Growth factor induction in intervertebral disc tissue:

Observations in basic mechanisms of disc degeneration and rearrangement

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Academic Dissertation

To be publicly discussed, by the permission of the Medical Faculty of the University of Helsinki,
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Abstract

The intervertebral disc is composed of concentrically arranged components: annulus fibrosus, the transition zone, and central nucleus pulposus. The major disc cell type differs in various parts of the intervertebral disc. In annulus fibrosus a spindle shape fibroblast like cell mainly dominates, whereas in central nucleus pulposus the more rounded chondrocyte-like disc cell is the major cell type.

At birth the intervertebral disc is well vascularized, but during childhood and adolescence blood vessels become smaller and less numerous. The adult intervertebral disc is avascular and is nourished via the cartilage endplates. On the other hand, degenerated and prolapsed intervertebral discs are again vascularized, and show many changes compared to normal discs: Including, nerve ingrowth, change in collagen turnover, and change in water content. Furthermore, the prolapsed intervertebral disc tissue has a tendency to decrease in size over time.

Growth factors are polypeptides which regulate cell growth, extracellular matrix protease activity, and vascularization. Oncoproteins c-Fos and c-Jun heterodimerize, forming the AP-1 transcription factor which is expressed in activated cells.

In this thesis the differences of growth factor expression in normal intervertebral disc, the degenerated intervertebral disc and herniated intervertebral disc were analyzed. Growth factors of particular interest were basic fibroblast growth factor (bFGF, FGF-2), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGFβ). Cell activation was visualized by the expression of the AP-1 transcription promoters c-Fos and c-Jun. The expression was shown with either mono- or polyclonal antibodies by indirect avidin-biotin-peroxidase immunohistochemical staining method. The normal control material was collected from a tissue bank of five organ donors. The degenerated disc material was from twelve patients operated on for painful degenerative disc disease, and herniated disc tissue material was obtained from 115 patients operated on for sciatica.

Normal control discs showed only TGFβ immunopositivity. All other factors studied were immunonegative in the control material. Prolapsed disc material was immunopositive for all factors studied, and this positivity was located either in the disc cells or in blood vessels. Furthermore, neovascularization was noted. Disc cell immunoreaction was shown in chondrocyte-like disc cells

or in fibroblast-like disc cells, the former being expressed especially in conglomerates (clusters of disc cells). TGFβ receptor induction was prominent in prolapsed intervertebral disc tissue.
In degenerated disc material, the expression of growth factors was analyzed in greater detail in various parts of the disc: nucleus pulposus, anterior annulus fibrosus and posterior annulus fibrosus. PDGF did not show any immunoreactivity**,** whereas all other studied growth factors were localized either in chondrocyte-like disc cells, often forming clusters, in fibroblast-like disc cells, or in small capillaries. Many of the studied degenerated discs showed tears in the posterior region of annulus fibrosus, but expression of immunopositive growth factors was detected throughout the entire disc. Furthermore, there was a difference in immunopositive cell types for different growth factors.
The main conclusion of the thesis, supported by all substudies, is the occurrence of growth factors in disc cells. They may be actively participating in a network regulating disc cell growth, proliferation, extracellular matrix turnover, and neovascularization. Chondrocyte-like disc cells, in particular, expressed growth factors and oncoproteins, highlighting the importance of this cell type in the basic pathophysiologic events regarding disc degeneration and disc rearrangement.

The thesis proposes a hypothesis for cellular remodelling in intervertebral disc tissue. In summary, the model presents an activation pattern of different growth factors at different intervertebral disc stages, mechanisms leading to neovascularization of the intervertebral disc in pathological conditions, and alteration of disc cell shape, especially in annulus fibrosus. Chondrocyte-like disc cells become more numerous, and these cells are capable of forming clusters, which appear to be regionally active within the disc. The alteration of the phenotype of disc cells expressing growth factors from fibroblast-like disc cells to chondrocyte-like cells in annulus fibrosus, and the numerous expression of growth factor expressing disc cells in nucleus pulposus, may be a key element both during pathological degeneration of the intervertebral disc, and during the healing process after trauma.

Contents Page

1. List of original publications

This thesis is based on the following publications:

I Tolonen J, Grönblad M, Virri J, Seitsalo S, Rytömaa T, Karaharju E (1995) Basic fibroblast growth factor immunoreactivity in blood vessels and cells of disc herniation. Spine 20:271-276

II Tolonen J, Grönblad M, Virri J, Seitsalo S, Rytömaa T, Karaharju E (1997) Platelet-derived growth factor and vascular endothelial growth factor expression in disc herniation tissue: an immunohistochemical study. Eur Spine J 6:63-69

III Tolonen J, Grönblad M, Virri J, Seitsalo S, Rytömaa T, Karaharju E (2001) Transforming growth factor β receptor induction in herniated intervertebral disc tissue: an immunohistochemical study. Eur Spine J 10:172-176

IV Tolonen J, Grönblad M, Virri J, Seitsalo S, Rytömaa T, Karaharju E (2002) Oncoprotein c-Fos and c-Jun immunopositive cells and cell clusters in herniated intervertebral disc tissue: an immunohistochemical study. Eur Spine J 11:452-458

V Tolonen J, Grönblad M, Vanharanta H, Virri J, Guyer RD, Rytömaa T, Karaharju E (2006) Growth factor expression in degenerated intervertebral disc tissue. An immunohistochemical analysis of extracellular matrix turnover regulators transforming growth factorβ, fibroblast growth factor and platelet-derived growth factor. Eur Spine J 15:588-596

The above publications will be referred to in the text by their Roman numerals.

2. Abbreviations

ABC	Avidin-biotin-peroxidase complex
AF	Annulus fibrosus
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
aFGF	Acidic fibroblast growth factor (=FGF-1)
AP-1	Activating protein 1
bFGF	Basic fibroblast growth factor (=FGF-2)
BMP	Bone morphogenetic protein
c-fos	oncogene *c-fos*
c-Fos	Oncoprotein c-Fos
CI	confidence interval
c-jun	oncogene *c-jun*
c-Jun	Oncoprotein c-Jun
CML	n-carboxymethyllysine
DAB	Peroxidase substrate solution

DN	Normal intervertebral disc tissue
DHT	Herniated intervertebral disc tissue
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EGP	Epiphyseal growth plate
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor –1
IL-1	Interleukin 1
kD	Kilo-Dalton
LAP	Latency associated peptide
LTBP1	Latent TGFß binding protein-1
mAb	Monoclonal antibody
MAPK	Membrane activated protein kinase
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging

mRNA	Messenger RNA
NGF	Nerve growth factor
NP	Nucleus pulposus
OA	Osteoarthritis
PBS	Phosphate-buffered saline solution
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
pAb	Polyclonal antibody
RA	Rheumatoid arthritis
r.t	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
TGFβ	Transforming growth factor β
TIMP-1	Tissue inhibitor of matrix metalloproteinase-1
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor (=VPF/vascular permeability factor)

3. Review of the literature

Intervertebral discs stabilize the spine by anchoring adjacent vertebral bodies to each other. They absorb and distribute the gravitational loads applied to the spine and, at the same time, give flexibility allowing the movement between the vertebral bodies. Like other connective tissues, the intervertebral discs are composed of different cell types surrounded by the extracellular matrix components and water. The mechanical properties of the disc depend on the extracellular macromolecules and their interaction with water.

The intervertebral disc is composed of four concentrically arranged components (Buckwalter 1982): 1) the outer annulus fibrosus, a ring of highly oriented densely packed collagen fibrils, 2) the fibrocartilaginous inner annulus fibrosus, 3) the transition zone, a thin zone between annulus fibrosus and nucleus pulposus, and 4) the central nucleus pulposus. Vertebral endplates, composed of hyaline cartilage, form the superior and inferior boundaries from the intervertebral disc to the adjacent vertebral bodies.

The outer annulus fibrosus contains fibroblast-like or fibrocyte-like cells, whereas the inner annulus and the transition zone contain more chondrocyte-like cells (Buckwalter 1982). The nucleus pulposus initially contains primarily notochordal cells, but in early adulthood they disappear leaving only chondrocyte-like cells (Buckwalter 1982, Troutt et al.1982a and b).

Collagens and proteoglycans are the primary structural components of the extracellular matrix in intervertebral disc tissue (Eyre et al. 1989). Proteoglycans interacting with water give stiffness to the tissue, and resilience to compression, while collagens give the tensile strength. In the outer annulus fibrosus of young people, collagens account for 70% of the dry weight, whereas in the nucleus pulposus they account for only 20%. In contrast, proteoglycans account for only a few percent of the dry weight in the outer annulus, but 50% in the nucleus pulposus. The type of collagen also varies in different parts of the intervertebral disc. The outer annulus fibrosus consists almost entirely of type I collagen (80%) and only of small amounts of type V collagen (3%) (Buckwalter 1995). Inside the outer annulus fibrosus the amount of type II collagen increases

toward the centre reaching 80% in the nucleus pulposus, where type I collagen is entirely absent. The nucleus pulposus contains small amounts (3%) of type XI collagen, and both the nucleus pulposus and the annulus fibrosus less than 2% type IX collagen. Type VI fine collagen filaments account for 10% in the nucleus pulposus and 15 % in the annulus fibrosus.

Throughout the disc tissue also exists a variety of noncollagenous proteins and small amounts of elastin (Buckwalter 1982, Eyre et al. 1989).

3.1 Disc growth

In general, it is very difficult to see the difference between ”normal aging” and ”pathological” degeneration. Simplifying, the intervertebral disc behaves in a genome-coded manner. This means, that the development and growth processes are mainly primarily similar to those seen in response to traumatic tears, or degeneration. Indeed, it is easier to understand pathological responses by first viewing normal growth and aging.

In fetal discs the number of lamellar bundles is higher in the anterior annulus than in the posterior annulus (Tsuji et al. 1993). The posterior middle annulus has a very complex structure, with a high percentage of incomplete/discontinuous lamellar bundles and larger fibre-interlacing angles. Furthermore, a loose connection of the lamellar bundles of posterior outer annulus was observed in fetal discs.

Chondromodulin-I (an antiangiogenic factor) is highly expressed during the gestational period and decreases after maturation (Takao et al. 2000). It is detected in both extracellular matrix and chondrocytes in the zone of hypertrophic cartilage, the zone of proliferative cartilage and the zone of resting cartilage in fetal discs. It is also present in annulus fibrosus, nucleus pulposus, and end-plate cartilage in mature discs. In degenerative discs, chondromodulin-1 expression tended to be elevated in the remaining chondrocytes.

At birth the disc tissue is separated from the vertebral bodies by hyaline cartilage endplates (Buckwalter 1995). Small blood vessels could be detected especially in the posterolateral regions of the disc, occasionally penetrating the inner annulus fibrosus. Nucleus pulposus composes nearly half of the disc and consists of soft gelatinous matrix and clusters of notochordal cells (Troutt et al. 1982a). Collagen fibrils have a nearly uniform small diameter. Furthermore, annular proteoglycan aggregates from newborn and infants have the same structure as hyaline cartilage. In nucleus pulposus only 1/3 resemble the large aggregates, the remaining 2/3 consist of aggrecan clusters lacking a visible central hyaluronate filament (Buckwalter 1985).

During childhood and adolescence disc volume markedly increases, and at the same time blood vessels become smaller and less numerous. At this time nucleus pulposus still composes nearly half of the total disc, but the fibrocartilaginous annulus fibrosus increases in size with time (Buckwalter 1995). In nucleus pulposus the number of notochordal cells decreases, and chondrocyte-like cells appear in this area (Troutt et al. 1982b). The mean diameter and the variability in diameter of collagen fibrils increase. At the same time the proteoglycan aggrecan size decreases, and large proteoglycan aggregates similar to that in articular cartilage disappear (Buckwalter et al. 1985, Pearce 1993). Clusters of short aggrecan molecules and no aggregated proteoglycans almost entirely create the proteoglycan population in the nucleus pulposus.

In adult life many of the remaining peripheral blood vessels disappear. The size of the outer annulus fibrosus remains the same, but the fibrocartilaginous inner annulus expands at the expense of the nucleus pulposus (Buckwalter 1995). In annulus fibrosus normal collagen fibril organization is lost, creating fissures and cracks. Nucleus pulposus changes from soft, translucent tissue to firm white tissue, and concentration and diameter of collagen fibrils increase. The number of viable cells decreases, especially in the most central regions of the disc (Troutt et al. 1982a and b). At this time nucleus pulposus consists almost entirely of chondrocyte-like cells (Troutt et al. 1982b) and at the same time the water content and the amount of proteoglycans decrease (Urban and McMullin 1988), and noncollagenous protein components increase (Dickson et al. 1967). Dense granular material accumulates in matrix tissue especially in regions immediately surrounding the cells (Troutt et al. 1982a and b).

Taken together, after skeletal maturity, age related changes in intervertebral disc tissue appear to decrease the structural integrity thereby increasing the probability of disc herniation (Andersson 1993).

In the elderly, the entire disc inside the outer annulus fibrosus becomes a stiff fibrocartilaginous plate (Buckwalter 1995). The disc loses its height, and the nucleus pulposus region becomes smaller in diameter, with less densely and less organized packed collagen fibrils, and few remaining viable cells. The spine loses mobility, and degeneration of facet joints and spinal stenosis may develop (Andersson 1993).

Development stages of the intervertebral disc is complex, involving several different connective tissue types, yet little is known of the developing extracellular matrix (ECM). The earliest collagen detected is type III, which is subsequently replaced by type II in the cartilaginous inner annulus and joined by type I in the fibrous outer annulus (Hayes et al. 2001). In the outer annulus type IV collagen appears, associated with myofibroblast-like cells of the orienting collagenous lamellae. In later stages extracellular matrix proteins laminin and fibronectin co-distribute here, although overall they have a wider distribution. Another extracellular matrix protein aggrecan occurs in the early nucleus pulposus and then appears in the inner annulus, in association with cartilage differentiation. Extracellular matrix protein versican appears later in the inner annulus, and also in the dorsal region of the outer annulus. Comparisons of glycosaminoglycan and proteoglycan label allowed extrapolations to be made as to likely glycosaminoglycan components of the large proteoglycans, and of other proteoglycans that may be present. Thus, differential distribution of aggrecan and keratan sulphate label suggested the presence of fibromodulin and/or lumican. Functionally aggrecan would confer compression resistance to cartilaginous structures (Hayes et al. 2001).

Type I collagen is seen in normal annulus fibrosus and in degeneratively altered nucleus pulposus, but not within the endplate, regardless of degenerative changes (Freemont et al. 1998). Type II and IX collagens occur in normal nucleus pulposus, inner annulus fibrosus and the end-plate. The type II and IX collagen expression seems to be enhanced in areas of minor degenerative lesions, but reduces in advanced lesions and in the degenerated endplate. Type III and VI collagens are significantly increased in areas of minor to advanced degeneration in all anatomical settings, while type V collagen showed only minor changes in its expression pattern.
Furthermore, type I collagen is seen abundantly in the outer zone and outer lamellas of the inner zone of the annulus fibrosus (Schollmeyer et al. 2000). In longitudinal sections, type I collagen distribution takes the shape of a wedge. In horizontal sections, its positive area takes the shape of a ring that is wider anteriorly than posteriorly. This suggests that the three-dimensional shape of the type I collagen-positive tissue in annulus fibrosus can be described as a doughnut that is wider anteriorly than posteriorly. Schollmeyr et al showed that type II collagen was present in the entire

inner area of the annulus fibrosus, but not in the outer zone. In addition, type II collagen was found in the cartilaginous endplates. Type III collagen showed some codistribution with type II collagen, particularly in pericellular locations in areas of spondylosis, at the endplates, vertebral rim, and insertion sites of annulus fibrosus (Schollmeyer et al. 2000).

There appears to be no distinct border between central nucleus pulposus and inner annulus fibrosus, the main difference being in their fibrous structure (Humzah and Soames 1988). Fluid space is important in nutrition of the disc, showing plastic deformation, and recovery characteristics. Humzah and Soames have stated that the structural elements, both macroscopically and microscopically, together with biochemical elements, are intimately related to function. The intervertebral disc should not be thought of as a homogeneous and static structure. It has a heterogeneous composition and responds dynamically to applied loads. Neither should it be considered an isolated structure because it interacts with vertebral bodies, together constituting the vertebral unit. Furthermore, changes within the disc can, and do, have dramatic effects on vertebral column kinematics. The intervertebral disc is not inactive, but is capable of self-maintenance. In injury it can repair itself and has considerable regenerative properties (Humzah and Soames 1988).

Synthesis in intervertebral discs, measured as the content of types I and II procollagen markers, is highest in the neonatal and 2-5-yr age groups (Antoniou et al. 1996). Antoniou and co-workers have showed that the contents of these epitopes/molecules progressively diminished with increasing age. In the oldest age group (60-80 yr) and in highly degenerated discs, however, type I procollagen epitope level increased significantly. The percentage of denatured type II collagen, assessed by the presence of an epitope that is exposed with cleavage of type II collagen, increased two-fold from the neonatal discs to the discs of young 2-5 yr age group. Thereafter, the percentage progressively decreased with increasing age, however, it still increased significantly in the oldest group and in highly degenerated discs. Three matrix turnover phases have been identified. Phase I (growth) is characterized by active synthesis of matrix molecules and active denaturation of type II collagen. Phase II (maturation and ageing) is distinguished by a progressive drop in synthesis activity and a progressive reduction in denaturation of type II collagen. Phase III (degeneration and fibrotic) is characterized by evidence of a lack of increased synthesis of aggrecan and type II procollagen, but also by an increase in collagen type II denaturation and type I procollagen synthesis, both dependent on age and grade of tissue degeneration (Antoniou et al. 1996).

Age-related changes have been classified by Boos et al. (2002) A diminished blood supply of the end-plate has been demonstrated, resulting in tissue breakdown beginning in nucleus pulposus and starting in the second life decade. Specific markers for disc alterations with age have been noted, allowing better correlation with disc function. Perhaps the best marker for ongoing age-related changes is the deposition of n-carboxymethyllysine (CML), a biomarker for oxidative stress (Nerlich et al. 1997). This is first observed after age 10 in nucleus pulposus, and increases significantly with age. In the elderly, both annulus fibrosus and nucleus pulposus show extensive CML deposition. Furthermore, Nerlich and co-workers detected that the nuclear collagen pattern was changed after the occurrence of CML in nucleus pulposus. First, collagen types II, III and VI were raised, followed by a decrease in type II, the occurrence of type I, and at the same time persistence of high levels of types III and VI. Finally, types III and VI decreased again. Nucleus pulposus cells of adolescents and young adults stained exclusively for collagen type IV. At a higher age with advanced degenerative changes collagen type X was expressed by these same cells.

3.2 Disc degeneration

Two major morphological features occur during aging and degeneration of the intervertebral disc: 1. nucleus pulposus decreases in volume, 2. the nucleus becomes histologically less distinguishable from annulus fibrosus. (Brown 1971, Hirsch and Schajowicz 1953, Donohue 1939)

From age 30 the cell type in the nucleus pulposus changes (Pritzher 1977). In addition to the former fibrocyte-like cell two new cell types appear . First, groups of large chondroid cells, chondrocyte-like cells appear in increasing numbers. Second, groups of small chondroid cells (minichondrons) are seen, but only in association with microfracture of the cartilage endplate. In annulus fibrosus, cells appear as mature fibrocytes, oriented in the direction of surrounding collagen fibres. With aging the cells near the innermost annular fibres appear identical to the adjacent giant chondrons of the nucleus pulposus (Pritzher 1977).

Potential causes of the age-related degeneration of intervertebral discs include declining nutrition, loss of viable cells, cell senescence, post-translational modification of matrix proteins, accumulation of degraded matrix molecules, and fatigue induced failure of matrix tissue (Buckwalter 1995). The most important of these mechanisms appears to be decreasing nutrition of the central disc that allows for accumulation of cell waste products and degraded matrix molecules, impairing cell nutrition, and causing a fall in pH levels that further compromises cell function and may cause cell death.

In 1981 Lyons and co-workers showed that degenerate disc proteoglycans were of larger average hydrodynamic size and had a higher glucosamine to galactosamine ratio (Lyons et al. 1981). It has been suggested that a large proportion of proteoglycans from normal disc have undergone a degree of degradation in the hyaluronate binding region and that proteoglycan synthesis in this tissue is slower than in degenerate tissue.
By interacting with cell surface integrins fibronectin helps to organize the extracellular matrix and provides environmental cues. In other tissues, its synthesis is elevated in response to injury. Fibronectin fragments can stimulate cells to produce metalloproteases and cytokines and inhibit matrix synthesis. Fibronectin is elevated in degenerated discs and frequently present as fragments (Oegema et al. 2000). Elevated levels of fibronectin suggest that disc cells are responding to the altered environment. Fibronectin fragments resulting from normal or enhanced proteolytic activity could be a mechanism that induces the cell to further degrade matrix tissue.

The rate of collagen biosynthesis in degenerated human annulus fibrosus has also been estimated (Kääpä et al. 2000). The turnover of collagen was found to be faster compared to normal annulus fibrosus. However, this process did not seem to cause any significant changes to its mature pyridinium crosslink concentrations.
Endplate injury has been reported to be strongly associated with disc degeneration (Kerttula et al. 2000). In this study no correlation was, however, seen between previous vertebral fracture and back pain.

An animal model was developed to test the hypothesis that discrete peripheral tears within the annulus lead to secondary degenerative changes in other disc components (Osti et al. 1990). Although the outermost annulus showed ability to heal, the defect induced by the cut initially led to deformation and bulging of the collagen bundles, eventually to inner extension of the tear and finally to complete failure. These findings suggest that discrete tears of the outer annulus may have

a role in the formation of concentric clefts and in accelerating the development of radial clefts. Peripheral tears of annulus fibrosus therefore, may play an important role in the degeneration of the intervertebral joint complex (Osti et al. 1990).
The tenascins are a family of extracellular matrix proteins with repeated structural domains homologous to epidermal growth factor, fibronectin type III and fibrinogens. In young discs, tenascin was by Gruber et al found to be localized throughout the annulus. In the nucleus, localization was confined to pericellular matrix (Gruber et al. 2002). In normal adult and degenerating disc specimens, tenascin in the annulus was localized primarily in pericellular matrix regions encircling either single cells or clusters of disc cells. In rare instances localization was more diffuse in the intraterritorial matrix.

