also found in the discocytes (Fig. 4), but TGF-β and TGF were not found in situ in the nucleus pulposus of human IVDs (Figs 3A and 3B).

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Fig. 3

Immunohistochemical avidin-biotin-peroxidase complex (ABC) staining of TGF-β and EGF. Discocytes do not contain any TGF-β. Figure 3A – Staining with monoclonal mouse anti-human TGF-β antibodies which recognise the β1, β2 and β3 isoforms of TGF-β (×500). EGF staining gave similar results (not shown). Figure 3B – A semi-serial section showing that the field in panel A contains a chondron and individual discocytes embedded in the extracellular matrix (×500) (haematoxylin counterstain). Figs 3C and 3D – Positive sample controls stained for TGF-β and EGF, respectively. Fibrous tissue of the capsule of the hip, obtained at a total hip replacement operation performed for a primary osteoarthritis, was used as a positive sample control (×625).
the synthesis of PG, namely TGF and EGF. There have been no studies on the eventual local production of urokinase-type plasminogen activator (uPA), collagenases' (MMPs) and a low capacity of the nucleus pulposus of herniated IVD. We conclude that these two events together, namely depletion of inflammation-induced ‘proteoglycanases’/PG and a low capacity of the nucleus pulposus of herniated IVD to compensate for the PG loss, make the nucleus pulposus vulnerable. These observations, however, do not allow any conclusions with regard to healthy human disc tissue because the exposure of the nucleus pulposus to rupture may significantly influence the expression of mRNAs and the presence of growth factors in herniated IVD tissue. It is also of interest that TGF-β suppresses some MMP synthesis, but increases the synthesis of their endogenous inhibitors. Furthermore, TGF-β inhibits the production of urokinase-type plasminogen activator (uPA) which exerts a protective influence against degradation of PG. This combined effect on MMP and plasminogen systems would be particularly detrimental, because plasmin is able to activate proteolytically the proMMPs to the corresponding active enzyme species. In the nucleus pulposus the lack of an anabolic effect seems to be combined with a lack of an anticytotoxic effect.

All discocytes stained for TGF-α. There is thus an apparent discrepancy between the local expression of TGF-α mRNA (3/10) and its translation product (10/10). This might indicate a short half-life for the TGF-α mRNA and/or a long half-life for the corresponding protein. Alternatively, it is possible that exogenous TGF-α is deposited in the discocyte. Because the discocytes did not contain mRNA coding for the TGF-α receptor (=EGF-R), they would seem to be inherently non-responsive to TGF-α. TGF-α and EGF share a common receptor, EGR-R, which was not locally produced, nor was TGF-β type-II receptor. Thus, the situation prevailing in the nucleus pulposus could be analogous to that in degenerating osteoarthritic cartilage in which catabolic cytokines like IL-1, TNF-α and IL-6, are produced in excess compared with anabolic cytokines, e.g., TGF-β, bFGF and IGF. Lack or low level of expression of anabolic growth factors and their respective receptors is probably a significant risk factor predisposing to degeneration of the IVD and thus herniation. This is further augmented by the low cell-to-extracellular-matrix ratio prevailing in the nucleus pulposus, which contains only approximately 4000 to 5000 cells/mm.

Routine histological examination of the samples showed the typical morphological features of the nucleus pulposus. More or less cell-rich chondrons were found embedded in an amorphous extracellular matrix. Usually no spindle-shaped, fibroblast-like cells or fibrillar collagen were seen. This finding confirms that the samples were indeed from the nucleus pulposus and not from the annulus fibrosus. To obtain such a clear-cut dissection of these two elements of the IVDs, we used an isolation protocol for mRNA based on the use of tissue sections and superparamagnetic oligo (dT) beads. An internal standard with β-actin and a positive control using capsular tissue from the hip confirmed the validity of our methods. If there had been any production of growth factor and receptor in situ, it would have been detected by the current protocol for isolation, probing and amplification of mRNA. To a large extent, parallel results were obtained using immunohistochemistry.

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References