In an experimental model of disc degeneration metaplasia into fibrocartilage originating from the cells along the margins of the annular wound, showed that proliferation of cells change almost the entire disc space into fibrocartilage (Lipson and Muir 1981). A vertebral osteophyte occurred through an endochondral ossification sequence. Aggregating proteoglycans had two periods of repletion in the early course of degeneration. The water content of the disc was rapidly but only transiently restored in the first two days after herniation, and the changes in the total proteoglycan content of the disc paralleled these changes. Hyaluronic acid content decreased rapidly after herniation, but the size of the proteoglycan monomers did not change with degeneration. It was suggested that loss of confined fluid mechanics signals an abortive repair attempt rather than that of biochemical changes in proteoglycans which initiate disc degeneration (Lipson and Muir 1981).

Nerve ingrowth has been observed in degenerated intervertebral discs (Freemont et al. 1997). In the healthy back only the outer third of annulus fibrosus of the intervertebral disc is innervated. Observation of isolated nerve fibres that express substance P deep within diseased intervertebral discs and their association with pain suggests an important role for nerve growth into the intervertebral disc in the pathogenesis of chronic lower back pain.

A field of its own in intervertebral disc degeneration is the regulation of the extracellular matrix by proteinases. A correlation is found between increasing levels of matrix metalloproteinase 2 and 9 and the grade of degenerative disc disease. In addition, the levels of these enzymes show a differential expression across the scoliotic disc with the highest levels in samples taken from the convexity of the curve (Crean et al. 1997). The difference between the concave and convex side of the scoliotic curve indicates that mechanical loads might influence the expression of these enzymes.

The increased expression of these enzymes in both degenerative disc disease and scoliosis strongly suggests that they may affect the progressive nature of these diseases.

Healthy human intervertebral discs contain relatively few cells and these are sparsely distributed. A characteristic feature of disc degeneration, however, is the appearance of cell clusters, particularly in damaged areas. How these clusters form is currently unknown. Excised pathological human discs have been investigated for evidence of cell proliferation (Johnson et al. 2001). In this study disc sections were immunostained for the proliferating cell nuclear antigen (PCNA) and the proliferation-associated Ki-67 antigen. PCNA immunopositive cells occured within degenerated discs, commonly though not exclusively, in cell clusters. Cells immunopositive for the Ki-67 antigen were less prevalent than those for PCNA, but similarly, were frequently observed within clusters in degenerated discs. In contrast, immunopositivity for these markers was not common in less degenerate discs or in areas of the disc where cell clusters did not occur. These observations suggest that disc cell proliferation is associated with disc degeneration (Johnson et al. 2001).

Chondrocyte markers have been compared in normal and degenerated human intervertebral discs (Sive et al. 2002). Chondrocyte markers SOX9 and type II collagen expression occurred in a similar pattern in normal and degenerated discs, in nucleus pulposus cells, and was absent or weak in annulus fibrosus cells. In addition, aggrecan was expressed strongly in normal nucleus pulposus, and not in normal annulus fibrosus. In degenerated discs aggrecan was absent in both these areas of the disc.

To help evaluate the degree of degeneration on MRI scans a classification method has been developed in which the degree of degeneration is divided into five categories (Pfirrmann et al. 2001). The benefit of this method is good reproducibility and reliability.

Genetic links for degenerative disc disease have also been observed (Annunen et al. 1999). In the COL9A2 gene, which codes for one of the polypeptide chains of collagen type IX a putative disease-causing sequence variation converting a codon for glutamine to one of tryptophan has been identified. The logarithm of the odds ratio score was 4.5, and subsequent linkage disequilibrium analysis conditional on linkage, gave an additional logarithm odds ratio score of 7.1.
In a large population based study (Jim et al. 2005) especially the Trp2 allele of COL9A2 gene is a significant risk factor for disc degeneration. The association was age-dependent and the risk was

especially high (4-fold) at age 30-39. In addition, the Trp2 allele was also associated with more severe degeneration.
Furthermore, mutations of type IX collagen may cause certain forms of degenerative disease in the spine as well as in joints (Kimura et al. 1996).

In genetic studies, subjects with shorter variable numbers of tandem repeat length of the aggrecan gene have a risk of developing multilevel disc degeneration at an early age (Kawaguchi et al. 1999). Two intragenic polymorphisms of the vitamin D receptor gene revealed an association with disc degeneration (Videman et al. 1998). The specific vitamin D receptor alleles associated with intervertebral disc degeneration as measured by T2-weighted signal intensity in MRI.

Experimental attempts to delay the degeneration process have been undertaken, including trying reinsertion of autogenous nucleus pulposus (Okuma et al. 2000). The direct reinsertion of activated nucleus pulposus cells into the disc offers a promising line of investigation for delaying intervertebral disc degeneration. However, these results obtained with notochordal cells may not necessarily apply when using mature central nucleus pulposus cells (Okuma et al 2000).
Discs receiving an intact nucleus pulposus showed least degeneration, followed by discs receiving nucleus pulposus cells, both of which were better than no treatment. These findings correlated directly with the intensity of immunohistochemical staining for type II collagen. Allogenic grafts did not induce any appreciable host-versus-graft response. Injection of nucleus pulposus and nucleus pulposus cells retarded intervertebral disc degeneration. Injection of intact nucleus pulposus was, however, more effective than injection of nucleus pulposus cells alone (Nomura et al. 2001).

3.3 Disc herniation

The histological difference between degenerated and herniated discs is small. Basically, the same kind of cellular response is seen in both pathological states. Avascularity is essential in the normal intact adult intervertebral disc. After disc herniation, of particular importance is the contact of herniated disc with surrounding tissues, producing inflammatory responses, neovascularization and tissue degradative responses. Disc herniation can be divided into subgroups (Spengler et al. 1990): Protrusion, in which annulus fibrosus bulges but remains continuous and its periphery remains

attached to the vertebral body bony rim; Extrusion, in which intervertebral disc herniates through the posterior longitudinal ligament into the epidural space, however, continuity occurs with the remaining disc; Sequestration, in which one or several separate disc fragments occur in the epidural space.
Disc herniation can also be classified differently. In contained disc herniation the herniated disc material remains inside the posterior longitudinal ligament. In non-contained herniation this ligament is broken and the disc material has penetrated through it.

Disc herniation tissue contains vascular ingrowth, promoting granulation tissue formation (Doita et al. 1996; Hirabayashi et al. 1990; Hirsch 1957; Leao 1960; Lindblom and Hultqvist 1950; Weidner and Rice 1988; Yasuma et al. 1993). Inflammation, either from direct chemical irritation (Marshall et al. 1977; McCarron et al. 1987; Saal et al. 1990), or secondary to an autoimmune response to the nucleus pulposus (Bobechko and Hirsch 1965; Elves et al. 1975; Gertzbein et al. 1975; Gertzbein 1977; Naylor et al. 1975; Pennington et al. 1988; Woertgen et al. 2000), is suggested to be involved in this granulation tissue formation. Furthermore, it is suggested that if the nucleus pulposus portion of the herniated mass perforates the posterior longitudinal ligament, it may be subject to a stronger neovascularization reaction (Ozaki et al. 1999). In addition, at the stage when the posterior longitudinal ligament is still intact, these vessels are observed to have difficulty reaching the inner disc portion.

The prolapsed disc tissue tends to diminish in size with time (Maigne et al. 1992; Saal et al. 1990). Furthermore, proteolytic activity has been demonstrated in herniated intervertebral discs (Ng et al. 1986). It is also suggested that there are enhanced apoptotic events (Ahsan et al. 2001), possibly caused by oxygen stress. In addition, inflammatory processes have been discussed (Goupille et al. 1998; Guiot and Fessler 2000; Hicks et al. 2002; Lipets 2002).

3.4 Fibroblast growth factor superfamily (FGF)

The fibroblast growth factors (FGFs) constitute a family of several structurally related polypeptides (Jave et al. 1986; Abraham et al. 1986; Dickson and Peters 1987; Taira et al. 1987; DelliPovi et al. 1987; Zhan et al. 1988; Marics et al. 1989; Finch et al. 1989). Primarily the family consists of two closely related isoforms (basic and acidic FGF: bFGF and aFGF) which were identified as embryonic inducers (Slack et al. 1987; Kimmelman and Kirschner 1987; Knochel and Tiedemann 1989).They share common receptors (Neufeld and Gospodarowicz 1986), and have similar effects on a wide variety of tissues.

Fibroblast growth factors (FGF) were first isolated from bovine pituitary gland (Gospodarowicz et al. 1975) and soon after from bovine brain (Westall et al. 1978). To date the FGF family consists of 22 members (Dailey et al. 2005). FGF signals through a single-pass transmembrane receptor with tyrosine kinase activity. FGF/FGF-receptor binding and dimerization requires the presence of heparin/heparin sulphate proteoglycans (Mohammadi et al. 2005)

Angiogenesis is one of the most important effects induced by FGF (Gospodarowicz et al. 1977). It induces capillary endothelial cells to invade extracellular matrices and form capillaries, the development of which is proportional to the amount of FGF present (Mignatti et al. 1986). Repeated application of FGF to a rat cell culture produces increased formation of granulation tissue (Buntrock et al. 1982). Increase in angiogenesis seems to suggest that in the studied *in vivo* model FGF proves to be, first of all, a factor of angiogenesis rather than a factor of fibroblast growth (Buntrock et al. 1982).

Both local and systemic FGF-1 (former acidic FGF) increase new bone formation and bone density. Systemic FGF-1 also appears to restore bone microarchitecture and prevent bone loss associated with oestrogen-withdrawal (Hamada et al. 1999).

Fibroblast growth factor has been highlighted in chondrocytes and chondrogenesis. Expression of Fgf-18 gene by adenovirus-mediated gene transfer in murine pinnae resulted in a significant increase in chondrocyte number. Furthermore, the addition of FGF-18 to the culture media of

primary articular chondrocytes increased the proliferation of these cells and increased their production of extracellular matrix (Ellsworth et al. 2002).

Fgf-18 gene is expressed in and required for osteogenesis and chondrogenesis in mouse embryos (Ohbayashi et al. 2002). It appears to regulate cell proliferation and differentiation positively in osteogenesis and negatively in chondrogenesis.

Indeed, mice lacking Fgf-18 display delayed ossification and decrease expression of osteogenic markers, phenotypes not seen in mice lacking Fgfr-3. These data demonstrate that FGF-18 signals through another FGF-receptor (FGFR) to regulate osteoblast growth. Signalling to multiple FGFRs FGF-18 coordinates chondrogenesis in the growth plate with osteogenesis in cortical and trabecular bone (Ohbayashi et al. 2002).

In cell culture, multiple members of the fibroblast growth factor (FGF) family (FGFs-2, -4, and -9) have significantly stimulated H-thymidine uptake by chondrocytes grown in an adherent serum-free culture system (Rousche et al. 2001).

Furthermore, in the rat chondrocyte-derived cell line FGF induces tyrosine kinase activity (Rozenblatt-Rosen et al. 2002). Upon FGF stimulation, FGFR-3 is selectively removed from local adhesions, which is followed by their disassembly and disruption of the organized cytoskeleton. Induced genes include c-jun, JunD, cyclin-D1, NFkappaB1 and plasma-membrane microdomain morphology, such as ezrin. The transcription factor Id1 is down regulated, consistent with exit of the cells from the mitotic cycle. Moreover, following FGF stimulation, levels of FGFR-3 mRNA and protein decline, as does downstream signalling through the MAPK pathway (Rozenblatt-Rosen et al. 2002).

In an additional experimental model, treatment of chondrocytes with bFGF (FGF-2) (1-100 ng/ml) stimulated the mRNA expression of bFGF and TGF-beta up to 121-604% and 130-220% respectively, at 12 h compared to controls (Shida et al. 2001). On the other hand, the treatment of chondrocytes with bFGF (1-100 ng/ml) suppressed IGF-1 mRNA expression 47-79% at 12 h.

Differences between FGFs have been noted. FGFs -2, -4, and -9 strongly stimulate avian chondrocyte proliferation, while FGFs -6 and -8 stimulate proliferation to a lesser extent (Praul et al. 2002). Assessment by RT-PCR has indicated that FGF-2 and FGF-4 are expressed in the postnatal avian epiphyseal growth plate, while FGF-8 and FGF-9 are not. Thus, FGF-2 and FGF-4 have been observed to stimulate chondrocyte proliferation, and both to be present in the epiphyseal growth plate.

The effects of FGF-18 closely resemble those of FGF-2, whereas FGF-10 affects none of these cells. FGF-18 may, therefore, compensate for the action of FGF-2 on bone and cartilage (Shimoaka et al. 2002).

In fracture healing, injected recombinant human bFGF (FGF-2) into the fracture site immediately after the fracture, has produced size enlargement of cartilage (Nakajima et al. 2001). Maturation of chondrocytes and replacement of the cartilage by osseous tissue, however, were not enhanced by exogenous bFGF.

Fibroblast growth factor (FGF) and its receptor (FGFR) are thought to be negative regulators of chondrocytic growth, as exemplified by achondroplasia and related chondrodysplasias, which are caused by constitutively active mutations in FGFR-3. FGF's growth inhibitory effects of chondrocytes appear to be mediated, at least partially, through p21 induction and the subsequent inactivation of cyclin E-Cdk2 and activation of pRb (Aikawa et al. 2001).

Recent experiments have established that Sox9 is required for chondrocyte differentiation. FGFs markedly enhance Sox9 expression in mouse primary chondrocytes, as well as in C3H10T1/2 cells that express low levels of Sox9. FGFs also strongly increased the activity of a Sox9-dependent chondrocyte-specific enhancer in the gene for collagen type II (Murakami et al. 2000).

In the neonatal rat, immunolocalization of FGFR-1, -2, and -3 was found in the middle of the condylar cartilage, mainly in the hypertrophic zone of the tibial cartilage (Dunstan et al. 1999). At three weeks of age, the three FGFRs were broadly observed in both the tibial and femoral cartilages. At eight weeks, localization of FGFR-3 was absent in the hypertrophic cell layer of the condyle, whereas it was still broadly observed in tibial growth plate cartilage. At the same stage, FGFR-1 and FGFR-2 showed similar localization in both cartilages compared to that at three weeks of age. All these observations suggest that FGFRs play an important role in the differential growth pattern of condylar cartilage.

3.5 Platelet-derived growth factor (PDGF)

Platelet-derived growth factor (PDGF) is structurally a dimer of A chains (17kD) and B chains (16kD). It appears either in the form of an A-A or a B-B homodimer or as an A-B heterodimer (Johnsson et al. 1984). It stimulates growth of vascular endothelial cells (Ross et al. 1974), and fibroblasts (Stiles 1983). Furthermore, it acts as a chemotactic signal for fibroblasts, even at very low concentrations (Postlethwaite et al. 1987). Additionally PDGF promotes the migration of smooth muscle cells, and acts as a true chemoattractant for vascular smooth muscle cells (Grotendorst et al. 1982).

In cartilage, PDGF has been suggested to increase the level of intracelluar calcium ions in chondrocytes (Fukuo et al. 1989), to stimulate DNA and proteoglycan synthesis in cartilage tissue (Fukuo et al. 1989), and increase chondrocyte proliferation (Guerne et al. 1994). Furthermore, PDGF has been shown to stimulate cell replication and generalized protein synthesis by differentiated cells (Canalis 1985).

Treatment with recombinant human platelet-derived growth factor (rh-PDGF-BB) has also been evaluated concerning fibroblast migration. The presence of rh-PDGF-BB resulted in a significantly greater fibroblast invasion as compared to untreated scaffolds (Gosiewska et al. 2001). In meniscal cells PDGF has been demonstrated to increase cell migration and DNA synthesis in a dose-dependent manner (Bhargawa et al. 1999). In cartilage PDGF-BB enhanced matrix production, but at the same time prevented progression of cells along the endochondral maturation pathway (Kieswetter et al. 1997).

In different age groups chondrocytes respond differently to PDGF. Cells from young donors (ages 10-20) responded better to platelet-derived growth factor, AA chain homodimer (PDGF-AA) than to TGF beta-1, while the inverse pattern was seen in cells from adult donors. This decrease in the response to PDGF-AA correlated significantly with increasing skeletal maturity (Guerne et al. 1995).

In osteoarthritic cartilage, more intense PDGF-AA and PDGF-BB expression has been compared to that in healthy cartilage. Activated chondrocytes were located in the middle and partly in the deeper layer of cartilage (Moos et al. 1999).

Interaction between different growth factors in cartilage has been observed. Human articular chondrocytes released TGF β activity. PDGF up-regulated TGFβ -1 and TGF β-3 mRNAs with a corresponding increase in protein secretion (Villiger and Lotz 1992).
Wroblewski and Edwall (1992) analyzed the role of two isoforms (PDGF AA and PDGF BB) of platelet derived growth factor either alone or in combination with insulin-like growth factor I, in the regulation of proliferation and differentiation of the rat rib growth plate chondrocytes. Both homodimers play an important role in chondrocyte differentiation and, together with IGF-I interact in the regulation of longitudinal bone growth.

3.6 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF), first known as a vascular permeability factor (VPF), is a dimeric heparin-bonding protein with a molecular weight of 45 kD (Ferrara and Henzel 1989; Ferrara et al. 1991a). It is expressed in various tissues including brain, kidney, pituitary gland, lung, adrenal gland, heart, liver, stomach, and ovary as well as in various tumours (Berse et al. 1992; Ferrara et al. 1991b). New family members are being identified, for example VEGF-A (also known as VPF or VEGF), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (Tammela et al. 2005). Of the three different VEGF-receptors, VEGFR-2 is the main signal transducer of VEGF-mediated angiogenesis (Tammela et al.2005).
VEGF regulates endothelial differentiation including vascular growth and blood vessel repair (Peters et al. 1993). VEGF shares homologies of about 21% and 24%, with the A and B chains of PDGF (Tischer et al. 1989). It also promotes blood vessel hyperpermeability, endothelial cell growth, and enhanced glucose transport (Connolly 1991). In addition, it is suggested to be involved in hypoxia-induced angiogenesis (Schweiki et al. 1992).

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) induces endothelial cell migration and proliferation in culture and is strongly angiogenic in vivo (Carlevaro et al. 2000). VEGF synthesis occurs in both normal and transformed cells and it is actively responsible for hypertrophic cartilage neovascularization through a paracrine release by chondrocytes, with

invading endothelial cells as a target. Furthermore, VEGF receptor localization and signal transduction in chondrocytes, strongly support the hypothesis of a VEGF autocrine activity also in morphogenesis and differentiation of a mesoderm derived cell (Carlevaro et al. 2000).
VEGF-mediated capillary invasion is an essential signal that regulates growth plate morphogenesis and triggers cartilage remodelling. Thus, VEGF is an essential coordinator of chondrocyte death, chondroclast function, and extracellular matrix remodelling, angiogenesis and bone formation in the growth plate (Gerber et al. 1999).

Vascular endothelial growth factor (VEGF)-mediated angiogenesis is an important part of bone formation. Expression of VEGF (164) and/or VEGF (188) is important for normal endochondral bone development, not only to mediate bone vascularization but also to allow for normal differentiation of hypertrophic chondrocytes, osteoblasts, endothelial cells, and osteoclasts (Maes et al. 2002).

VEGF is expressed by articular chondrocytes in normal and OA human knee cartilage (Pfander et al. 2001). The percentage of VEGF immunopositive chondrocytes significantly increases in late stages of the OA.

VEGF has recently been shown to play an important role during endochondral bone formation in hypertrophic cartilage remodelling, ossification, and angiogenesis. Apart from its production in hypertrophic chondrocytes, VEGF is also produced in chondrocytes of OA cartilage. While the splice variant VEGF (189) binds to extracellular matrix proteoglycans, VEGF (121) is diffused freely. Both proteins are believed to contribute to the inflammatory process by autocrine/paracrine stimulation of chondrocytes, chemotaxis of macrophages, and promotion of angiogenesis (Pufe et al. 2001).

Angiogenesis shown to be essential for the replacement of cartilage by bone during growth and repair.
VEGF was absent in chondrocytes in the resting zone and only weakly expressed by occasional chondrocytes in the proliferating region. In the hypertrophic zone the number of chondrocytes stained and the intensity of staining for VEGF increased with chondrocyte hypertrophy, with maximum expression of VEGF being observed in chondrocytes in the lower hypertrophic and mineralised regions of the cartilage (Horner et al.1999).

3.7 Transforming growth factor β superfamily (TGFβ)

The transforming growth factor β (TGFβ) superfamily is composed of several growth factors: mammalian TGFβs -1, -2, -3, more than 20 BMPs, activins/inhibins, Nodal, myostatin and anti-Mullerian hormone (Derynck et al. 1985; Miyazano et al. 2005). TGFβ has been found in almost all tissues studied (Assoian et al. 1983; Moses et al. 1981; Roberts et al. 1981). It regulates cellular growth and stimulates extracellular matrix protein incorporation as well as collagen, hyaluronic acid, and fibronectin production (Ignotz and Massagues 1986; Raghow et al. 1987). Furthermore, subcutaneous injection of TGFβ in mice results in rapid induction of fibrosis and angiogenesis (Roberts et al. 1986). In cartilage, TGFβ has been demonstrated to stimulate the production of tissue inhibitor of metalloproteinase (TIMP) (Wright et al. 1991), thus participating in the control of connective tissue degradation. Furthermore, it is involved in articular inflammation (Lotz et al. 1992; Pujol et al. 1991), chondrogenesis and osteogenesis (Frenz et al. 1994; Joyce et al. 1990).

TGFβs are secreted from cells in a latent form, which does not have the capability to bind to TGFβ receptors. The latency-associated peptide is removed through enzymatic degradation by proteases, resulting in the activation of TGFβ (Burmester et al. 1993; Flaumenhaft et al. 1993; Massaque 1990; Sato and Rifkin 1989). Latent TGFβ binding proteins allows fore more specificity and control on TGFβ action (Rifkin 2005).
The actions of TGFβ are mediated via binding to cell surface receptors (Wrana et al. 1992). TGFβ binds to the type II receptor, which recruits and phosphorylates the type I receptor (Bachmann and Park 2004). This leads to activation of the kinase domain of the type I receptor and the subsequent regulation of the SMAD proteins. Activated SMAD complexes assembly transcriptional complexes for target genes (Massague et al. 2005). There is strong evidence that absence of the type II receptor can completely abrogate TGFβ signalling (Bachmann and Park 2004). The type III receptor, also known as betaglycan, can exist in soluble forms and has the capacity to strongly bind two TGFβ molecules. This can allow a greater concentration of TGFβ molecules to be present at the cell surface (Lopetz et al 1993). Of the other cell surface receptors (type IV, type V, and type VI), especially the type V receptor has been demonstrated to inhibit cell growth (Huang and Huang 2005).

Transforming growth factor beta-1 (TGFβ-1) is secreted in a biologically inactive form and stored in extracellular matrix as a 290 kD complex consisting of mature TGFβ-l homodimer (Mr 25 kD), latency-associated peptide (LAP; Mr 75 kD), and latent TGFβ-l binding protein-1 (LTBP1; Mr 190 kD) (Maeda et al. 2002). Latent TGFβ-l, composed of these three components, is known as the "large latent TGFβ-1 complex." In contrast, latent TGFβ-l without LTBP1 is known as "small latent TGFβ-1." For all latent forms, dissociation of the TGFβ-l homodimer from LAP is necessary for growth factor activation and acquisition of biological activity. Matrix vesicles contain enzymes, especially MMP-3, which are responsible for release of TGFβ-1 from matrix, most of which is in latent form. Further, data suggest that release of the large complex occurs via cleavage at several novel sites in the 130 kD LTBP1 molecule. Since matrix vesicle MMP-3 is also able to activate small latent TGFβ-1, these results suggest that the large latent TGFβ-1 complex protects against activation of small latent TGFβ-1. Thus, data suggest that release of the large latent TGFβ-l complex from matrix and activation of the latent growth factor are only two steps of what must be at least a three-step process (Maeda et al. 2002).

Chondrocytes from normal human cartilage, when cultured in gelled agars, have shown correlations between the expression of TGFβR-II/TGFβ-1 and intracellular levels of TIMPs, indicating that a TGFβ autocrine pathway may contribute to homeostasis of ECM in normal cartilage. The relations between MMPs, TIMPs, and ECM molecules support the idea that a physiological balance between MMPs and TIMPs results in a well-controlled matrix turnover in normal cartilage (Wang et al. 2002).
Indeed, TGFβ has a proven role in chondrocyte maturation and endochondral ossification. TGFβ production, release, and activation are developmentally regulated in chondrocytes (Dangelo et al. 2001).

TGFβ and bone morphogenetic protein-2 induce chondrophyte formation at the joint margins. Studies using scavenging TGFβ soluble receptor identified endogenous TGFβ involvement in spontaneous cartilage repair and chondrophyte and subsequent osteophyte formation in arthritic conditions (van den Berg et al. 2001).
Furthermore, overexpression of active TGFβ-1 in the knee joint results in OA-like changes and suggests synovial lining cells to be contributing to the chondro-osteophyte formation (Bakker et al. 2001).

TGFβ has different actions in mesenchymal precursor cells for chondrocytes, osteoblasts, and osteoclasts in different stages (Bostrom and Asnis 1998). Although TGFβs appear to stimulate proliferation of precursor cells, it seems that TGFβs have an inhibitory effect on mature cell lines. *In vivo* studies indicate the presence of TGFβ protein and TGFβ gene expression in normal fracture healing, whereas exogenous TGFβ administration stimulates recruitment and proliferation of osteoblasts in fracture healing (Bostrom and Asnis 1988).

Furthermore, TGFβ is present in a latent form in matrix of costochondral chondrocytes. It is suggested that chondrocytes are able to regulate both temporal and spatial activation of latent TGFβ, even at sites distant from the cell, in a relatively avascular environment (Pedrozo et al. 1998).

TGFβ-1 has been shown to stimulate synthesis of both DNA and proteoglycan in a bimodal fashion (Qi and Scully 1997). The presence of extracellular type-II collagen increased the rate of DNA and proteoglycan synthesis in a dose-dependent fashion in cultures stimulated by TGFβ-1, whereas heat-inactivated type-II collagen abrogated the effects observed with type-II collagen for synthesis of both DNA and proteoglycan. Type-II collagen has a specific role in chondrocyte regulation and mediates chondrocyte response to TGFβ-1.
Furthermore, the responsiveness to TGFβs changes as cells differentiate and these changes in TGFβ receptor profile may account for some of these differences (Moses and Serra 1996).
TGFβ is particularly dependent upon the context in which it acts, eliciting seemingly opposite effects under different experimental conditions. TGFβ and other factors interact to modulate their respective actions, creating effector's cascades and feedback loops of intercellular and intracellular events that control articular chondrocyte functions (Trippel 1995).

In addition, TGFβ has a dual effect on proliferation of joint chondrocytes. In medium with a low serum concentration, it inhibits cell growth, while in medium supplemented with 10% fetal calf serum it stimulates cell growth (Pujol et al. 1994).

Exogenous TGFβ-1 has a stabilizing effect on the prehypertrophic epiphyseal chondrocytes (Ballock et al. 1993). It reversibly has been shown to prevent terminal differentiation of epiphyseal chondrocytes into hypertrophic cells. It maintains gene expression for the cartilage matrix proteins,

type II collagen, and aggrecan core protein, as well as inhibits expression of genes encoding metalloproteases collagenase and stromelysin.

Using immunohistochemical staining analysis with specific TGFβ antibodies (Thorp et al. 1992), TGFβ -1, -2, -3 were identified to be localized in hypertrophic chondrocytes, chondroclasts, osteoblasts and osteoclasts in developing growth plate, epiphysis and metaphysis.

TGFβ has been suggested to have a potentially important autocrine function in modulating chondrocyte proliferation and matrix synthesis in endochondral calcification (Rosier et al. 1989).

In regulating the extracellular matrix, TGFβ has in cell culture, had an inhibitory effect on the level of TIMP-2 and membrane type 1matrix metalloproteinase (MT1-MMP) (Pattison et al. 2001).

The responsiveness of cultured human articular chondrocytes to TGFβ appeared reduced with age (Hickery et al. 2003). The responsiveness tended to rise up to the age of 20, and there after reduced linearly.

3.8 Oncoproteins c-Fos and c-Jun

Proto-oncogenes encode proteins with three main sites of action: the cell-surface membrane, cytoplasm and nucleus (Angel et al. 1988). First observed in cancer research (Bohmann et al. 1987), the *jun* oncogene is expressed as a 65-kD protein Jun (Bos et al. 1988). It is a member of the early activating protein (AP-1) family of transcription factors, which mediate regulation of gene expression in response to extracellular signalling (Bohmann and Tijan 1989). Stimulation of quiescent murine fibroblasts by growth factors has resulted in a rapid and transient transcriptional activation of proto-oncogenes (Jahner and Hunter 1991). In addition, transient inhibition of protein synthesis has induced expression of proto-oncogenes and stimulated resting cells to enter the cell cycle (Rosenwald et al. 1995).

The proto-oncogene protein products (c-Fos and c-Jun) heterodimerize through their leucine zippers consisting of AP-1 transcription factor (Papavassiliou et al. 1992). The transcriptional activity of this heterodimer is regulated by signal-dependent phosphorylation and dephosphorylation.

The effect of *c-fos* on human chondrocytes and in cartilage destruction of rheumatoid arthritis (RA) have been highlighted (Tsuji et al. 2000). Introduction of *c-fo*s in chondrocytes decreased

endogenous transcription of Type II collagen and TIMP-1, and increased that of MMP-1. MMP-1 promoter was clearly activated by Jun related proteins as well as Fos/Jun related protein heterocomplex. On the other hand, *c-fos* combined with any of the Jun related proteins failed to stimulate the TIMP-1 promoter, although it was activated by Fra-1 or Fra-2/Jun related protein heterocomplexes. Expression of *c-fos* mRNA was detected in chondrocytes in the mid and deep layers of cartilage in 11/15 patients (73%) with RA, but only in the superficial layer of cartilage from 2/10 patients (20%) with OA (Tsuji et al. 2000).
Furthermore, c-Fos combined with any of the Jun-related proteins failed to stimulate the TIMP-1 promoter, though the collagenase promoter was effectively activated by any Fos/Jun-related protein heterocomplex (Tsuji et al. 1996). This may indicate that the *c-fos* expression may govern cartilage metabolism and hence may play an important role in the pathogenesis of joint destruction in arthritis.

In cell culture, induction of *c-fos* has resulted in the concomitant increase in the expression of *fra-1* and *c-jun*, further highlighting the importance of AP-1 transcription factors in chondrocyte differentiation. Indeed, *c-fos* overexpression directly inhibits chondrocyte differentiation *in vitro* (Thomas et al. 2000).

In an attempt to define molecular influences on rat interstitial collagenase gene expression in cartilage, the promoter function was characterized using a transient transfection assay, electrophoresis mobility shift assay, and genetic analysis in isolated growth plate chondrocytes (Grumbles et al. 1997). One of the factors involved was identified as the nuclear protein, c-Jun. Indeed, c-Jun directed antisense oligonucleotides reduced rat interstitial collagenase mRNA.

In addition, basic fibroblast growth factor (bFGF, FGF-2) has been demonstrated to have an inductive effect on *c-fos* in primary cultures of rat rib growth plate chondrocytes (Wroblewski and Edwall-Arvidsson 1995).
Furthermore, Zarafullah et al. (1992) has showed that normal and osteoarthritic human articular cartilage chondrocytes, released enzymatically in the presence of 0.5% fetal calf serum, display constitutive expression of early response activating protein (AP-1) genes, *c-fos*, *c-jun*, and *jun-B*. Among the late AP-1 responsive genes, total metallothionein (MT) and stromelysin mRNAs are expressed at high levels in both normal and OA chondrocytes, while collagenase and hMT-IIA mRNA levels are elevated only in OA individuals.

3.9 Growth factors in intervertebral disc tissue

During the past decade intervertebral disc research has focused both on the expression of growth factors in normal discs and in pathological conditions. The rationale is based on understanding of disc cell function. That function has been considered one of the key elements in degenerative processes in the intervertebral disc (Gruber and Hanley 2003). Growth factor research has mainly followed two pathways: the neovascularization process and regulation of extracellular matrix formation and remodelling.

In cell cultures, TGFβ, FGF, and IGF-I and EGF have been demonstrated to be potent cellular proliferation stimulators (Thompson et al. 1991). Cells from nucleus pulposus and the transition zone reacted more than annulus fibrosus cells. Greatest stimulation was gained by TGFβ and EGF. IGF-I stimulated the cells from nucleus pulposus, but not annular or transition zone cells.
In addition, TGFβ-1 and IGF-I regulate matrix metalloproteinase production in nucleus pulposus cells (Pattison et al. 2001). They both decrease the level of active gelatinase-A (MMP-2). Furthermore, the surface levels of metalloproteinase inhibitors have been decreased by these factors.
In nucleus pulposus cell culture, proteoglycan synthesis is stimulated in a dose-dependent manner by IGF-I. The expression of IGF-I and its receptor is especially noted in young nucleus pulposus (Osada et al. 1996).
This is supported by the finding that IGF-I receptor expression is clearly decreased with age (Okuda et al. 2001). Furthermore, proteoglycan synthesis is reduced and IGF-I binding protein-1 is more strongly expressed with age.

In annulus fibrosus cell culture, apoptosis is significantly reduced by IGF-I and PDGF (Gruber et al. 2000a). In addition, IL-1β sensitizes annular cells to fluid-induced shear stress resulting in increased Ca-intake (Elfervig et al. 2001). Furthermore, annulus fibrosus cells grown in three-dimensional agarose or alginate culture assumes a rounded phenotype and forms colonies. In the presence of

TGFβ-1 there is first a significant enhancement of proliferation, and after 10 days of exposure to TGFβ, a lessened mitogenic response (Gruber et al. 1997). In addition, in human annulus cell cultures, higher age, female gender, more advanced disc degeneration, and surgical derivation diminishes the proliferation potential of the cells (Gruber et al. 2001).

Co-culturing murine peritoneal macrophages and human herniated intervertebral disc tissue, a marked increase in macrophage VEGF expression and an increase in neovascularization was shown by Haro et al. in herniated tissue (Haro et al. 2002). TNF-α was required for induction of VEGF. The induction was strongly inhibited by anti-VEGF antibody, and enhanced by recombinant VEGF. Co-culturing intervertebral disc tissue and peripheral blood mononuclear cells, revealed significant numbers of mononuclear cells attached to extruded disc tissue, compared to control discs (Doita et al. 2001). In addition, cytokine incubation of isolated cells from herniated discs, showed significantly higher production of MMP-1 and MMP-3.
Furthermore, VEGF has been localized in extruded and sequestrated intervertebral discs, in the spindle shaped cells og the granulation tissue area (Koike et al. 2003)

By using collagen/hyaluronan scaffold, biomaterial has been evaluated as to supporting the viability and function of disc cells (Alini et al. 2003). Nucleus and annulus cells behaved in a similar manner. Production of proteoglycan was greatest with the presence of TGF-β1. Proteoglycan production, however, never exceeded 10% of that present in mature nucleus pulposus, and the production was not uniform within the scaffold. Instead it was increased near its periphery.

In animal models, growth factor expression has especially been demonstrated in growing animals. TGFβ expression decreased with age in a senescence-accelerated mice model (Matsunaga et al. 2003; Nagano et al. 2000). Degenerative changes were recognized at 50 weeks. In young mice, TGFβ-1, -2, -3 and receptor types I and II were localized to both fibrocartilaginous annulus cells and notochord-like nucleus pulposus cells. Furthermore, in senescence-accelerated mice, BMP-2/4 and its receptors, were localized in hyaline cartilaginous cells within the end-plate in young mice (Takae et al. 1999). With age, the expression moved to fibrous cells of annulus fibrosus and to calcified cartilage.
In an experimental mouse spondylosis model, BMP-4 and BMP receptors have at the early stage been localized mainly in cells of the anterior margin (Nakase et al. 2001). At the late stage, BMP-4 expression decreased, whereas growth and differentiation factor (GDF)-5 and BMP-6 expression

increased, and was localized in cells undergoing chondrogenesis. The expression of BMP receptors remained the same.

In a recent experimental study, using a real-time PCR technique Sobajima et al. (2005) showed that during degenerative process in intervertebral disc, TGFβ had a double peak characteristic in its expression. The first early peak increased by week 3, and the second late peak at 24 weeks. BMP-2, BMP-7, and IGF-I only had a late peak expression.

In an experimental rabbit model that mimics the sequestration type intervertebral disc herniation, bFGF stimulated neovascularization and proliferation of inflammatory cells (Minamide et al. 1999). Indeed, the degradative process was enhanced and the effect was dose-dependent.
In an experimental degeneration model induced by annular laceration of rabbit intervertebral disc, a profile of possibly important genes was observed (Anderson 2002). There was notable up regulation of the genes for collagen Type I, collagen Type II, fibronectin, Fas, MMP-1, MMP-9 and MMP-13 in these degenerated discs. Degeneration induced down regulation of the gene for decorin. The expression level of genes IL-1α, BMP-2, and TNF-α was not affected.

In degenerated rat intervertebral discs bFGF and its receptor have been shown to be localized mostly in chondrocyte-like rounded cells. Proliferation capacity of these cells exceeded that of normal annular spindle-shaped cells (Nagano et al. 1995).

When comparing injured annulus fibrosus of merinos to intact ones, bFGF (FGF-2), TGF-β and osteonectin were strongly localized in blood vessels and cells in the vicinity of the annular lesion (Melrose et al. 2002). The reaction was maximal 12 month after the operation, and diminished by 26 months. Osteonectin was also elevated in outer annulus fibrosus cells distant to the lesion site. In control discs, the expression of FGF-2, TGF-β, and osteonectin was localized to sparsely distributed cells in annulus fibrosus.

Epidural injection of bFGF has been demonstrated to facilitate resorption of the intervertebral disc, relocated to the epidural space (Minamide et al. 1999).
Intradiscal injection of osteogenic protein-1 in normal adolescent rabbits has increased both disc height and proteoglycan content in nucleus pulposus (An et al. 2004).

In an *in vivo* model, TGFβ-1 gene has been transferred to rabbit intervertebral disc. The accumulation of TGFβ-1 into disc cells was analyzed immunohistochemically, and in cell culture TGFβ-1 production and proteoglycan synthesis were also analyzed (Nishida et al. 1999). Adenovirus-mediated TGFβ-1 gene transfer exhibited intensive and extensive positive immunostaining of TGFβ-1 in disc cells. Furthermore, nucleus pulposus cells showed a 30-fold increase of inactive TGFβ-1 production and a five-fold increase in total (latent and active) TGFβ-1 production. The overall proteoglycan synthesis exhibited a 100% increase.
In addition, human intervertebral disc cells were treated successfully with adenovirus-containing marker genes (Moon et al. 2000). The adeno associated viral vector has proved to be a valuable method in experimental analysis of transgene expression (Lattermann et al. 2005). This method is known to be less immunogenic than the commonly used adenoviral vectors.

Of special interest is a recent study by Steck et al. (2005) showing that incubation of human adult bone marrow mesenchymal stem cells with TGFβ produces induction of stem cells to chondrocyte-like cells. The phenotype of these cells more closely resembles native intervertebral disc cells than native joint cartilage cells.

During development of the human intervertebral disc, there are certain changes in type II procollagen expression (McAlinden et al. 2001). At day 54 of gestation, the developing disc is divided into outer annulus fibrosus containing collagen types I and III, inner annulus fibrosus containg type IIA procollagen, and the notochord containing all three fibrillar collagens. Moreover, at day 54 of gestation type IIA N-terminal propeptide is localized in extracellular matrix, whereas, at day 101 of gestation the localization of this propeptide is in cytoplasm of inner annular cells. This domain of type IIA procollagen binds to BMP-1 and TGF-β.

In painful degenerative human intervertebral discs, neovascularization from adjacent vertebral bodies, has been causally linked to nociceptive nerve ingrowth with the production of NGF (Freemont et al. 2002). Such NGF expression has only been identified in discs painful at discography, not in painless discs.

In extruded or sequestrated human intervertebral disc herniations, IL-1 and bFGF have been localized particularly in the granulation tissue area at the surface of the herniated disc (Doita et al.

1996). In addition, this study showed that bFGF expression was far more intense in extruded or sequestrated discs compared to protruded ones, and enhanced the proliferation of endothelial cells. In the protrusion type of disc herniation chondrocyte-like disc cells have been demonstrated to express TGFβ-1, IGF-I, IL-6, IL-6R and fibronectin (Specchia et al. 2002).

As demonstrated by reverse transcription-polymerase chain reaction, TNF-α, IL-8, IL-1α, IL-10 and TGFβ have been expressed in herniated lumbar intervertebral discs (Ahn et al. 2002). IL-8 mRNA expression was associated with the development of radicular pain by back extension, and short symptom duration (average 3.8 weeks). This is supported by the finding that IL-8 levels in cerebrospinal fluid were higher in patients operated on for sciatica and short duration of pain, and in more pronounced herniations (extrusion or sequestration) (Brisby et al. 2002). With respect to serum levels of IL-8 there were no differences as compared to the normal control group. Furthermore, the cerebrospinal concentration of IL-1β, IL-6, IFN-γ and TNF-α was similar in sciatica patients and controls.

In one study, mRNA coding for TGF-α, EGF, TGFβ-1, TGFβ-3, EGF-R, and TGFβ type-II receptor, were found only occasionally in herniated human intervertebral discs (Konttinen et al. 1999).

Apoptosis in herniated disc cells has also been evaluated (Kohyama et al. 2000). This paper showed that apoptotic cell death was significantly higher in herniated disc cell than in control disc cells. Furthermore, culturing disc cells revealed a release of nitric oxide from disc cells, and the suppressive action of this agent.

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Table 1 summarizes the comparative observed effects on disc and cartilage cells of the growth factors present in this thesis.

Table 1. Comparison of growth factor effects on disc - and cartilage cells.

	Disc cells	**Cartilage cells**
TGFβ	Cell proliferation (Thompson et al. 1991) Proteoglycan synthesis/ matrix turnover (Alini et al. 2003) MMP-2 ↓ (Pattison et al. 2001)	Cell proliferation (Fukumoto et al. 2000; Olney 2004) Proteoglycan synthesis/ matrix turnover (van Beuningen et al. 1994) TIMP-1 and TIMP-3 ↑ (Hickery et al. 2003)
bFGF	Cell proliferation (Thompson et al. 1991) Neovascularization (Minamide et al. 1999) Disc degradation, extruded disc resorption (Minamide et al. 1999)	Cell proliferation (varying effects by various FGF isoforms) (Fukomoto et al. 2000; van der Eerden et al. 2003; Dailey et al. 2003; Veilleux et al. 2005; Henson et al 2005)
PDGF	Apoptosis ↓ (Gruber et al. 2000a)	Cell proliferation (Olney et al. 2004)

VEGF	Neovascularization	Neovascularization Proliferation, differentiation, chondrocyte survival (van der Eerden et al 2004; Maes et al. 2004; Zelzer et al. 2004)

TGFβ = transforming growth factor β, bFGF = basic fibroblast growth factor, PDGF = platelet-derived growth factor, VEGF = vascular endothelial growth factor; MMP = matrix metalloproteinase, TIMP = tissue inhibitor of matrix metalloproteinase, ↓ = expression decreased, ↑ = expression increased.

4. Aims of the study

The objective was to analyze the differences in growth factor expression in normal, degenerated, and herniated intervertebral disc tissues. The rationale for this is based on the importance of growth factors in cell growth, in cell activation, and in regulation of extracellular matrix turnover, as well as, in neovascularization.
Cellular activation was observed by the putative expression of the transcription promoters c-Fos and c-Jun in disc tissue cells.

Specific aims:

1. To study the expression of growth factors involved in neovascularization, cell activation and control of extracellular matrix turnover in herniated disc tissue and in normal control discs. In addition, to study differences in growth factor expression in order to gain deeper understanding of the basic pathophysiological events leading to disc herniation and disc rearrangement. TGFβ and FGF are well documented cell activation regulators, and extracellular matrix regulators in many tissues including cartilage, whereas PDGF and especially VEGF promote vascularization. The role of these factors in intervertebral disc tissue was not well documented in 1994, when this work commenced. Special interest was focused on identifying the disc cell types expressing growth factors, and characterizing possible differences between normal and herniated discs (I-III).

2. To evaluate whether cell activation markers, oncoproteins c-Fos and c-Jun, are expressed differently in herniated disc tissue, and control disc tissue. Furthermore, if expressed, in which cell type? To determine whether there is activation centres in intervertebral discs during pathological processes (IV)?

3. To study growth factor expression patterns in degenerated versus normal control disc tissue and to compare the results with those obtained from herniated disc tissue in order to understand basic

pathophysiology involved in intervertebral disc degeneration and disc rearrangement. To study the expression of growth factors in different degenerated disc regions, and to investigate in more detail which cell types show signs of activation (V).

4. To create a theoretical model for pathophysiological mechanisms of human disc degeneration and the disc tissue remodelling process on basis of results obtained regarding growth factor expression and expression of oncoproteins

5. Material and methods

5.1 Operations

5.1.1 Disc herniation tissue (DHT) (I-IV)

DHT material was obtained from a total of 115 patients operated on for sciatic radicular pain. Clinical records of the patients described their age, sex, duration of preoperative radicular pain period, straight leg raising (SLR), the level of disc herniation and the type of herniation.
After removal, the DHT material was rapidly frozen to –70^{o}C in the operation theatre. The clinical data of DHT material is summarized in Table 2.

(I) DHT material was obtained from 27 discectomy patients. The age of patients varied from 25 to 68. There were eight female patients. Preoperative radicular pain duration ranged from 0.5 months to 16 months. The operating surgeon classified thirteen of the DHT samples as sequesters (free disc material in the epidural space, and the disc has lost its continuity). Eleven were extrusions, i.e. disc tissue was exposed to the epidural space, but there was, however, still continuity with the rest of the disc. Three were protrusions, i.e. the annulus fibrosus bulged abnormally, but remained intact and the periphery of the annulus fibrosus remained attached to the vertebral body bony rim.

(II) DHT material was obtained from 50 discectomy operations. The age of the patients ranged from 24 to 71. There were 26 male and 24 female patients. Mean preoperative pain duration was 4.12 months. Twenty-five DHT were classified as extrusions, twenty were sequesters, two were not reported and the remaining three were protrusions.

(III, IV) DHT material was obtained from 38 discectomy operations. The age of the patients ranged from 20 to74. Mean preoperative radicular pain duration was 6.9 months (range 3 weeks-36 months). Twelve were sequesters, 24 were extrusions, and two were protrusions. There were 22 male and 16 female patients.

Table 2. Clinical data of the disc herniation tissue (DHT) obtained from the patients operated on for sciatic pain. Roman numbers I-IV refer to different substudies of the thesis.

	I	II	III-IV
Patients (n)	27	50	38
Gender (F/M)	8/19	24/26	16/22
Age (years)	25-68	24-71	20-74
(mean)	45.4	43.2	41.6
Preoperative pain			
duration (months)	0.5-16	0.25-24	0.75-36
(mean)	4.10	4.12	6.9
Prolapse type (n)			
Not reported	-	2	-
Protrusion	3	3	2
Extrusion	11	25	24
Sequester	13	20	12

5.1.2 Degenerated intervertebral disc tissue (DD) (V)

Sixteen degenerated intervertebral discs were obtained from 12 patients operated on for degenerative disc disease (Table 3.). In the operation the anterior fusion method was used and the degenerated disc was removed. After removal, anterior annulus fibrosus, posterior annulus fibrosus, and nucleus pulposus were each rapidly frozen to –70^{o} C in the operating theatre.

Table 3. Clinical data of the patients operated on for degenerative disc disease

Patients (n)	12
Gender (F/M)	5/7
Age (years)	29-63
(mean)	44.8

5.1.3 Normal control disc tissue (DN)

As a normal control, disc material from eight discs from a tissue bank of five organ donors was used (Table 4.). None of the donors had a history of lower back pain. No sign of autolysis was observed, i.e. all discs looked intact and they all showed a normal morphology, macroscopically. The mean age of the donors was 36.2 (range, 13-53).

Table 4. Clinical data of the normal control disc patients (organ donors)

Patients (n)	5
Gender (F/M)	3/2
Age (years)	13-53
(mean)	36.2

5.2 Immunohistochemistry

All tissue material was treated identically. After removal the tissue material was immediately rinsed in phosphate-buffered saline (PBS) and frozen in liquid nitrogen to –70°C in the operating theatre. The tissue material was kept in a –70°C freezer until analysis.

Using the avidin-biotin-peroxidase (ABC) method (Hsu et al. 1981) 8 μm thick cryostat sections were subjected to indirect immunohistochemistry. Prior to immunostaining, slides were fixed in ice cold (4°C) acetone at room temperature. In the TGFβ study (III) some specimens were prefixed in Zamboni solution, to detect a possible difference in immunoreaction between prefixed and section-fixed samples. Prior to treatment with primary antibody, non-specific binding was blocked by treatment with non-immune serum (horse normal serum).
Endogenous peroxidase was inhibited by incubation in 0.3% H_2O_2 in 70% methanol-PBS at room temperature for 30 minutes, before treatment with the ABC reagent.
Control sections were stained omitting the primary antibody. Furthermore, preincubation with the corresponding antigen was performed (at 1:10). As a positive control, tissue material from rheumatoid synovia was used.

5.2.1 Detailed protocol for immunostaining method of frozen sections using avidin-biotin-peroxidase complex (ABC) (Hsu et al. 1981) (I-V)

Incubations were carried out at room temperature in a humid chamber. The staining sequence was as follows:
1) Incubation with diluted normal serum for 20 min, 2) blotting out of excess serum and incubation with primary antibody overnight at 4°C, 3) rinsing section slides in PBS for 10 min, 4) incubation with diluted biotinylated antibody for 30 min, 5) rinsing in PBS for 10 min, 6) inhibiting endogenous peroxidase activity by incubation for 30 min in 0.3 % H_2O_2, 7) rinsing for 20 min in PBS, 8) incubation of sections for 30-50 min with diluted Vectastain ABC reagent, 9) rinsing in PBS for 10 min, 10) incubation for 2-7 min with peroxidase substrate solution (DAB), 11) rinsing

in tap water for 5 minutes, 12) 1-2 min counterstaining with haematoxylin, 13) rinsing in tap water for 5 min, and finally, 14) embedding in Aqua mount (BDH Limited, Poole, UK).

Table 5. Summary of the antibodies used in these studies (I-V)

Antibody specificity	Antibody type	Studies in which used	Source
bFGF	pAb	I,V	R & D Systems Inc, Minneapolis, Minn, USA
c-Fos	mAb	IV	Santa Cruz Biotechnology, Santa Cruz, CA, USA
c-Jun	mAb	IV	Santa Cruz Biotechnology, Santa Cruz, CA, USA
PDGF	pAb	II, V	R & D Systems Inc., Minneapolis, Minn, USA
TGFβ-I	pAb	III, V	Santa Cruz Biotechnology, Santa Cruz, CA, USA
TGFβ-II	pAb	III, V	Santa Cruz Biotechnology, Santa Cruz, CA, USA
TGFβ receptor type II	pAb	III, V	Santa Cruz Biotechnology, Santa Cruz, CA, USA
VEGF	pAb	II	Santa Cruz Biotechnology, Santa Cruz, CA, USA
vWF	pAb	I,II	Dakopatts, Copenhagen, Denmark

bFGF: basic fibroblast growth factor, c-Fos: oncoprotein c-Fos, c-Jun: oncoprotein c-Jun, mAb: monoclonal antibody, PDGF: platelet-derived growth factor, pAb: polyclonal antibody, TGFβ:

transforming growth factor β, VEGF: vascular endothelial growth factor, vWF: von Willebrand factor

5.3 Statistical analyses

Statistical analysis was done either using non-parametric Mann-Whitney test (III) or Chi-square and Fisher exact tests (IV, V). Mann-Whitney test was performed using SOLO statistical software program (BMDP, Los Angeles, CA, USA) (III) and the Chi-square and Fisher exact tests either with the same program (IV) or the Sigma Stat Version 1.0 (Statistic software, Jandel Scientific GmgH, Erlerath, Germany) (V). Level of statistical significance was set at $P < 0.05$.

5.4 Quantitation by immunohistochemistry

The number of immunopositive cells was estimated semiquantitatively by using the following grades: -/immunonegative, no cells, (+)/(immunopositive), a few scattered cells, and +/immunopositive, abundant cells (more than 20 immunopositive cells in total). Two observers performed the cell counting independently and analyzed three different sections, per antibody. If the result differed between sections, we chose the highest immunopositive cell estimate.

The TGFβ study required a more detailed immunoquantitation. (III) Again two observers performed the analyses in blind review. Positive staining for TGFβ antibodies was quantified as the ratio of positive disc cells per cross sectional area compared to all disc cells. We took five random microscopic fields at the magnification 250x from all control discs and ten herniated discs, and used the mean cell counts obtained by the two observers. Altogether, counts included 50 fields from herniated disc samples and 40 fields from normal controls. Different groups were then compared statistically, as previously described.

6. Results

Tables 6 and 7 summarize the immunostaining results of studied growth factors in normal control disc tissue, in degenerated intervertebral disc tissue, and in disc herniation tissue. Table 6 shows the total occurrence of these factors, and Table 7 differentiates between disc cell immunopositivity and blood vessel associated immunopositivity.

Table 6. Occurrence of growth factors in normal and pathological disc tissues.

	bFGF	**PDGF**	**VEGF**	**TGFβ1**	**TGFβ2**	**TGFβrec**
DN	-	-	-	100%	100%	100%
DD	100%	-	N.A.	94%	94%	100%
DHT	81%	78%	88%	100%	100%	100%

DN = normal disc, DD = degenerated disc, DHT = disc herniation tissue; bFGF = basic fibroblast growth factor, PDGF = platelet-derived growth factor, VEGF = vascular endothelial growth factor, TGFβ = transforming growth factor beta, TGFβrec = transforming growth factor beta receptor type II. N.A. = not analyzed.

Table 7. Occurrence of growth factors in disc cells (DC) and blood vessels (BV) in normal and pathological disc tissues.

	bFGF	**PDGF**	**VEGF**	**TGFβ1**	**TGFβ2**	**TGFβrec**
DN						
DC:	-	-	-	100%	100%	100%
BV:	-	-	-	-	-	-
DD						
DC:	100%	-	-	94%	94%	100%
BV:	some	-	N.A.	some	some	some
DHT						
DC:	67%	38%	-	100%	100%	97%
BV:	48%	54%	88%	58%	37%	37%

DN = normal disc, DD = degenerated disc, DHT = disc herniation tissue; bFGF = basic fibroblast growth factor, PDGF = platelet-derived growth factor, VEGF = vascular endothelial growth factor, TGFβ = transforming growth factor beta, TGFrec = transforming growth factor beta receptor type II. N.A. = not analyzed.

6.1 Basic fibroblast growth factor (bFGF) immunoreactivity in blood vessels and cells of disc herniations (I)

Histologically, 11/27 DHT samples contained only annular tissue, 9/27 contained both annulus fibrosus and nucleus pulposus, whereas 5 contained only nuclear tissue, and two of the DHT samples remained undefined. The classification was made by morphology.
Altogether, 22/27 (81%) samples showed bFGF immunopositivity. bFGF immunopositive capillaries were found in 13/27 (48%) of the DHT samples, and 18/27 (67%) of the disc cells were also bFGF immunopositive. The latter finding often formed small clusters (Figure 1 in I). The capillary bFGF (Figure 3b in I) immunopositivity was confirmed by colocalization of von Willebrand factor (vWF) immunoreactivity (Figure 3a in I). All capillaries, however, were not bFGF immunopositive.

All disc protrusions contained bFGF immunopositive capillaries. Eight (62%) sequesters showed vascular bFGF immunoreactivity, and two of the extrusions contained bFGF immunopositive vascular ingrowth. Furthermore, nine (69%) of the sequesters showed bFGF immunopositive disc cells, and seven out of eleven (64%) extrusions contained bFGF immunopositive disc cells.

Sections stained, omitting the primary antibody, did not show any bFGF immunoreactivity (Figure 4 in I). The five normal control discs did not show any bFGF immunoreactivity either.

No clear evident relationship existed between the preoperative radicular pain duration and the localization of the bFGF immunoreaction, vascular or cellular. For all patients the median duration of such pain was 3.5 months (range 0.5-16 months). For those exhibiting both vascular and cellular bFGF immunoreactivity median pain duration was 3 months (range 1.5-9 months), and for those exhibiting only cellular immunoreactivity it was 3.5 months (range 1.5-16 months). In addition, for the patients totally immunonegative the median duration of sciatica was 1.5 months, the range being wide (0.5-12 months).

6.2 Platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) expression in disc herniation tissue: an immunohistochemical study (II)

Altogether, 38 DHT samples (78%) showed PDGF immunopositivity. In 27 (54%) this immunoreactivity was located in capillaries (Figure 1a in II), whereas in 19 (38%) disc cells were immunopositive (Figure 2 in II). Six samples (12%) also showed PDGF immunopositive fibroblasts (Figure 3 in II). VEGF immunopositive capillaries were noted in 44/50 (88%) of the DHT samples (Figure 1b in II). Once again, this capillary immunoreactivity was confirmed by immunolocalization of vWF to the same capillaries (Figure 1c in II).

The five normal control discs did not show any immunoreactivity for either of the growth factors (Figure 4 in II). Furthermore, sections stained omitting the primary antibody, did not show any immunoreactivity.

VEGF or PDGF immunopositivity did not show any dependence on preoperative pain duration.
In all DHT patients the mean pain duration was 4.12 months (median 3 months). In the VEGF immunopositive group this was 4.51 months (median 3 months), and for the PDGF immunopositive group 4.16 months (median 3 months). In the VEGF immunonegative group mean duration of pain was 4.08 months (median 4 months), and in the PDGF immunonegative group 3.54 months (median 3 months).
More specifically, in the group where disc cells showed VEGF immunopositivity the mean duration of preoperative radicular pain was 5.46 months (median 4 months), and the PDGF immunopositive capillary group 4.13 months (median 3.5 months).

When comparing PDGF and VEGF immunoreactivity in different prolapse types, it was noted that 23/25 extrusions showed VEGF immunoreactivity and 21/25 PDGF immunoreactivity. PDGF immunopositivity was located in 11/25 extrusions of disc cells, in 16/25 in capillaries, and in 3/25 in fibroblasts.

In sequesters VEGF immunopositivity was detected in all 20 studied samples. PDGF immunoreactivity was observed in 16 samples. In eight discs this finding was cell associated, in 10 discs blood vessel associated, and in three discs present in fibroblasts.
Of the protrusions, one showed immunoreactivity to both PDGF and VEGF. Two of the DHT samples remained undefined by the operating spine surgeon.

When comparing different age groups, VEGF did not show any age dependence, and the finding was present in more than 80% of the samples. Blood vessel associated PDGF immunoreactivity was more common in younger patients (under 40 years of age).

6.3 Transforming growth factor (TGF) β receptor induction in herniated intervertebral disc tissue: an immunohistochemical study (III)

All discs studied, both DHT and DN, showed TGFβ-1 (Figure 1a in III), TGFβ-2 (Figure 1c in IIII, Figure 2a in III) and TGFβ receptor type II immunopositivity (Figure 1b in IIII). The immunoreaction was mainly disc cell associated, located in the cytoplasm of the cells. In some DHT samples, TGFβ immunopositive capillaries also existed.
All studied DHT samples showed TGFβ-1 and TGF β-2 immunopositive disc cells, whereas only one such sample lacked such immunoreactivity for TGFβ receptor type II.
Capillary immunoreactivity was observed in 22/38 (TGFβ-1), 14/38 (TGFβ-2) and 14/38 (TGFβ receptor type II) DHTs.
We did not see any difference between different prolapse types with respect to immunoreactivity.

Sections stained omitting the primary antibody, did not show any immunoreactivity. Furthermore, preincubation with the corresponding antigen blocked the immunoreactivity (Figure 2b in IIII). The positive rheumatoid arthritic synovia controls showed immunopositivity for all three antibodies, and the comparison between pre-fixed and section-fixed samples did not show any differences in immunoreactivity.

Normal control discs (n=8) showed fewer TGFβ and TGFβ receptor immunopositive disc cells than DHT samples. When comparing the two groups by Mann-Whitney test, significant differences ($p<0.0001$) were calculated for all three antibodies.

6.4 Oncoproteins c-Fos and c-Jun immunopositive cells and cell clusters in herniated intervertebral disc tissue (IV)

All studied control discs were immunonegative for both c-Fos and c-Jun. Rheumatoid arthritic synovia and dermal samples, studied as positive controls, showed c-Fos and c-Jun immunopositive cells. Sections stained omitting the primary antibodies, did not show immunoreactivity, neither did sections preabsorbed with the corresponding antigen.

c-Fos immunoreactivity was observed in 15/38 (39%) of DHT samples, and c-Jun in 28/38 (74%). Immunopositive clusters of disc cells (conglomerates, i.e. ≥ 3 cells) stained in 7/28 (25 %) of the total immunopositive samples. The immunoreaction in these clusters was mainly of the c-Jun type.

No gender related differences could be observed regarding immunoreactivity. Furthermore, there were no statistically significant differences regarding proto-oncogene immunoreactions in extrusions compared with sequestrations. The total number of protrusions (n=2) was too small to make any statistical analysis.

No statistically significant oncoprotein expression difference existed between the radicular pain duration groups. Furthermore, the presence of immunoreaction in cell groups (conglomerates) did not show any statistical difference between different radicular pain duration groups.

6.5 Growth factor expression in degenerated intervertebral disc tissue. An immunohistochemical analysis of TGFβ, FGF and PDGF (V)

According to the Dallas Discogram Description the degree of degeneration was calculated as level 3 in five discs and level 2 in six discs. In one disc the degree of degeneration was level 1, and discography data were missing from three discs.

TGFβ-1 (Figure 1b in V) and TGFβ–2 immunoreactivity occurred in 15/16 discs, whereas all discs were TGFβ receptor type II immunopositive (Figure 1c in V). Furthermore, all discs were bFGF immunopositive, but none showed any PDGF immunoreactivity (Figure 1a in V).

In normal control discs no PDGF or bFGF immunoreactivity was observed, whereas TGFβ-1, TGFβ–II, and TGFβ receptor type II immunoreactivity did occur.
Sections stained, omitting the primary antibody and sections preabsorbed with the corresponding antigen, were totally immunonegative.

In degenerated discs, immunoreactivity was observed in either fibroblast-like disc cells, mainly seen in the annular region of the disc, or in chondrocyte-like disc cells, scattered more evenly throughout the disc, but especially noted in nucleus pulposus. Fibroblast-like disc cells were spindle shaped, often in lines, whereas chondrocyte-like disc cells were more rounded, often forming cell clusters.
We also detected immunopositive blood vessels.

When statistically comparing the immunostaining results for anterior annulus fibrosus, posterior annulus fibrosus and nucleus pulposus, specific differences could be observed:
In the anterior part of the annulus, bFGF immunopositivity was far more common in chondrocyte-like disc cells than in fibroblast-like disc cells ($p=0.0063$). In posterior annulus fibrosus, such overexpression of immunopositive chondrocyte-like disc cells was observed for both bFGF and TGFβ-2 ($p=0.0001$ and $p=0.0092$). TGFβ-1 immunopositive chondrocyte-like disc cells were more common in posterior annulus fibrosus than in the anterior part. Comparing nucleus pulposus with anterior annulus fibrosus, chondrocyte-like disc cell immunopositivity was statistically more often

observed in nucleus pulposus for TGFβ-1, TGFβ-2, and TGFβ receptor type II (p=0.016, p=0.0006 ,and p=0.012).

TGFβ receptor type II immunopositivity in fibroblast-like disc cells was statistically observed more often in the anterior part of annulus fibrosus than in nucleus pulposus (p=0.016).

Furthermore, in nucleus pulposus, immunoreactivity for all growth factors (bFGF, TGFβ-1, TGFβ-2, and TGFβ receptor type II) was far more often located in chondrocyte-like disc cells than in fibroblast-like disc cells (p=0.00000006, p=0.0011, p=0.00002, and p=0.000003)

7. Discussion

7.1 FGF in herniated disc tissue (I)

Blood vessels are not usually observed in adult intervertebral disc. Yasuma et al (1993) has demonstrated vascular invasion as a sign of intervertebral disc aging. In their cadaver study, they have seen capillary invasion only in patients over 40.
FGF has been demonstrated to be a potent inducer of angiogenesis (Buntroc et al. 1982, Gospodarowicz et al. 1977) as well as chondro- and osteogenesis (Ellsworth et al. 2002, Ohbyashi et al. 2002).

FGF research on intervertebral disc tissue has started with a novel finding of the stimulatory effect of this growth factor in *in vitr*o disc cells (Thompson et al. 1991). By now, this finding has immunohistochemically been located (Doita et al. 1996; Melrose et al. 2002; Nagano et al. 1995) both in degenerated and in herniated intervertebral disc tissue. Furthermore, FGF effect on the intervertebral disc has been analyzed in an experimental model in which FGF was injected into the epidural space (Minamide et al. 1999). FGF enhanced the degradative process in a dose-dependent manner.

We observed bFGF immunopositivity (Tolonen et al. 1995) in 22/27 (81%) herniated discs (Table 6.). This immunoreactivity was especially abundant in chondrocyte-like disc cells (67%), but also in blood vessels (48%) (Table 7). Small capillaries were identified by immunostaining with vWF, a marker for capillary endothelial cells. All vWF immunopositive vessels did not stain for bFGF. Colocalization of bFGF in some of these vWF immunopositive capillaries in parallel slides, led to the postulation of an active neovascularization process in herniated disc tissue. It should also be noted that FGF immunoreaction in blood vessels may show particularly active blood vessel formation from capillary endothelial cells (Eguchi 1992). We also observed bFGF immunopositivity in the granulation tissue area near the surface of the prolapsed disc. Doita et al (1996) noted similar result. They reported especially intense staining in extruded disc samples compared to protruded ones. Furthermore, in an animal model mimicking the disc degeneration process, bFGF was localized especially in cells and vessels near the trauma area (Melrose et al. 2002). Interestingly, the peak of this expression occurred after 12 months, and thereafter

diminished. In addition, FGF has shown direct enhancement of proteolytic activity in tissues, even though it also induces the pericellular activator inhibitor (Lucore et al. 1988; McCarron et al. 1987). The FGF-dependent endothelial cell invasion, however, is inhibited by metalloproteinase inhibitors, such as TIMP, which counteracts stromelysin and collagenases (Edwards et al. 1987; Mignatti et al. 1986; Mignatti et al. 1989). Moreover, FGF has been observed to enhance the expression of TIMP regulating the proteolytic activity in extracellular matrix, and to regulate plasminogen activator activity (Laiho and Keski-Oja 1989). In addition, FGF stimulates plasminogen activator and interstitial collagenase production (Moscatelli et al. 1986). Plasminogen activation is the most effective inducer of extracellular matrix proteolysis (Saksela et al. 1988). Plasminogen is activated to an effective serine protease plasmin, which degrades most extracellular glycoproteins and protein parts of the glycosaminoglycans. Furthermore, plasmin activates latent collagenase, possibly together with stromelysin (He et al. 1989). Previously, Ng et al (1986) demonstrated such proteolytic activity in disc herniation tissue. We mainly localized FGF immunoreactivity to newly formed capillaries and in chondrocyte-like disc cells. Thus, FGF may also regulate the proteolytic activity in disc herniation tissue. This is further supported by the finding that these same chondrocyte-like disc cells are immunopositive to stromelysin (Grönblad et al. 1994).

In our study all protruded discs were immunopositive to FGF, but sample number (n=3) were too small to make any further conclusions. The prolapse type did not otherwise show any statistical difference with respect to the immunolocalization of FGF, either cellular or vascular. Furthermore, the preoperative radicular pain duration did not show any clear relationship to the expression of FGF. In totally immunonegative samples, however, pain duration was the shortest with a mean time of only 1.5 months. In discs showing only a vascular reaction the mean preoperative radicular pain duration was 3 months and for both the vascular and the cellular expression group it was 3.5 months. The total number of studied discs was, however, too small to give further statistical power. Furthermore, a too wide range of preoperative radicular pain durations in different groups, existed.

An earlier observation of the FGF peak expression at 12 months after trauma in an animal model (Melrose et al. 2002), and the immunolocation of FGF in disc cells and vessels in herniated discs in our study, especially the lack of FGF in normal control discs, suggests that FGF is inactive in normal avascular adult intervertebral disc. After some kind of trauma, either mechanical, as in experimental models, or with herniation into the epidural space, an active neovascularization of the disc is initiated. This first starts in the granulation tissue area, either near the trauma, or near the surface of prolapsed disc, and thereafter spreads throughout the disc, thereby accelerating the

resorption process. This can be noted by follow-up studies of disc herniation (Maigne et al. 1990; Saal et al. 1990).

Intense expression of FGF in disc cells and capillaries, the activation of neovascularization and proliferation induced by FGF in an experimental model, and especially the stimulation of disc resorption after disc herniation (Minamide et al. 1999), confirm a central role for FGF in intervertebral disc tissue.

The presence of bFGF in disc cells and capillaries in disc herniated tissue, and the total absence of bFGF in normal control disc tissue, underlines the importance of bFGF in disc cell metabolism, and also in vascular endothelial cell proliferation.

7.2 VEGF and PDGF in herniated disc tissue (II)

VEGF is a potent inducer of angiogenesis, both *in vitro* (Ferrara and Henzel 1989; Connolly 1991) and *in vivo* (Carlevaro et al. 2000). In addition to our study on human herniated disc tissue (Tolonen II, 1997) there are two additional observations of this growth factor in intervertebral disc tissue. Haro et al (2002) showed that co-culturing human herniated discs with murine peritoneal macrophages, induced intense expression of VEGF in macrophages and neovascularization in herniated disc tissue. Koike et al. (2003) localized VEGF in herniated human lumbar discs in spindle-shaped cells in granulation tissue area. PDGF stimulates migration of smooth muscle cells, and is a true chemoattractant for vascular smooth muscle cells (Grotendorst et al. 1982). Additionally, it stimulates cell replication and protein synthesis (Canalis 1985) as well as invasion by fibroblasts (Gosiewska et al. 2001). Only three studies show PDGF in intervertebral disc tissue. Our group has located PDGF immunohistochemically in herniated disc tissue (Tolonen II, 1997) and analyzed PDGF in human degenerative discs (Tolonen V, 2005). In cell culture additional PDGF has been shown to reduce apoptotic activity (Gruber et al. 2000a).

Chondrocyte-related data of both these growth factors (PDGF and VEGF) are mainly from cartilage research. PDGF stimulates cell migration and DNA-synthesis in a dose-dependent manner (Kieswetter et al. 1997). Interestingly, especially young chondrocytes express PDGF intensely (Guerne et al. 1995). Furthermore, the expression of PDGF is intense in osteoarthritic chondrocytes

(Moos et al. 1999). VEGF, on the other hand, has been shown to be expressed in hypertrophic chondrocytes and osteoarthritic chondrocytes (Pufe et al. 2001). In cartilage VEGF has a prominent role during both growth and repair (Horner et al. 1999). It is, however, absent in chondrocytes of the resting zone, and expression is weak in the proliferation zone chondrocytes, whereas in the hypertrophic zone expression of chondrocytes is intense.

Several studies report an active neovascularization process in herniated intervertebral disc tissue (Freemont et al. 2002; Haro et al. 2002; Minamide et al. 1999; Ozaki et al. 1999; Tolonen et al. I; II; III). We observed VEGF expression in 44/50 (88%) of herniated discs, and PDGF in 38/50 (78%) (Tolonen et al. II). VEGF expression was located in the capillaries and PDGF was present in 54% in capillaries, in 38% in chondrocyte-like disc cells, and in 12% in fibroblast-like disc cells. Interestingly, PDGF immunopositivity was observed particularly in the youngest group of patients (< 40 years). This may suggest that the reactivity for PDGF decreases with age. Preoperative pain duration did not relate to the reactivity. Nor was any statistical difference between different prolapse types seen. Only one of the three protrusions showed immunoreactivity for PDGF and/or VEGF, whereas all sequesters were VEGF immunopositive and 23/25 extrusions showed VEGF immunoreactivity. In both sequesters and extrusions PDGF immunoreaction was located more often in capillaries than in disc cells. We also, however, noted disc cell associated reaction either in chondrocyte-like or in fibroblast-like disc cells.

The presence of active angiogenic growth factor VEGF in herniated disc tissue, both in capillaries (Tolonen et al. II) and in spindle-shaped cells (Koike et al. 2003) suggests an active role for this growth factor in the neovascularization process. Indeed, a linear correlation for cellular VEGF expression in endothelial cells and macrophages has been demonstrated (Koike et al. 2003).

The expression of PDGF both in disc cells and in capillaries in disc herniation may suggest an active role for this growth factor in the regulation of intradiscal cells and vascular endothelial cells. On the other hand, normal control discs were totally immunonegative for this growth factor.

7.3 Transforming growth factor beta in herniated disc tissue (III)

TGFβ stimulates extracellular matrix component formation and regulates cellular growth and extracellular proteolysis (Campbell et al. 1994; Ignotz and Massagues 1986; Laiho and Keski-Oja 1986; Raghow et al. 1987; Roberts et al. 1986; Wright et al. 1991). Furthermore, TGFβ participates in angiogenesis and in the early phase of inflammation (Lotz et al.1992; Pujol et al. 1991; Roberts et al. 1986). Such proteolytic activity also exists in herniated disc tissue (Ng et al. 1986).

TGFβ has a well-documented role in chondrocyte maturation (Dangelo et al. 2001). Furthermore, it is postulated that TGFβ in chondrocytes can by an autocrine route regulate the contents of extracellular matrix (Wang et al. 2002). It inhibits matrix metalloproteinase inhibitors (Pattison et al. 2001). Especially intense expression has been noted during development. For example, in the developing growth plate, hypertrophic chondrocytes, chondroclasts, osteoblasts and osteoclasts expressed TGFβ (Thorp et al. 1992).

In addition, TGFβ has a dual role, it is stimulatory for precursor cells and inhibitory for mature cell lines (Bostrom and Asnis 1998). In cell culture with low serum concentration, TGFβ also shows inhibitory effects on cell growth, whereas addition of 10% calf serum changes the action of TGFβ stimulatory for cell growth (Pujol et al. 1994). Chondrocytes have a specific mechanism in TGFβ function (Pedrozo et al. 1999). They can activate the latent TGFβ from extracellular matrix.

It is notable that in our study (Tolonen et al. III) all samples, both herniated discs (n=50) and normal control discs (n=8), were immunopositive to TGFβ-1, TGFβ-2 and TGFβ receptor Type II. This reaction was located in chondrocyte-like disc cells and in some samples in capillaries. The most important finding was that by immunoquantitation assay the number of immunopositive cells for both TGFβ and receptor Type II was statistically ($p<0.0001$) higher in herniated discs than in control discs. The 95% CI did not overlap at all. The key finding was that the number of TGFβ receptor Type II positive cells increased. This would suggest that TGFβ can autoregulate its receptor and promotes receptor induction in herniated intervertebral discs. It could be that as in cartilage, TGFβ may be latent in extracellular matrix, and that chondrocyte-like cells can respond as chondrocytes and that TGFβ may in an autocrine way activate disc cells and latent TGFβ. The neovascularization seen after disc herniation allows another route to TGFβ for disc cell stimulation.

In intervertebral disc tissue TGFβ is, in addition to lymphokines, the most studied cytokine. During development and growth its expression decreases with age (Matsunaga et al. 2003; Nagano et al.

2000). Both fibrocartilaginous cells in annulus fibrosus and notochord-like cells in nucleus pulposus, show intense TGFβ expression in young discs. Furthermore, Thompson et al (1991) showed that especially TGFβ stimulates disc cells in cell cultures. Cells from nucleus pulposus and the transition zone were more reactive than cells from annulus fibrosus. In later studies, an intense stimulation of both nucleus pulposus and annulus fibrosus cells has been demonstrated (Alini et al. 2003).

In an experimental animal model for the intervertebral disc degeneration process, TGFβ was located in disc cells and vessels in the granulation tissue area near the annular tear (Melrose et al. 2002). Interestingly, the investigators also noted scattered TGFβ immunopositive cells in control discs. Furthermore, TGFβ-1 gene transferred into a rabbit intervertebral disc shows 100% elevation in proteoglycan synthesis and a 30-fold more active TGFβ-1 presentation in nucleus pulposus cells (Nishida et al. 1999).

Our study was mainly concerned with extruded and sequestrated disc herniations. In disc protrusions TGFβ-1 is located in chondrocyte-like disc cells (Specchia et al. 2002). Konttinen et al (1999) only occasionally observed TGFβ -1, TGFβ–3 and TGFβ receptor Type II. This difference in results could be due to the small total number (n=10) of herniated discs in their study. Nor did they report the type of prolapse or preoperative pain duration. In that study RT-PCR for TGFβs and receptor were also negative, but another study demonstrated RT-PCR positivity in human herniated disc specimens (Ahn et al. 2002). Ahn et al. (2002) observed TGFβ in 5/10 studied disc samples. Thus, the intervertebral disc appears to be a difficult tissue for modern molecular biological techniques. Perhaps the main reason for this is the small number of total cells in disc tissue and the huge overexpression of extracellular matrix and its components compared to disc cells. Nevertheless, a need exists for further investigation of the role of active and latent TGFβ and their location in the extracellular matrix, as well as the total activation process in intervertebral disc tissue.

We showed marked statistical difference between TGFβ and its receptor type II immunopositivity in disc herniation tissue compared to such immunopositivity in normal control disc tissue, highlighting the activation of TGFβ. Especially the statistical difference in TGFβ receptor type II expression suggests receptor induction in disc herniation tissue. Such an extended expression in disc herniation tissue, compared to normal control disc tissue may suggest pronounced local production of TGFβ and positive loop control of the production: An elevated level of TGFβ may stimulate the receptor production and the enhanced receptor activation further stimulate the production of TGFβ.

TGFβ is present and active during the development and growth of the intervertebral disc. During maturation and after disc tissue has become avascular, TGFβ does not disappear. It is still present in cells of avascular tissue and perhaps also in a latent form in extracellular matrix, being again activated after some kind of trauma, either mechanical trauma affecting the annular area or degeneration and herniation of the intervertebral disc. Furthermore, TGFβ allows for autoregulation of the cells through its receptor induction.

7.4 Oncoprotein c-Fos and c-Jun expression, cellular activation and cell clusters in herniated disc tissue (IV)

A healthy adult disc contains relatively few cells distributed sparsely throughout the disc. In contrast, in damaged or degenerated disc the cell number has increased. Disc cell clusters are typically seen (Pritzher 1977), particularly in damaged areas (Johnson et al. 2001). The proliferation markers PCNA (proliferation cell nuclear antigen) and Ki-67 have immunohistochemically been located especially in these clusters. PCNA is expressed more than Ki-67. These markers were not common in less degenerated discs or in areas of the degenerated discs where no clusters occurred (Johnson et al. 2001).

In addition, in herniated discs proliferation activity marker Ki-67 has been localized in disc cells (Paajanen et al. 1999). Interestingly, in that study Ki-67 occurred in first-time prolapses but was absent in recurrent ones. This may indicate that enhanced extracellular matrix proliferation is not associated with recurrent prolapses.

We observed components of the early transcription factor AP-1 (c-Fos and c-Jun) in human herniated discs (Tolonen et al. IV). Nuclear oncoproteins c-Fos and c-Jun (or their related proteins JunB or JunD) represent the AP-1, and they act as intracellular messengers converting short-term signals generated by extracellular stimulators into long-term changes in cell phenotype. This is done by regulating the expression of downstream genes that possess an AP-1 binding site (Angel et al. 1991).

The importance of AP-1 transcription factors in chondrocyte differentiation was highlighted *in vitro* by Thomas et al (2000). In particular, the induction of c-Fos resulted in concomitant increase in the expression of fra-1 and c-jun. The overexpression of c-Fos also directly inhibited chondrocyte

differentiation and it has previously been observed that the growth-stimulatory effect of TGFβ-1 in chondrocytes is mediated by activation of the c-fos oncogene (Osaki et al. 1999). This is mediated by protein kinase activation (Yonekura et al. 1999).

Interestingly, our normal control discs did not express these AP-1 factors. In herniated discs c-Jun was expressed more frequently than c-Fos, in 28/38 (74%) and in 15/38 (39%). This may indicate that in disc cell activation, after the disc herniation, c-Jun plays a more important role. Observation in cell culture studies show that either *c-fos* or *c-jun* alone, or a combination of these is able to stimulate matrix metalloproteinase –1 (MMP-1) transcription activity (Tsuji et al. 2000). Moreover, *c-fos* alone decreases the transcription activity of Type II collagen and TIMP-1. Furthermore, in another cell culture study the combination of c-Fos and c-Jun did not affect the transcription activity of TIMP-1, but stimulated that of MMPs (Tsuji et al. 1996). We did not observe lone c-Fos immunopositivity, whereas lone c-Jun immunoreactivity occurred as well as the immunoreactivity to both oncoproteins. This may suggest that proteolytic activity, in herniated disc also, is controlled by expression of c-Fos and c-Jun.

We saw no correlation when focusing on preoperative pain duration or gender related differences in oncoprotein expression. Furthermore, prolapse type did not seem to matter either.

Oncoprotein immunopositive cell clusters occurred in 7/28 (25%) of the studied herniated discs. Interestingly, that expression was mainly of the c-Jun type, possibly indicating, that c-Jun may be more important than c-Fos in the disc cell clusters. Indeed, turnover of matrix components is established with an intricate balance between synthesis and degradation of associate molecules, such as MMP and TIMP. Previously, *c-fos* had been observed to be degradative (Tsuji et al. 2000), whereas *Jun* related proteins stimulated both MMP and TIMP (Tsuji et al. 2000). This may indicate that disc cell clusters participate in the turnover of the extracellular matrix. Regulation of the previously noted degradative enzyme activity (Ng et al. 1986) and activation of matrix metalloproteinases (Roberts et al. 2000) in herniated disc tissue could also be partly mediated by AP-1 protein in disc tissue.

This presence of c-Jun, and also to some extent c-Fos, demonstrates that these clusters exhibit transcriptional activity, being active components in the herniated discs. The formation of such clusters is associated with degenerative disease (Pritzher 1977; Johnson et al. 2001). The pattern of proliferation cell nuclear antigen (PCNA) and proliferation associated antigen Ki-67 positivity in degenerated disc tissue samples, suggests that disc cell clusters arise through increased cell proliferation (Johnson et al. 2001). This is further supported by our observation of AP-1 proteins in disc cell clusters in herniated disc tissue. Furthermore, a previous observation of colony formation

in vitro, by annular cells from patients with degenerative disc disease, and from young normal discs, exists (Desai et al. 1999).

7.5 Cellular remodelling in different areas of degenerated intervertebral disc tissue (V)

Degenerated discs have lost their normal architecture, and changes occur both in nuclear and annular parts of the disc. Annulus cells change markedly (Pritzher 1977). Spindle-shaped fibroblast-like cells become more rounded chondrocyte-like cells. At the same time they are surrounded by unusual accumulations of extracellular matrix components (Gruber and Hanley 2000b).

We observed growth factor immunopositivity in both fibroblast-like disc cells and chondrocyte-like disc cells. Interestingly, PDGF immunopositivity was, however, entirely absent. One benefit of our study was the ability to compare different parts of the degenerated discs: nucleus pulposus, anterior annulus fibrosus and the ruptured posterior annulus fibrosus. Some important differences in growth factor expression in these areas exist. As could be expected in nucleus pulposus an intense staining occurred for all immunopositive growth factors in chondrocyte-like cells. Posterior and anterior annulus fibrosus (AF) differed from each other and from nucleus pulposus (NP). Posterior AF somewhat resembled NP. An intense immunopositivity was seen in chondrocyte-like disc cells for bFGF, TGFβ-2, and TGFβ receptor type II.

An intense immunopositivity was observed in anterior annulus fibrosus for bFGF and TGFβ receptor type II in chondrocyte-like cells and for TGFβ receptor type II in fibroblast-like cells. Many of these degenerated painful discs showed posterior or posterolateral ruptures within annulus fibrosus, and in some small protrusions. A few discs were totally ruptured. This growth factor expression result suggests that the important site for the degenerating process is the border between nucleus pulposus and posterior annulus fibrosus. Intense staining for growth factors in posterior AF in chondrocyte-like disc cells compared to fibroblast-like disc cells demonstrates that especially chondrocyte-like disc cells are probably activated. Clusters of these chondrocyte-like disc cells

were also observed. The activation of growth factors also in the anterior part of annulus fibrosus means that in the degeneration process the whole disc is involved. The signal spreads to the entire disc. In the anterior AF only bFGF showed an intense immunoreaction in chondrocyte-like disc cells. This may suggest that FGF is activated before TGFβ, in the cellular remodelling process. Interestingly, TGFβ receptor type II showed an intense reaction in both chondrocyte-like and fibroblast-like disc cells in anterior AF. Moreover, in fibroblast-like disc cells this was the only very intense immunopositive reactivity observed. Indeed, this could highlight the importance of TGFβ. It may be that at this stage TGFβ is activated from a latent form in extracellular matrix and that cellular activation comes later. Altogether, the observed growth factor immunoreactivity in disc cells suggests that these cells may be actively regulating the turnover of extracellular matrix components. An interesting subgroup was the most degenerated discs (degree of degeneration level 3 in the Dallas Discogram Description) (Sachs et al. 1987). None of these samples showed fibroblast-like disc cell immunoreactivity. In nucleus pulposus all samples showed chondrocyte-like disc cell immunopositivity, whereas in the annular area such immunoreactivity could only be observed for TGFβ receptor type II. Unfortunately the total number of such severely degenerated discs was too small for further statistical analyses.

In an experimental animal model the degeneration process could be delayed with reinsertion of autogenous activated nucleus pulposus (Okuma et al. 2000). This activation was produced by co culturing nucleus pulposus cells with annulus fibrosus cells. During the co-culture both cell types were proliferating. In addition, reinsertion of activated nucleus pulposus, especially delayed the formation of clusters of chondrocyte-like disc cells. We observed such clusters in both nucleus pulposus and annulus fibrosus. Furthermore, injection of intact nucleus pulposus is far more effective in delaying degeneration than injection of only nucleus pulposus cells (Nomura et al. 2001), thus highlighting the importance of the extracellular matrix. In addition, in chondrocyte-like disc cell clusters marked matrix metalloproteinase activity has been demonstrated (Roberts et al. 2000). Interestingly, such enzyme activity was particularly intense in herniated discs (Roberts et al. 2000). The presence of growth factors (Tolonen et al. I; II; III; V), matrix metalloproteinases (Roberts et al. 2000) and oncoproteins (Tolonen et al. IV) suggest that these particular chondrocyte-like disc cells have been activated in pathological processes (including degeneration and herniation) of the intervertebral disc tissue. They may regulate the turnover of extracellular matrix.

Since a time gap between discography and the operation existed in our study, the possible local irritation caused by the discography procedure was not present at the time when tissue samples were taken, for further analysis. Of note, we observed growth factor immunopositivity throughout the whole discs, not only restricted to the disrupted posterior annulus fibrosus.

Comparing the results of growth factor expression in degenerated intervertebral disc to that of herniated discs revealed some differences. Firstly, PDGF is expressed in herniated discs, but not in degenerated discs. Furthermore, normal control discs were also totally immunonegative for PDGF, possibly indicating that until PDGF is activated it may be in a latent form. Alternatively, PDGF is delivered together with inflammatory cells. The key element may be the posterior longitudinal ligament. We hypothesize that PDGF is not activated until the disc has ruptured through the posterior longitudinal ligament. Bulging through this ligament exposes the disc to the epidural space and inflammatory responses may proceed, and PDGF is delivered to the disc. One source of delivery may be macrophages. Nevertheless, the nature of PDGF activation will require further research.

8. Conclusions

The cell proliferative and angiogenic FGF can be concluded to be expressed in herniated intervertebral disc tissue, both in disc cells and in small capillaries. Furthermore, normal adult disc tissue does not express this growth factor. Thus, FGF most likely participates in neovascularization of herniated disc tissue and is part of a more extensive growth factor network regulating extracellular matrix turnover and cell proliferation. PDGF appears to have a similar expression pattern as FGF in herniated intervertebral disc tissue and in normal adult discs. VEGF is expressed in capillaries of herniated intervertebral disc tissue, but not in normal discs. The lack of expression of FGF, PDGF and VEGF, in normal disc tissue suggests that these growth factors are activated after disc herniation and may be participants in the trauma healing process. They may also have a role in disc tissue resorption after herniation. TGFβ differs from the previous growth factors. It is expressed in both normal and herniated disc tissue. The observed expression in normal adult disc tissue may suggest a specific role for TGFβ in the maintenance and control of normal intervertebral extracellular disc tissue matrix turnover. A clear receptor type II induction, in herniated disc tissue, and a statistically marked increase in expression of TGFβ compared to normal disc tissue exits, thus, suggesting TGFβ activation in herniated disc tissue.

Thus, one can conclude that PDGF, FGF, VEGF and TGFβ are all part of a disc growth factor network which crosstalks between cell surface, surrounding extracellular matrix, and the intracellular signal cascade system. Presently it is not known which growth factor is initially activated and which is the most important. TGFβ can be highlighted, however, also for its expression in normal adult disc tissue.

In herniated disc tissue the main cell type which expresses PDGF, FGF and TGFβ appears to be the chondrocyte-like disc cell. Because of the often undefined nature of herniated disc material, it is

difficult to determine whether true nucleus pulposus cells are the active cells or the spindle shaped cells from annulus fibrosus, that have remodelled in to a more chondrocyte-like cell type.

In disc herniation tissue cell activation is prominent. Oncoprotein c-Fos or c-Jun expression could not be shown in normal discs. In herniated disc tissue the expression was more often of the c-Jun type. The expression was mainly located in chondrocyte-like disc cells, and interestingly, we detected conglomerates (groups of immunopositive disc cells). This highlights the importance of chondrocyte-like disc cells and conglomerates acting as activation centres, in herniated disc tissue.

Different parts of the intervertebral disc tissue could separately be observed from the intervertebral disc material, of patients operated on for painful degenerative disc disease. Nucleus pulposus chondrocyte-like disc cells expressed FGF, TGFβ-1, TGFβ-2, and TGFβ receptor type II. In posterior annulus fibrosus such expression was observed for FGF, TGFβ-2, and TGFβ receptor type II in chondrocyte-like disc cells, whereas in anterior annulus fibrosus expression was shown for FGF and TGFβ receptor type II expression in chondrocyte-like disc cells and for TGFβ receptor type II in fibroblast-like disc cells. Even in the annulus fibrosus, chondrocyte-like cells are the main cells expressing growth factors. Interestingly, PDGF was totally absent in degenerated intervertebral disc tissue. PDGF could only be observed in herniated intervertebral disc tissue. Since PDGF is important in vascularization and tissue healing, its total absence in degenerated and normal disc tissue may partly explain poor healing properties of disc ruptures and fissures.

In the intervertebral disc tissue, growth factors actively participate in a network regulating disc cell growth, proliferation, extracellular matrix turnover and neovascularization. The cell type expressing growth factors and also oncoproteins, is the chondrocyte-like disc cell, highlighting the importance

of this particular cell type in the basic pathophysiologic events relating to disc degeneration and rearrangement.

9. Hypothesis of cellular remodelling in intervertebral disc tissue

A simplified hypothesis of the role played by various growth factors in intervertebral disc remodelling, from a normal vascular developing and growing disc tissue to an avascular stage, followed by pathological alterations induced by degeneration and disc herniation may be presented.

During development and growth the intervertebral disc is a vascularized tissue (A in Figure 1). Vascularity disappears during the second decade of life, during which time the disc obtains its normal avascular structure. At this stage an animal model reported intense expression of TGFβ (Matsunaga et al. 2003; Nagano et al. 2000) and IGF-1 (Osada et al. 1996). Interestingly, this expression decreased with age.

At the adult stage (B in Figure 1) disc cell nutrition is mainly supplied to the disc through the end-plates and disc cells are at a resting stage. Nucleus pulposus cells are rounded chondrocyte-like cells, and annulus fibrosus cells are mainly spindle-shaped fibroblast-like cells. At this stage disc cells express TGFβ-1, TGFβ–2 and TGFβ receptor type II. Despite the disappearance of vascularity during growth, disc cells may remain capable of reacting to possible neovascularization. Cell responses after trauma appear to be similar to those observed in growing disc tissue.

Mechanical trauma, overload, and genetic predisposition may produce the susceptibility for disruption of the circular collagen lamellae in annulus fibrosus (C in Figure 1), and activation of disc cells. The essential site may be the border between nucleus pulposus and inner zone of the posterior annulus fibrosus. Disc cells begin to produce, besides TGFβs, other growth factors influencing themselves, extracellular matrix, and the neovascularization process. Growth factors can act basically by three different routes (Figure 2): 1. Endocrine, in which the growth factor is delivered to the area by vascular supply, 2. Paracrine, the growth factor affects surrounding cells,

and 3. Autocrine, when the growth factor affects the producing cell itself. In a delayed paracrine route growth is activated from extracellular matrix, where the growth factor is stored.
An alteration of disc shape also exits. Additional annular cells become chondrocyte-like cells and the disruption of extracellular matrix proceeds, producing more serious tissue damage. This cellular remodelling may spread from the initial starting point, the border area between nucleus pulposus and posterior annulus fibrosus, to the entire disc. Different growth factors are expressed at various time points of this process. At the disc degeneration stage (C in Figure 1), nerve ingrowth is also coupled with neovascularization and, although debated, some authors have suggested that this process may be painful (Coppes et al. 1997; Freemont et al. 1997).

Later on, mechanical stress is more intense in the posterior region of annulus fibrosus due to more pronounced cellular remodelling and collapse of normal lamellar architecture of collagen. This may in the end lead to disc herniation (D in Figure 1), producing sciatica. At this stage (D) the neovascularization process and cellular remodelling still proceed. New elements also appear. Bulging of disc tissue through the posterior longitudinal ligament exposes the disc to the epidural space. Being there it can irritate surrounding tissue (Ozaki et al. 1999) and at the same time be affected itself. Inflammatory cells, for example macrophages, present in the granulation tissue area may deliver totally new growth factors to the intervertebral disc tissue. Growth factors may be part of an important network consisting of cytokines, lymphokines, proteolytic enzymes and their regulators. These factors affect the surrounding tissue and at the same time will be affected by other local factors. Furthermore, it may be that the key element, ” the most important growth factor” has not yet been recognized. Nevertheless, the disc cell, and especially the remodelling of annular disc cells may play an essential role in intervertebral disc metabolism and in maintenance of the structure of the disc.

With time the size of the herniated disc becomes smaller (Maigne et al. 1992; Saal et al. 1990). The cellular remodelling process continues over time in the prolapsed intervertebral disc tissue. The same process takes place in remaining intervertebral disc. This process may end in a steady-state situation (E in Figure 1), characterized possibly by a slow reorganization of the disc structure, or in reherniation. The disc degeneration process (C) may also end in a reorganized end-state (E).

Figure 1. Schematic showing the pattern of growth factor expression in normal young and adult intervertebral disc (A and B), in degenerated intervertebral disc (C) and in herniated intervertebral disc (D). (E) shows reorganization of disc tissue.

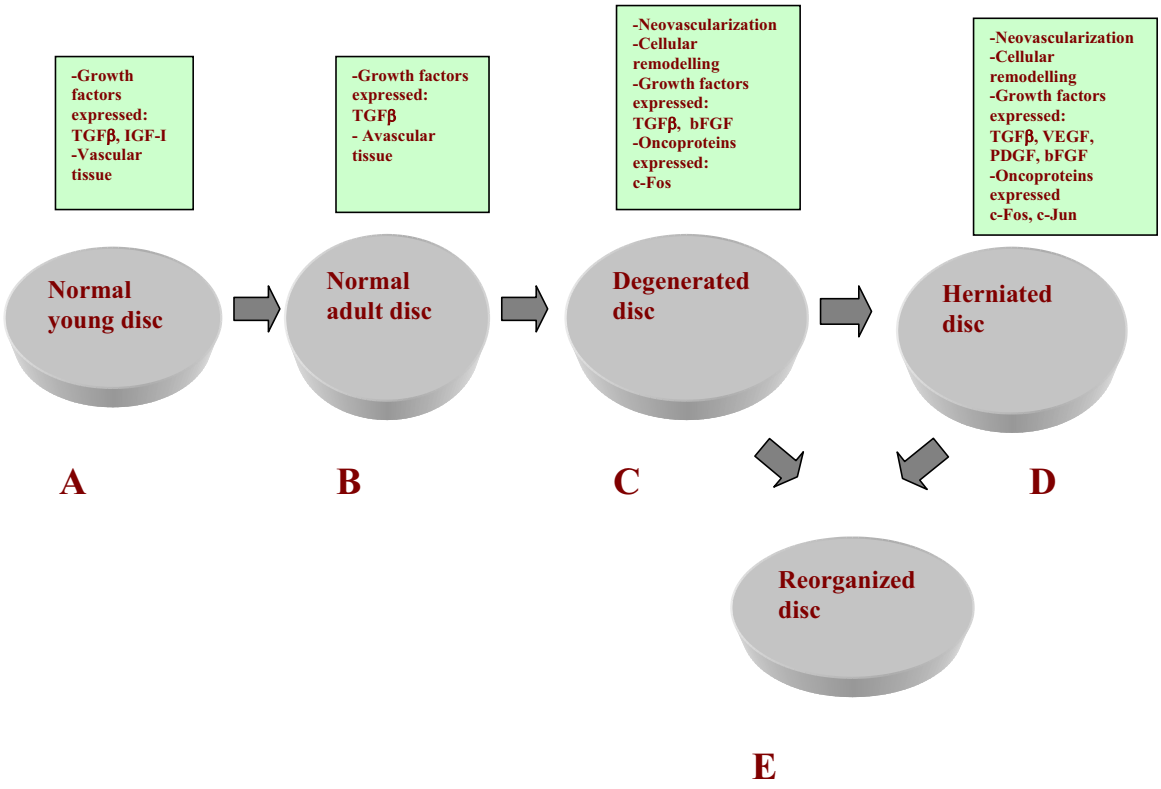
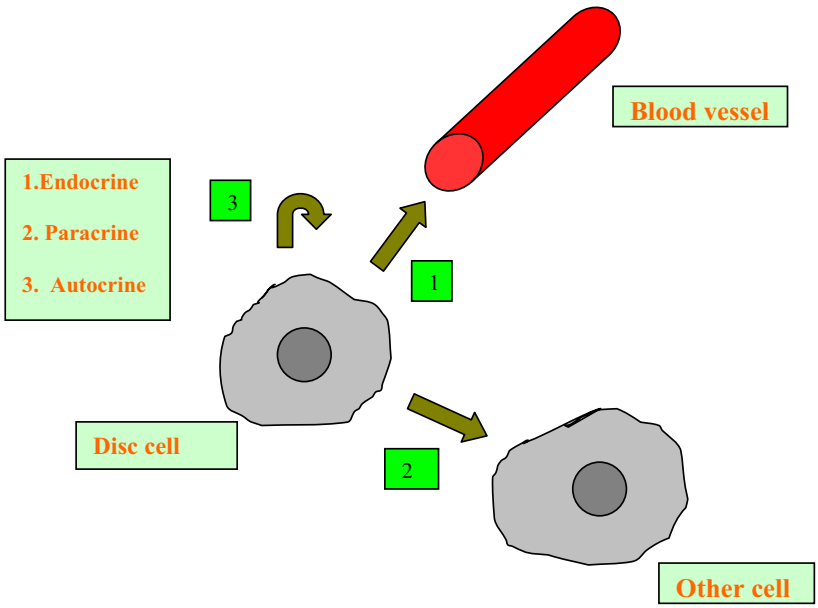


Figure 2. Schematic showing different routes of growth factor action.



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Basic Fibroblast Growth Factor Immunoreactivity in Blood Vessels and Cells of Disc Herniations

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Study Design. Basic fibroblast growth factor immunoreactivity was studied in disc herniation tissue.

Objectives. The first objective was to analyze in which tissue components, if any, fibroblast growth factor is expressed in the disc herniation. The second objective was to compare such expression with that in fresh cadaver disc tissue.

Summary of Background Data. Disc herniation tissue contains vascular ingrowth, which promotes the formation of granulation tissue. Fibroblast growth factor is a potent inducer of angiogenesis and also regulates extracellular proteolysis.

Methods. Twenty-seven disc herniation tissue and five macroscopically normal fresh cadaver discs were treated with an identical immunohistochemical protocol. Serial frozen sections were stained with a polyclonal basic fibroblast growth factor antibody and a polyclonal antibody to von Willebrand factor, which localizes endothelial cells. The immunostaining data were compared with relevant clinical data.

Results. Histologically, 74% of the samples contained anulus fibrosus and 59% nucleus pulposus. Basic fibroblast growth factor immunoreactivity was detected in 81% of the samples. There were immunopositive small blood vessels and scattered immunopositive disc cells (67%). Not all observed blood vessels were basic fibroblast growth factor immunopositive. In control discs, no immunoreactivity was observed.

Conclusions. The observed presence of fibroblast growth factor in small blood vessels suggests an active angiogenesis as a result of disc injury. Cellular expression of fibroblast growth factor may be linked to proteolytic activity in disc extracellular matrix. [Key words: fibroblast growth factor, disc herniation, intervertebral disc] **Spine 1995;20:271–276**

It has been estimated that disease of the intervertebral disc is responsible for 23% of the cases of low back pain.[1] The intervertebral disc may herniate posteriorly in three basic patterns. In protrusion, there is abnormal bulging of the the anulus fibrosus, which remains continuous. The periphery of the anulus fibrosus remains attached to the vertebral body bony rim. In extrusion disc, tissue is exposed to the epidural space. However, there is continuity with the disc. The third type of herniation is disc sequestration. In this type, there is free-disc tissue material in the epidural space that is no longer in continuity with the remaining disc.[39]

Disc herniation tissue has been suggested to contain vascular ingrowth, promoting granulation tissue formation.[18,20,22,43,44] Inflammation, either from direct chemical irritation[25,26,34] or secondary to an autoimmune response to the nucleus pulposus,[7,10,12,13,30,33] has been suggested to be involved in this granulation-inflammatory response.[11,20] The origin of the capillaries observed in the disc herniation tissue has remained unclear.

Growth factors, especially fibroblast growth factor (FGF), may be important as inducers of capillary ingrowth in the disc. They are polypeptides with a potential for mitogenesis, cellular differentiation, and matrix synthesis. Research concerning growth factor response in cartilage has dealt mainly with articular[2–6] and growth plate cartilage.[32,41] Thompson et al studied growth factor response in intervertebral disc cell cultures.[40]

Acidic and basic FGF (bFGF) are proteins (M_r 15,000–18,000) produced especially by tissues with high angiogenic activity.[14] They are potent stimulators of vascular growth and growth of fibroblasts and chondrocytes.[14] Thus, the effect of FGF is multifunctional. Fibroblast growth factor stimulates in angiogenesis endothelial cell migration and invasion.[14]

Fibroblast growth factor induces capillary endothelial cells to invade extracellular matrices and form capillaries, the development of which is proportional to the amount of FGF present.[27] Also, FGF regulates cellular functions. Therefore, it is significant to demonstrate whether FGF is expressed in disc herniation tissue. The goal of the present study was to investigate in detail FGF

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Table 1. Clinical Characteristics of Patients With Disc Herniation

Number	Age	Sex M/F	Duration of Pain (mo)	Operation Level
1.	41	F	9	L4–L5
2.	37	M	4, 5	L4–L5
3.	47	M	3	L4–L5
4.	43	M	3, 5	L5–S1
5.	28	M	16	L5–S1
6.	48	F	2	L4–L5
7.	44	M	4	Central L4–L5
8.	36	F	7	L5–S1
9.	32	M	3	L5–S1
10.	41	M	2, 5	L4–L5
11.	25	F	4	L5–S1
12.	40	M	3	L4–L5
13.	53	M	3, 5	L4–L5
14.	54	F	1, 5	L5–S1
15.	58	M	4	L4–L5
16.	53	M	1, 5	L4–L5
17.	36	M	4	L4–L5
18.	43	F	5	L5–S1
19.	52	F	6	L4–L5
20.	43	M	1	L3–L4
21.	46	M	1, 5	L4–L5
22.	55	F	12	L4–L5
23.	68	M	0, 5	L4–L5
24.	37	M	4	L5–S1
25.	59	M	0, 75	L4–L5
26.	53	M	2	L3–L4
27.	53	M	2	L4–L5

Table 2. Histologic Description of 27 Disc Herniation Tissue Samples

Number	Prolapse Type	Anulus/Nucleus	Location of FGF Immunoreactivity
1.	Sq	A	Blood vessels, cells
2.	Sq	A	Blood vessels, cells
3.	Sq	A	Blood vessels, cells
4.	P	A	Blood vessels
5.	Ex	A/N	Cells
6.	Sq	A	Cells
7.	Ex	A	Cells
8.	Sq	A	Blood vessels, cells
9.	Sq	A/N	Cells
10.	Ex	A	Blood vessels, cells
11.	Sq	A/N	Cells
12.	Ex	N	Cells
13.	Ex	A	Cells
14.	Sq	A/N	Blood vessels, cells
15.	Sq	A/N	Blood vessels, cells
16.	Ex	—	Cells
17.	Sq	A	Blood vessels
18.	Sq	A	Blood vessels
19.	Ex	N	No reaction
20.	Ex	A/N	Blood vessels
21.	Ex	N	No reaction
22.	Ex	—	No reaction
23.	Sq	N	No reaction
24.	Ex	A/N	Cells
25.	Sq	N	No reaction
26.	P	A/N	Blood vessels, cells
27.	P	A/N	Blood vessels, cells

Sq = sequester. P = protrusion. Ex = extrusion.

expression in disc herniation tissue and to compare it with vascular ingrowth. Fibroblast growth factor expression also was compared with patient clinical data. The hypothesis was that FGF expression may be more marked during the initial stages of clinical symptoms.

■ Material and Methods

Herniated disc material was obtained at surgery from 27 discectomy patients. Eleven of the herniated discs were extrusions, 13 were sequesters, and three were protrusions. The age of the patients varied from 25 to 68 years, and duration of the radicular leg pain symptoms before surgery was 0.5 months to 16 months (Table 1). For a normal control we used disc tissue from five fresh cadavers (31–53 years old). All of these discs appeared normal according to conventional histological staining. No signs of autolysis of tissue material were observed. Disc herniation tissues were rapidly frozen in the operating theater. Eight-micrometer-thick cryostat sections were fixed in ice-cold acetone and stained using ABC-peroxidase (Zymed, San Jose, CA and Vector Laboratories, Burlingame, CA) immunohistochemical staining kits.

A polyclonal bovine bFGF antibody was used (R & D Systems Inc., Minneapolis, MN) at the dilution of 1:500. The gene of this peptide is conserved and there is cross-reactivity between different species (manufacturer statement). The specificity of the immunoreaction was tested by pre-absorption of bFGF with the corresponding antigen at a final dilution of 1:10. We used polyclonal von Willebrand factor antibody (1:10.000) to visualize vascular endothelium (Dakopatts, Copenhagen, Denmark).[38] In addition to the control cadaver discs, sections also were treated omitting the primary antibody.

Obtained immunostaining results then were compared with patient clinical data (Table 1).

■ Results

Surgical data for all disc herniation tissue (DHT) samples are described in detail in Tables 1 and 2. Histologic data are presented in Table 2. Thirteen (48%) of the DHT samples were sequesters, 11 (41%) were extrusions, and three were protrusions. All protrusion samples contained bFGF immunopositive blood vessels. Eight (62%) sequesters showed vascular bFGF staining and nine (69%) showed disc cell-associated bFGF staining. Seven of eleven (64%) extruded DHTs showed bFGF immunopositive disc cells. Two of the extrusion samples contained bFGF immunopositive vascular ingrowth (Table 3).

For histologic identification, all samples were classified as containing only anular tissue, only nuclear tissue, or both by an independent pathologist unaware of patient clinical data. Of the 27 DHT samples studied, 11

Table 3. Vascular and Cellular Immunoreactivities in Different Types of Disc Herniation

	Vascular	Cellular
Protrusion	100% (3/3)	67% (2/3)
Extrusion	18% (2/11)	64% (7/11)
Sequester	62% (8/13)	69% (9/13)

(41%) contained only anular tissue, nine (33%) contained anulus and nucleus, five (20%) contained only nuclear tissue, and two remained undefined (Table 2). Altogether, 22 samples showed bFGF-positive immunohistochemical staining (81%). There were immunopositive blood vessels in 13 (48%) DHT and immunopositive disc cells in 18 (67%) of the DHT samples (Figure 1). Blood vessel-associated immunoreaction was endothelial. This vascularization appeared with granulation tissue, and the vessel ingrowth proceeded from the surface of the DHT. These blood vessels were specifically identified on the basis of their von Willebrand factor immunoreactivity. Consecutive tissue sections were stained with antibody to von Willebrand factor and bFGF (Figures 2 and 3).

Sections treated in the absence of the primary antibody did not show immunoreactivity. Neither was there immunoreactivity in sections treated with antiserum pre-absorbed with the corresponding FGF antigen. The five cadaver control disc samples also did not show bFGF immunoreactivity (Figure 4).

When clinical and immunohistochemical data were compared, there was no clear relationship between the period of radicular pain before operation and the localization of the bFGF immunoreaction, either vascular or cellular (Tables 1 and 2). Thus, for all 27 patients, the reported median duration of preoperative pain symptoms was 3.5 months (range, 0.5–16 months). For those with FGF immunoreaction in blood vessels and cells, it was 3 months (range, 1.5–9 months; Tables 1 and 2). The median pain duration for those with only vascular or cellular immunoreactivity was similar.

When FGF immunoreactivity in the oldest patient group (>50 years) and the youngest patient group (<40 years) was compared, it was noted that FGF immunoreactivity was present in all seven young patient samples. However, for the older patient group, six of 10 (60%) showed such immunoreactivity. Furthermore, there was no age-dependent difference in blood vessel-associated FGF immunoreaction, but such a difference was noted for the cellular immunoreaction (Table 4).

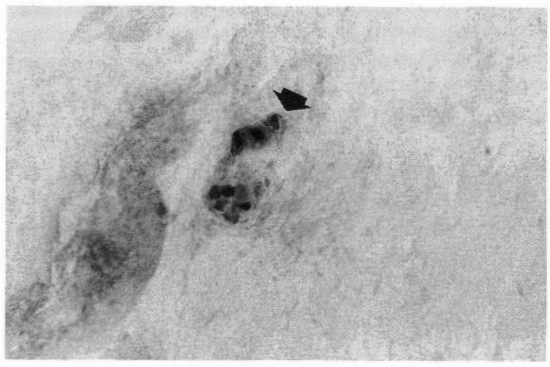

Figure 1. Higher magnification view of disc cells showing bFGF immunoreactivity (arrow). (Avidin biotin complex [ABC] immunostaining with hematoxylin counterstaining; original magnification ×370.)

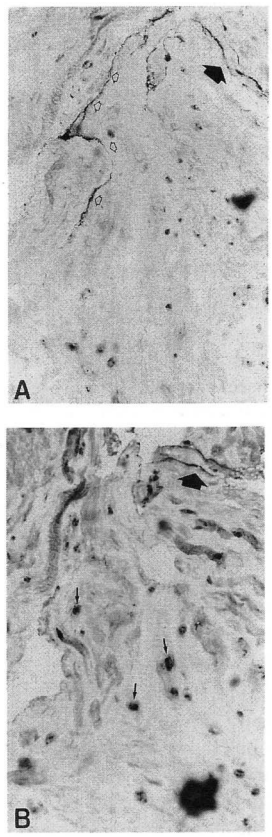

Figure 2. Low magnification view of disc herniation tissue. Serial tissue sections have been stained with antibody to (**A**) von Willebrand factor to visualize blood vessels and (**B**) basic fibroblast growth factor (bFGF). Two identical blood vessels stained with both antibodies (thick black arrow; shown at higher magnification in Figure 3). Blood vessels lacking bFGF immunoreactivity also can be observed (**A**, open arrows). Small black arrows indicate bFGF immunoreactive disc cells. (ABC immunostaining with hematoxylin counterstaining; original magnification ×93.)

■ Discussion

Our results confirmed the previous finding of granulation tissue formation and blood vessel formation in disc herniation tissue.[20,22,43,44] Fibroblast growth factor is a potent inducer of angiogenesis, as seen in various experimental models.[14] Vlodavsky et al showed that almost a third of the FGF produced in endothelial cell cultures is bound to the extracellular matrix, which acts as a storage site for this factor.[42]

Blood vessels are not usually observed in intervertebral disc. Yasuma et al demonstrated vascular invasion

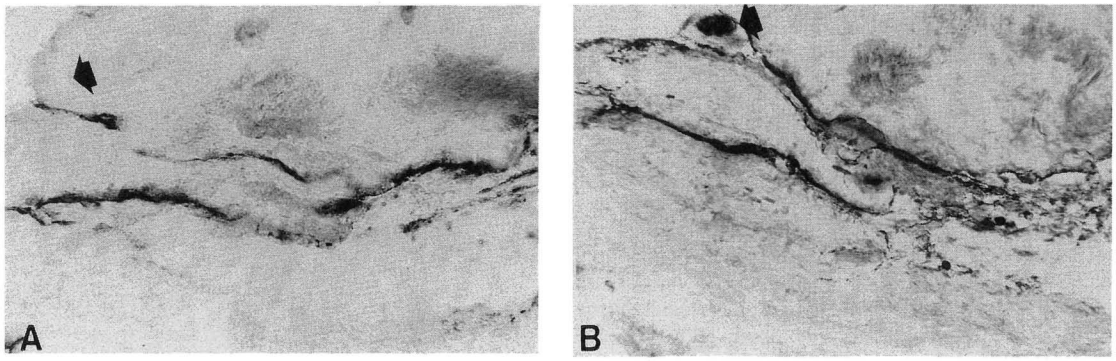

Figure 3. Higher magnification of the two bFGF immunoreactive blood vessels seen in Figure 2. **(A)** Stained with antibody to von Willebrand factor. **(B)** Stained with bFGF antibody. Thick arrow indicates disc cell that shows specific bFGF immunoreactivity but that lacks immunoreaction after staining with von Willebrand factor antibody. (ABC immunostaining with hematoxylin counterstaining; original magnification ×370.)

as a sign of aging of the intervertebral disc.[44] In their cadaver study, they noticed capillary invasion only in patients over 40 years old. In our study of disc herniations, total FGF expression was more prevalent in the younger patients. However, such an age-dependent difference was not noted with regard to blood vessel-associated FGF expression. The younger patients, however, more often exhibited disc cell-associated FGF immunoreaction. This may indicate stronger cellular reaction capability in younger subjects.

It has been discussed whether capillaries in disc herniation tissue are newly formed or are herniated with the intervertebral disc. Yasuma et al concluded in their study that most of the blood vessels in the prolapsed and extruded tissue are newly formed after herniation of the disc.[44] Previously, it was shown that dorsally displaced free disc fragments become vascularized.[17] In our study, vascular FGF immunoreactivity was more frequently observed in protrusions and sequesters than in extrusions. The few protrusions studied all exhibited vascular FGF immunoreactivity. It was shown by Yasuma et al that there are blood vessels in protrusions.[44] Whether there are newly formed blood vessels in protrusions, as suggested by the results of the present study, cannot be determined because of the small number of protrusions studied. Note that FGF immunoreaction in blood vessels may particularly show active blood vessel formation from capillary endothelial cells.[9] It could be hypothesized that the inflammatory reaction, which is coupled to angiogenesis, is more marked in protrusions and sequesters than in extrusions. Perhaps during the initial stage of disc injury (*i.e.*, protrusion stage) FGF expression is marked. Then, when disc material extrudes, vascular FGF expression may decrease. A totally displaced disc fragment (*i.e.*, sequestration stage) again may induce more inflammation coupled to a higher expression of FGF in blood vessels.

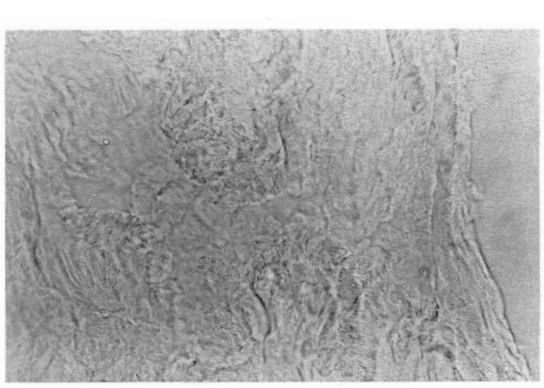

Figure 4. Control disc stained for bFGF. Note total lack of immunoreactivity. (ABC immunostaining with hematoxylin counterstaining; original magnification ×370.)

It also is known that prolapsed disc tissue tends to disappear with time.[24,35] The vascularization of the herniated disc may bring degrading agents to the disc. Fibroblast growth factor directly enhances proteolytic activity in tissues, even though it also induces the pericellular deposition of proteolytic activator inhibitor.[23,36] However, the FGF-dependent endothelial cell

Table 4. Comparison of Fibroblast Growth Factor Immunoreactivity in the Youngest and Oldest Age Groups

	Immunoreactivity		
	Total No. (%)	Blood Vessel No. (%)	Cellular No. (%)
Young group (<40 yr, n = 7)	7 (100)	3 (43)	6 (86)
Old group (>50 yr, n = 10)	6 (60)	4 (40)	4 (40)

invasion is inhibited by metalloproteinase inhibitors, such as tissue metalloproteinase inhibitor (TIMP), which counteracts stromelysin and collagenases.[8,27,28] Moreover, it has been demonstrated that FGF enhances the expression of TIMP, thus regulating the proteolytic activity in extracellular matrix. In addition, FGF is a potent regulator of plasminogen activator activity.[19] It stimulates plasminogen activator and interstitial collagenase production.[29] The most effective inducer of extracellular matrix proteolysis is this plasminogen activation.[37] Plasminogen is activated to an effective serine protease plasmin, which degrades most extracellular glycoproteins and protein parts of the glycosaminoglycans. Furthermore, plasmin activates latent collagenase, possibly together with stromelysin.[16]

In previous studies, Ng et al demonstrated such proteolytic activity in disc herniation tissue.[31] In the present study, the FGF immunoreactivity was localized presumably in mostly newly formed small blood vessels and in disc cells. Fibroblast growth factor also may regulate the proteolytic activity in disc herniation tissue. This is supported by the finding that these disc cells are immunopositive for a stromelysin antibody.[15] Interaction between proteinases and growth factors forms a cascade, in which a growth factor at the time may be stimulatory and inhibitory.[19] In the present study, in about two-thirds of all types of disc herniations, FGF expression in disc cells was noted. This may suggest activation of a proteolytic process, which may explain why disc herniation tissue can disappear with time.[24,35]

Recent evidence suggests that FGF injected in intact discs or in disc tissue cultures stimulates cell growth, mainly in the anular region.[21,40] This FGF response was markedly lower than the response to transforming growth factor beta. In the present study, we observed FGF immunopositive disc cells in the nuclear and the anular region of the DHT samples. However, immunopositive vessels were seen only in the anular region. These findings may indicate that in disc tissue physiology and pathophysiology, FGF simultaneously participates in angiogenesis and regulates cellular functions, such as cell growth and perhaps proteolysis.

Contrary to our original hypothesis—*i.e.*, regarding more marked FGF expression during the initial stages of nerve root pain—no clear relationship could be established between the preoperative duration of root pain symptoms and the site, either vascular or cellular, of bFGF immunoreactivity. A possible explanation may be continued irritation and inflammation until surgery. Such tissue reaction may continuously result in FGF expression in disc herniation tissue. A more detailed study on the time course of FGF expression—and perhaps on the expression of other growth factors—after disc tissue injury could further clarify the role of growth factors in disc tissue pathophysiology.

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Platelet-derived growth factor and vascular endothelial growth factor expression in disc herniation tissue: an immunohistochemical study

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Abstract Angiogenesis is essential in tissue growth and regeneration. There are several factors that are able to stimulate vascular endothelial cell growth, including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). Disc herniation tissue (DHT) contains vascular ingrowth, which promotes granulation tissue formation. In this study we observed 50 disc herniations for PDGF and VEGF immunoreactivity. PDGF immunopositivity was detected in 38 samples (78%). In 28 samples (56%) there were PDGF immunopositive capillaries, PDGF immunopositive disc cells were detected in 19 samples (38%) and PDGF immunopositive fibroblasts in 6 DHT samples (12%). VEGF immunopositive capillaries were identified in 44 DHT samples (88%). For neither growth factor was immunopositivity dependent on preoperative radicular pain duration. In extrusions ($n = 25$) VEGF immunopositive capillaries were detected in 23 samples (92%) and PDGF immunopositivity in 21 samples (84%). PDGF immunopositivity was more commonly associated with capillaries than with nuclei of disc cells. In sequesters ($n = 20$) VEGF immunopositive capillaries were identified in all samples and PDGF immunopositivity in 16 (80%). As in extrusions, PDGF immunoreaction was more prevalent in capillaries than in disc cells. Patient age did not relate to VEGF expression. In all age groups it was higher than 80%. Thus capillaries in disc herniation tissue are evidently newly formed and our results demonstrate that PDGF and VEGF participate in the neovascularization process. The presence of PDGF in fibroblasts and in disc cells suggests that this growth factor regulates the function of these cells, possibly the proliferation of the cells and the production of extracellular matrix components.

Key words Intervertebral disc · Disc herniation · Platelet-derived growth factor · Vascular endothelial growth factor

Introduction

Angiogenesis involves proliferation of endothelial cells, chemotaxis and enzymatic degradation of the basement membrane of local blood vessels [8, 12, 33]. This process is essential in tissue growth and regeneration. Several factors are able to stimulate vascular endothelial cell growth, including fibroblast growth factor (FGF) [10], angiogenin [7], transforming growth factor beta (TGF-beta) [25], platelet derived growth factor (PDGF) [26] and vascular endothelial growth factor (VEGF) [2, 4, 11, 16].

Platelet-derived growth factor (PDGF) is structurally a dimer of A chains (17 kD) and B chains (16 kD). It appears either in the form of an A-A or a B-B homodimer or

as an A-B heterodimer [15]. It stimulates the growth of vascular endothelial cells [26] and fibroblasts [29]. It also acts as a chemotactic signal for fibroblasts, even at very low concentrations [22]. In cartilage it has been demonstrated to increase the level of intracellular free calcium ions in chondrocytes [9]. Furthermore, it stimulates DNA and proteoglycan synthesis in cartilage tissue [9] and chondrocyte proliferation [13].

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a dimeric, heparin-bonding protein with a molecular weight of 45 kD [4, 5]. Its expression has previously been demonstrated in brain, kidney, pituitary gland, lung, adrenal gland, heart, liver, stomach mucosa and ovary, as well as in some tumours [1, 6]. VEGF is actually a family composed of four different species of VEGF [6]. It regulates endothelial differentiation, blood vessel growth and vascular repair [21], and it shares homologies of about 21% and 24%, respectively, with the A and B chains of PDGF [30]. VEGF also regulates plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells [20]. It promotes blood vessel hyperpermeability, endothelial cell growth, angiogenesis and enhanced glucose transport [3]. Hypoxia has been shown to induce VEGF, which may in turn mediate hypoxia-initiated angiogenesis [28].

Intervertebral disc tissue may herniate in three basic patterns. In protrusions it bulges posteriorly, but the annulus fibrosus remains intact. In extrusions disc material is exposed to the epidural space; there is still, however, a continuity with disc tissue. In sequesters there is a separate fragment of disc tissue material in the epidural space.

Disc herniation tissue (DHT) contains vascular ingrowth [14, 17, 32]. This neovascularization promotes granulation tissue formation. In a previous study we demonstrated basic FGF expression in DHT [31]. The aim of this study was to investigate PDGF and VEGF expression in DHT and compare it with vascularization.

Materials and methods

Our disc material was obtained from 50 discectomy operations. The age of the patients ranged from 24 to 71 years. There were 26 male and 24 female patients (Table 1). All the tissue material was rapidly frozen in the operating theatre and 8 μ thick cryostat sections were cut (2800 Frigocut, Reichert-Jung). The sections were fixed in ice-cold acetone and stained by the avidin biotin complex-(ABC-) peroxidase immunohistochemical staining method (Vectastain, Vector Lab, Burlingame, Calif.). Disc material was also obtained from organ donor patients as a control. In these specimens there was no sign of autolysis.

We used a polyclonal platelet-derived growth factor antibody (1 mg/ml) at a dilution 1:100 (R & D Systems, Minneapolis, Minn.) and a polyclonal vascular endothelial growth factor antibody (1 mg/ml) at a dilution 1:500 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Antigen absorption (1:10) was made to test the specificity of the antibodies. We used a polyclonal von Willebrand factor antibody (1:20000) to visualize vascular endothelium (Dakopatts, Copenhagen). In addition to studying control discs, sections were also stained omitting the primary antibody. The immunohisto-

Table 1 Clinical characteristics of the patients sampled for disc herniation tissue

Patient no.	Age (years)	Sex	Preoperative pain duration (months)
1	39	M	2.5
2	32	F	2
3	55	F	3
4	37	M	2.5
5	44	F	0.5
6	27	F	6
7	28	M	6
8	37	F	3.5
9	71	M	3
10	68	M	3
11	45	M	2.5
12	37	M	2.5
13	42	F	2
14	47	M	2
15	47	F	3
16	39	F	2
17	56	M	0.75
18	49	F	5
19	63	F	4
20	31	M	2
21	45	M	3.5
22	39	M	12
23	30	M	1
24	46	F	6
25	45	F	0.5
26	46	F	0.75
27	56	F	6
28	33	F	12
29	29	M	5
30	29	M	6
31	58	F	0.25
32	31	M	6
33	55	F	0.5
34	47	M	12
35	53	M	8
36	52	F	2
37	44	F	6
38	24	F	1
39	40	F	3.5
40	31	M	2.5
41	42	M	4
42	39	M	2.5
43	34	F	3
44	39	F	9
45	47	M	24
46	42	M	12
47	46	F	4
48	58	M	2.5
49	31	M	6
50	56	M	4

Table 2 Platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) immunohistochemical staining results (*nr* not reported)

Patient (no.)	Prolapse (type)	PDGF (immunopositivity)			VEGF (immunopositivity)
		Nuclear	Blood vessel	Fibroblast	
1	nr	–	–	–	–
2	nr	–	–	–	–
3	Extrusion	+	–	–	–
4	Extrusion	–	–	+	+
5	Sequester	–	–	–	+
6	Protrusion	–	+	–	–
7	Sequester	–	+	–	+
8	Sequester	–	+	–	+
9	Extrusion	–	–	–	+
10	Sequester	–	–	–	+
11	Extrusion	–	+	–	+
12	Sequester	–	–	–	+
13	Extrusion	–	–	–	+
14	Sequester	–	+	–	+
15	Extrusion	–	+	–	+
16	Extrusion	–	+	–	+
17	Sequester	–	+	–	+
18	Extrusion	–	+	–	–
19	Protrusion	–	–	–	+
20	Sequester	–	+	–	+
21	Extrusion	–	+	–	+
22	Extrusion	–	+	+	+
23	Extrusion	–	+	–	+
24	Extrusion	–	+	–	+
25	Sequester	–	+	–	+
26	Sequester	–	+	–	+
27	Sequester	–	+	–	+
28	Extrusion	–	+	–	+
29	Extrusion	–	–	–	+
30	Protrusion	–	–	–	–
31	Extrusion	+	+	–	+
32	Extrusion	+	+	–	+
33	Sequester	+	–	+	+
34	Sequester	+	–	–	+
35	Sequester	+	–	–	+
36	Sequester	+	+	+	+
37	Sequester	+	–	–	+
38	Sequester	+	+	–	+
39	Extrusion	+	+	+	+
40	Sequester	+	+	+	+
41	Extrusion	+	–	–	+
42	Extrusion	+	+	–	+
43	Sequester	–	–	–	+
44	Extrusion	–	–	–	+
45	Sequester	+	–	–	+
46	Extrusion	+	+	–	+
47	Extrusion	+	–	–	+
48	Extrusion	+	–	–	+
49	Extrusion	+	+	–	+
50	Extrusion	+	+	–	+

chemical results were then compared with the patient clinical data (Tables 1–4).

Results

Immunohistochemical staining results are described in detail in Table 2. Altogether, 38 DHT samples (78%) showed PDGF immunopositivity. In 27 DHT samples (54%) this immunoreactivity was detected in blood vessels (Fig. 1 A) and in 19 (38%) it was found in nuclei of disc cells (Fig. 2). Six samples (12%) also showed PDGF immunopositive fibroblasts (Fig. 3). Forty-four DHT samples (88%) showed VEGF immunopositive capillaries (Fig. 1 B). In Fig. 1 C capillary endothelial cells are identified with von Willebrand factor antibody. The five studied control discs did not show any PDGF or VEGF immunoreactivity (Fig. 4). Sections stained omitting the primary antibody did not show any immunoreactivity.

Mean preoperative pain duration for all patients was 4.12 months (median 3 months). VEGF and PDGF immunopositivity did not show any dependence on the duration of preoperative pain. Mean pain duration in the VEGF immunopositive group was 4.51 months (median 3 months). In the VEGF immunonegative group it was 4.08 months (median 4 months). Mean preoperative pain duration in the PDGF immunonegative group was 3.54 months (median 3 months); in the PDGF immuno-positive group it was 4.16 months (median 3 months). In the group where disc cell nuclei showed PDGF immunoreactivity the mean preoperative pain duration was 5.46 months (median 4 months). In the group where capillaries were immunoreactive for PDGF the mean preoperative pain duration was 4.13 months (median 3.5 months). Finally, in the group where fibroblasts stained for PDGF the mean preoperative pain period was 3.83 months (median 2.5 months). However, there were only six such DHT samples in the present material (Table 2).

Table 3 compares PDGF and VEGF immunoreactivity in the different prolapse types. Of the 25 extruded disc herniation samples studied, 23 (92%) showed VEGF immunoreactivity and 21 (84%) PDGF immunoreactivity. PDGF immunoreaction was located in disc cell nuclei in 11 samples (44%); in 16 samples (64%) it was associated with blood vessels and in 3 samples (12%) fibroblasts were PDGF immunopositive. Twenty of the DHTs were sequesters, VEGF immunopositive capillaries were detected in all of them. PDGF immunopositivity was detected in 16 samples (80%): in 8 of these immunoreaction was localized in disc cell nuclei, 10 (50%) showed PDGF immunopositive capillaries, and three (15%) PDGF immunopositivity in fibroblasts. In the three disc protrusions studied, one showed vascular PDGF immunoreactivity and VEGF immunoreactivity. For two of the tissue samples the prolapse type had not been defined by the operating spine surgeon.

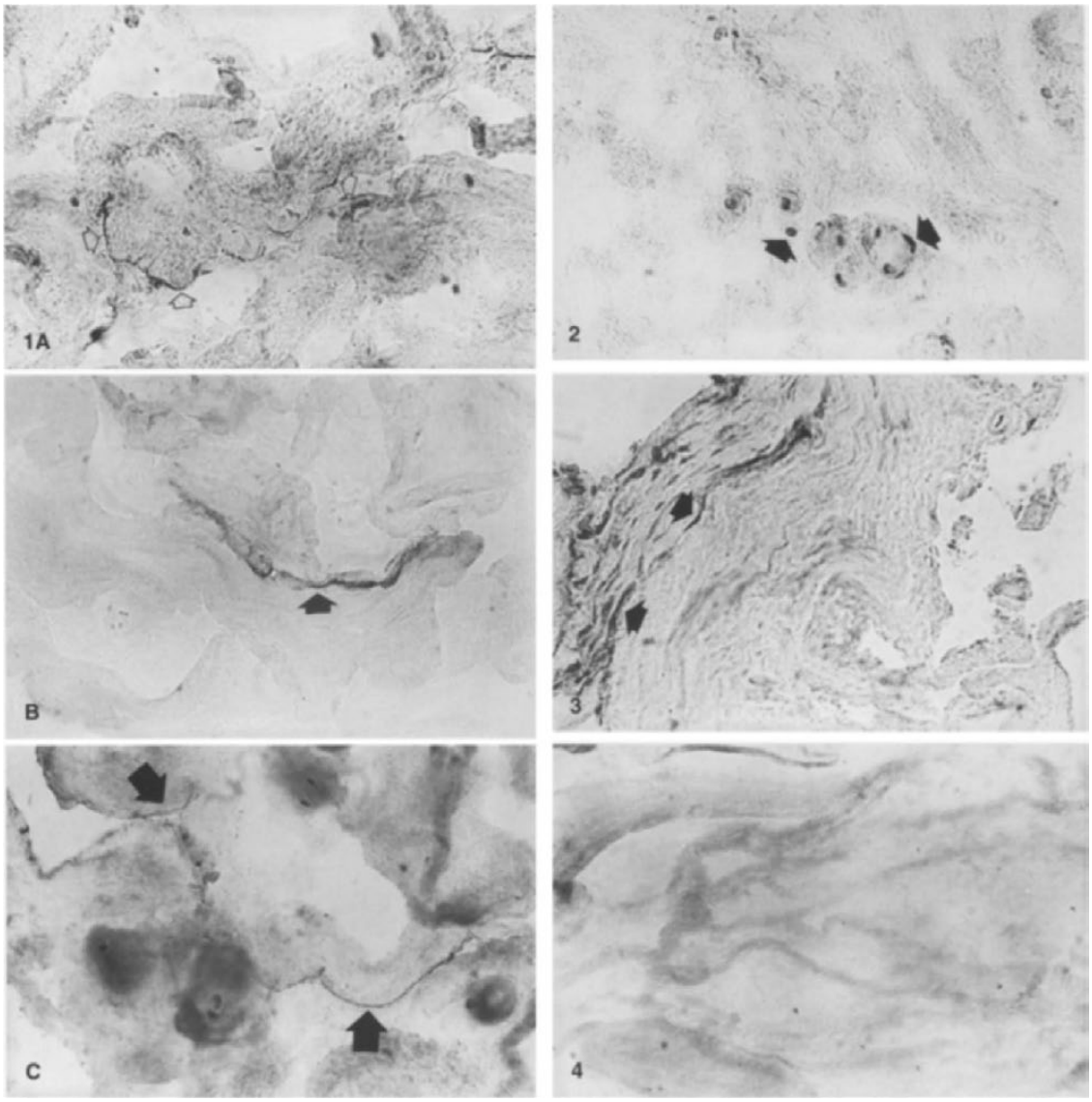

Fig. 1 **A** Platelet-derived growth factor (PDGF) immunopositivity in capillaries *(open arrows)* in an extrusion disc herniation tissue sample from a 39-year-old man. (Operation level L5–S1, avidin biotin complex- (ABC) immunostaining, haematoxylin counterstaining, original magnification × 370) **B** Vascular endothelial growth factor (VEGF) immunopositive capillaries *(black arrow)* in an extrusion from a 31-year-old man (Operation level L4–L5, ABC-immunostaining, haematoxylin counterstaining, original magnification × 250). **C** von Willebrand factor immunopositive capillaries *(black arrows)* in an extrusion disc herniation tissue sample from the same 39-year-old male patient as the sample in **A** (ABC-immunostaining, haematoxylin counterstaining, original magnification × 370)

Fig. 2 An extrusion from a 40-year-old woman. Note PDGF immunopositivity in the nuclei of disc cells *(black arrows)*. (ABC-immunostaining, haematoxylin counterstaining, original magnification × 370)

Fig. 3 PDGF immunopositive fibroblasts *(black arrows)* in a sequester from a 52-year-old woman (Operation level L5–S1, ABC-immunostaining, haematoxylin counterstaining, original magnification × 370)

Fig. 4 A normal control disc from a 50-year-old man. Note total lack of immunoreaction. (Level L4–L5, PDGF antibody, ABC-immunostaining, haematoxylin counterstaining, original magnification × 370)

Table 3 PDGF and VEGF immunoreactivity by prolapse type

Prolapse type[a]	PDGF immunopositivity				VEGF immunopositivity
	Total	Nuclear	Blood vessel	Fibroblast	
Protrusions (n = 3)	1/3	0/1	1/1	0/1	1/3
Extrusions (n = 25)	21/25 (84%)	11/21 (52%)	16/21 (76%)	3/21 (14%)	23/25 (92%)
Sequesters (n = 20)	16/20 (80%)	8/16 (50%)	10/16 (63%)	3/16 (19%)	20/20 (100%)

[a] Two samples were not classified

Table 4 PDGF and VEGF immunoreactivity by patient age group

Age group	PDGF immunopositivity				VEGF immunopositivity
	Total	Nuclear	Blood vessel	Fibroblast	
Below 40 years (n = 21)	14/21 (66.7%)	5/14 (23.8%)	13/14 (61.9%)	3/14 (14.2%)	17/21 (81.0%)
40–50 years (n = 17)	15/17 (88.2%)	7/15 (41.2%)	10/15 (58.8%)	1/15 (5.9%)	16/17 (94.1%)
Over 50 years (n = 12)	9/12 (75.0%)	7/ 9 (50.8%)	5/ 9 (41.6%)	2/ 9 (16.7%)	11/12 (91.7%)

Table 4 compares immunoreactivity for PDGF and VEGF among different age groups. VEGF immunoreactivity showed no clear age dependence and was present in more than 80% of all types of DHT (Table 4). Blood vessel associated PDGF immunopositivity was clearly more common than disc cell nuclear immunoreactivity in the youngest patients (under 40 years old). However, in the oldest age group PDGF immunoreaction appeared to be slightly more common in disc cells than in blood vessels (Table 4).

Discussion

DHT contains vascular ingrowth promoting granulation tissue formation [14, 17], PDGF is a potent chemotactic agent for fibroblasts [22] and it induces cellular growth of fibroblasts and stimulates their collagenase production [29]. PDGF is liberated from alpha-granules of platelets and macrophages [26, 29]. One finding of the present study was the demonstration of PDGF immunopositive fibroblasts in DHT, suggesting a regulative role for PDGF in fibroblast function, including in disc herniations. PDGF immunopositive disc cells were also demonstrated, the nuclei exhibiting strong immunoreactivity. The number of disc cells is greater in disc herniations than in normal discs (unpublished data). This could suggest that PDGF also regulates the function of disc cells and that in DHT PDGF stimulates extracellular matrix component production as has been observed for cartilage tissue [9, 23, 24]. PDGF may also be stimulative for disc cell proliferation. In a previous study we demonstrated disc cell cytoplasmic fibroblast growth factor (FGF) immunoreactivity [31], while in this study we noted disc cell nuclear PDGF immunoreactivity. This suggests that these growth factors are part of a greater network of growth factors and cytokines that contribute to overall disc tissue responses, governing repair of tissue damage.

DHT has a tendency to disappear or to decrease in size with time [18, 27]. Furthermore, it has been demonstrated that there is ongoing proteolytic enzyme activity in prolapsed discs [19]. PDGF stimulates collagenase production in fibroblasts [29] and has a chemotactic effect on them [22]. Our results may suggest a possible role for PDGF in regulation of proteolysis including in DHT. This proteolytic process is part of regeneration and may also act as a stimulator for angiogenesis.

Disc tissue is normally avascular and the appearance of capillaries is a sign of aging or tissue degeneration [32]. A neovascularization process also takes place following disc tissue injury and in disc herniation. The blood vessels in herniated disc tissue are mainly newly formed [32]. Several factors are able to stimulate such vascular endothelial cell growth. In this study we demonstrated PDGF and VEGF immunopositivity in disc herniation tissue capillaries. In a previous study we located FGF in these same capillaries [31]. These growth factors act synergistically in angiogenesis, and our results suggest that these factors also participate in the neovascularization process in DHT.

No relationship between VEGF or PDGF expression and the duration of preoperative pain before operation was noted. In the different prolapse types PDGF immunoreaction was detected slightly more often in capillaries than in nuclei of disc cells, but the difference was not marked (Table 3). VEGF immunopositive capillaries were highly prevalent. VEGF was expressed in all sequesters and in more than 90% of extrusions. In both sequesters and extrusions blood vessels were more often immunoreactive for VEGF than PDGF (Table 3).

VEGF expression in capillaries was similar across all age groups, whereas PDGF expression varied by age group, with a higher prevalence in capillaries than in disc cells being particularly characteristic of patients younger than 40 years (Table 4). In a previous study cellular FGF immunoreactivity was detected more often in younger pa-

tients and vascular FGF expression was found to be similar across all age groups [31].

Our results confirm that PDGF and VEGF participate in the neovascularization process. Furthermore, PDGF expression in disc cells and in fibroblasts suggests that this growth factor regulates the cellular function of these cells, mainly the production of the extracellular matrix components and proliferation of the cells.

Conclusion

The immunolocalization of the angiogenic agents PDGF and VEGF in capillaries of the herniated intervertebral disc supports the active neovascularization process. Furthermore, the presence of PDGF in disc cells suggests that this growth factor may be regulative for the production of extracellular matrix components and the proliferation of these cells. This disc cell activation is important in the pathogenesis of the intervertebral disc and needs further examination.

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ORIGINAL ARTICLE

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Transforming growth factor β receptor induction in herniated intervertebral disc tissue: an immunohistochemical study

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Abstract Transforming growth factor β (TGF-β) is a potent inducer of angiogenesis and fibrogenesis. There is presently little information about the pathophysiological function of TGF-β in herniated disc tissue. In order to analyze the cellular role and activation of TGF-β after disc herniation we immunostained frozen material from 38 disc herniation operations and from eight macroscopically normal discs from organ donors. Polyclonal TGF-β-I, TGF-β-II and TGF-β receptor type II antibodies were used with the avidin biotin complex (ABC-) immunoperoxidase method. All the herniated discs were TGF-β immunopositive. Such immunoreactivity was mainly associated with disc cells. In a few samples, capillaries were also TGF-β immunopositive. Immunopositivity was similarly observed in the control discs. To analyze possible differences between the two groups, we calculated the ratio of immunopositive disc cells. For all three antibodies, a statistically significantly (Mann-Whitney test, P=0.0001) higher number of disc cells showed immunopositivity in the herniated discs. The increase in TGF-β receptor immunopositivity suggested induction of TGF-β receptors in herniated discs. Our results support an active regulatory role for TGF-β in disc cell metabolism.

Keywords Herniated disc · TGF-β

Introduction

Both fibrotic and angiogenic reactions take place in disc herniations [7, 8, 29]. Herniated disc tissue has a tendency to decrease in size with time [14, 22], and it has proteolytic activity [17].

The TGF-β superfamily is composed of several growth factors [4]. TGF-β has been found in almost all cells studied [1, 16, 20]. It regulates cellular growth and stimulates extracellular matrix protein incorporation and collagen, hyaluronic acid, and fibronectin production [9, 19]. Subcutaneous injection of TGF-β in mice results in rapid induction of fibrosis and angiogenesis [20]. In cartilage, TGF-β stimulates production of tissue inhibitor of metalloproteinases (TIMP) [28], thus participating in the control process of connective tissue degradation. It also participates in inflammatory responses during articular inflammation [13, 18]. Furthermore, TGF-β has an effect on chondrogenesis and osteogenesis [6, 10].

TGF-βs are secreted from cells in the latent form, which does not bind to TGF-β receptors. The latency-associated peptide is removed by enzymatic degradation by proteases, resulting in activation of TGF-β [2, 5, 15, 23].

Table 1 Description of 38 disc samples from discectomy operations and eight normal controls (*DHT* disc herniation tissue, *DNT* normal disc control tissue, *S* sequester, *E* extrusion, *P* protrusion)

Tissue type	Sex	Prolapse type	Duration of radicular pain Mean (range)	Age Mean (range)
DHT (*n*=38)	22M/16F	S=12; E=24; P=2	6.9 mths (3wks–36 mths)	41.6 (20–74)
DNT (*n*=8, 5 patients)	4M/1F	–	–	36.2 (13–53)

The actions of TGF-β are mediated via binding to cell surface receptors [27].

Since TGF-β has been demonstrated to be a potent activator of disc cells in cell cultures [24] and in vivo [12], in the present study we wanted to analyze its role in herniated disc tissue: specifically, whether the number of TGF-β immunopositive cells is raised by herniation and whether the TGF-β receptor on disc cells is induced.

Materials and methods

Herniated disc material was obtained from 38 discectomy operations. As a normal control we used tissue from eight discs that had been obtained from a disc tissue bank (–70°C) of five organ donors (Table 1). None of the donors had a history of low back pain. After removal, tissue material was immediately frozen to –70°C in the operating theatre, and 8-µm-thick cryostat sections were fixed in ice-cold acetone. For some specimens we performed Zamboni prefixation to detect a possible difference in immunoreaction between prefixed and section-fixed samples. All immunoreactions were detected using an avidin biotin complex- (ABC-) peroxidase staining kit (Vectastain Elite, Vector Laboratories, Burlingame, Calif.). All tissue sections were counterstained by hematoxylin and eosin. Thus, all cells, including those showing no immunoreactivity, could be visualized and counted.

Table 2 Ratio of immunopositive (TGF-β-I, TGF-β-II and TGF-β receptor type II) to total disc cells in herniated disc tissue and control disc samples: values for the total sum of immunopositive disc cells to all disc cells in each group and the means, standard deviations (SD) and 95% confidence intervals (CI) for each group are given. Immunopositive disc cells were counted from eight normal discs and ten herniated discs and their number was then compared with the corresponding total cell number. For counting, five random microscopic fields from each tissue sample were used

Type of studied tissue	Ratio of immunopositive to total disc cells		
	TGF-β-I	TGF-β-II	TGF-β receptor type II
DHT (*n*=10)			
Total sum	550/858	437/787	520/864
Mean	0.64	0.56	0.60
SD	0.09	0.15	0.08
95% CI	0.58–0.69	0.45–0.64	0.54–0.64
DNT (*n*=8)			
Total sum	271/772	100/506	149/489
Mean	0.32	0.16	0.28
SD	0.10	0.11	0.12
95% CI	0.27–0.38	0.10–0.22	0.22–0.34
Group difference	$P<0.0001$	$P<0.0001$	$P<0.0001$

Antibodies

Polyclonal anti-human TGF-β-I, TGF-β-II and TGF-β receptor type II antibodies raised in rabbits were used (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), all at the dilution 1:50. There is no cross-reactivity between these three different antibodies. We chose antibodies to TGF-β-I and -II, in particular, since these are the most common members of the TGF-β superfamily. An antibody to TGF-β receptor type II was chosen, since this receptor is located in the cellular membrane, and it detects all members of the TGF-β superfamily. As a positive control for the immunostaining reaction, we used rheumatoid arthritic synovia tissue. Three sections were stained with each antibody from every specimen.

Immunohistochemical quantitation

All herniated and control disc samples studied consisted of nucleus pulposus tissue only. The immunohistochemical analyses were done in blind review by two observers. Positive staining for all three TGF-β antibodies (TGF-β-I, TGF-β-II, and TGF-β receptor type II) was quantified as the ratio of positive disc cells per cross-sectional area to all disc cells. We took five random microscopic fields at the magnification ×250 from all control discs and from ten herniated discs. The mean of the cell counts obtained by the two observers was used.

We counted 50 fields from herniated disc samples and 40 fields from all control samples, and standard deviations were found to be small (Table 2). Furthermore, when comparing groups, 95% confidence intervals did not overlap at all. Thus, we considered our counting material sufficient for statistical analysis.

For immunostaining control, sections were treated omitting the primary antibody in the staining sequence. For all antibodies (TGF-β-I, -II and receptor type II) preincubation with the corresponding antigen (1:10) was done.

Statistical analysis

Statistical analysis was done using the SOLO statistical software program (BMDP, Los Angeles, Calif.). Groups were compared using the nonparametric Mann-Whitney test. Level of statistical significance was set at $P<0.05$.

Results

The disc samples studied are described in detail in Table 1. Herniated disc samples were classified by the operating surgeon as previously described [25]. In control discs, no signs of autolysis were observed, i.e., all disc cells looked intact, and they all showed a normal morphology macroscopically.

All discs studied, both disc herniations and controls, showed TGF-β-I, -II and receptor type II immunopositivity. The immunoreaction for TGF-βs and TGF-β receptor was mainly disc cell associated, located in the cytoplasm

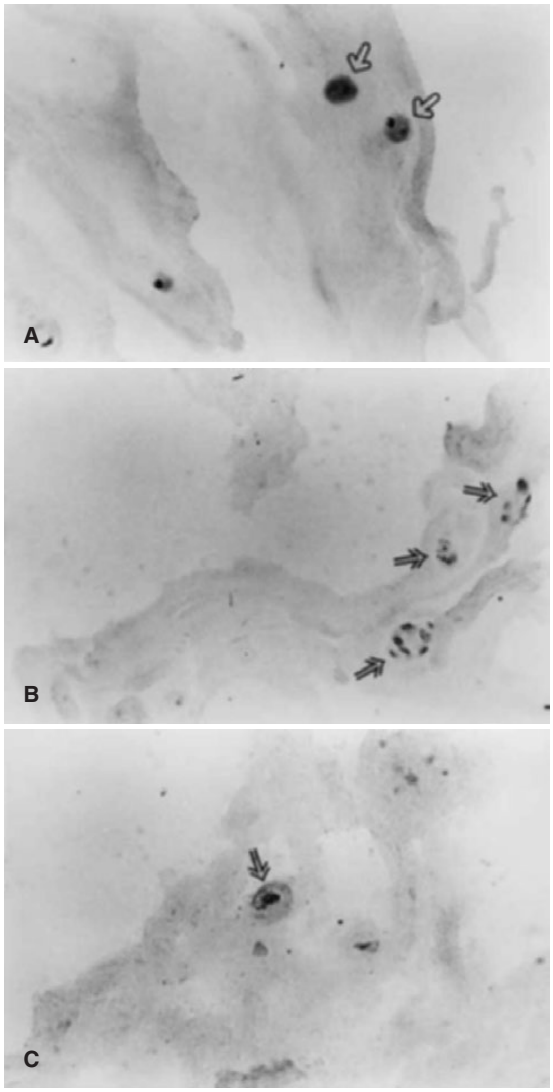

Fig. 1 **A** Transforming growth factor (TGF-)β-I immunopositive disc cells (*open arrows*) in nucleus pulposus of rapidly frozen herniated disc tissue from a 43-year-old male patient. Note the intense cytoplasmic immunoreaction around counterstained nuclei. The operation level of the sequestrated disc was L5-S1 [avidin biotin complex (ABC-) peroxidase immunostaining (Vectastain); hematoxylin counterstaining; original magnification ×241]. **B** TGF-β receptor type II immunopositivity in nucleus pulposus disc cell groups (*open arrows*) in rapidly frozen herniated intervertebral disc from a 40-year-old male patient. The operation level of the disc protrusion was L4-L5 (immunostaining as in **A**; original magnification ×241). **C** TGF-β-II immunopositivity (*open arrow*) in cytoplasm/cell membrane of disc cell in nucleus pulposus. Rapidly frozen herniated intervertebral disc from the same patient as in **B** (immunostaining as in **A**; original magnification ×241)

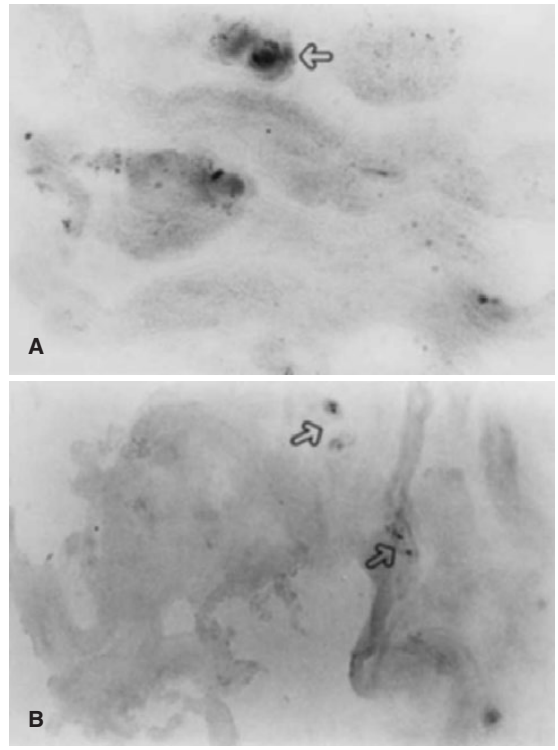

Fig. 2 **A** TGF-β-II immunopositive nucleus pulposus disc cell (*open arrow*) in a 43-year-old female organ donor (control disc). The disc level was L2-L3 (immunostaining as in Fig. 1A; original magnification ×241). **B** Immunostaining control. Antigen preabsorption for TGF-β-II. Note the pale nuclei of nucleus pulposus disc cells (*open arrows*) (immunostaining as in Fig. 1A, original magnification ×241)

of the cell (Fig. 1, Fig. 2 A). In some samples, we also noted blood vessel associated immunoreactivity. For TGF-β-I and TGF-β-II, immunoreactivity in disc cells was noted in all 38 disc herniation samples, whereas only one such sample lacked disc cell associated immunoreactivity for TGF-β receptor type II.

We did not see any difference in immunoreaction between different types of herniated tissue samples, i.e., sequesters, extrusions, and protrusions.

Blood vessel immunoreactivity was observed in 22/38 (58%)(TGF-β-I), 14/38 (37%)(TGF-β-II) and 14/38 (37%) (TGF-β receptor type II) disc herniations, respectively.

Sections stained omitting the primary antibody against corresponding antigen did not show any immunoreactivity. There was no immunoreaction after preincubation with the corresponding antigen for any of the three antibodies employed (Fig. 2B). The rheumatoid arthritis synovia sections were immunopositive for all the antibodies.

Comparison between prefixed and section-fixed samples did not show differences in immunoreactivity.

Control discs showed fewer TGF-β and TGF-β receptor immunopositive disc cells than the disc herniations (Table 2). When comparing the two groups by the Mann-Whitney test, significant differences ($P<0.0001$) were observed for all the three antibodies. A significant correlation (Spearman's $\rho=0.89$, $P<0.002$) between TGF-β-I immunopositivity and TGF-β receptor type II immunopositivity in herniated disc samples was also noted. The correlation between TGF-β-II and TGF-β receptor type II remained at a non-significant level ($\rho=0.45$, $P>0.05$).

Discussion

TGF-β stimulates extracellular matrix component formation and regulates cellular growth and extracellular proteolysis [3, 9, 11, 19, 21]. It participates in angiogenesis and in the early phase of inflammation [13, 18, 21]. Such proteolytic activity has also been observed in herniated disc tissue [17]. Thus TGF-β may be important in mechanisms of disc tissue ageing and repair, which are presently incompletely understood. New blood vessel formation (i.e., neovascularization), inflammation and the regulation of cellular growth and extracellular matrix (e.g. proteoglycan) formation and breakdown are all major processes in tissue healing and/or degradation.

TGF-β may participate in the regulation of extracellular proteolysis [11, 17, 28] in disc herniation tissue. This extracellular proteolysis and the suggested possible role in the regulation of matrix production may be important for the mechanisms of disappearance of prolapsed disc material with time [14, 22].

Yasuma and co-workers have recently noted angiogenesis to be a sign of ageing in disc tissue [29]. Whereas normally blood vessels are known to be sparse in disc tissue, in disc herniation tissue, the expression of growth factors, e.g., fibroblast growth factor (FGF) [25] and TGF-β, in small capillaries is suggestive of an ongoing active neovascularization process. Furthermore, such a neovascularization process is supported by the expression of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [26].

Conclusions

The marked statistical difference between immunoreactivity for TGF-β and its receptor type II in herniated intervertebral disc tissue as compared with control discs (Table 2) shows that this particular growth factor is activated. Furthermore, a statistical difference in TGF-β receptor type II immunoreactivity suggests receptor induction in disc herniation tissue, as compared with control disc tissue. We surmise that the increased immunopositivity in herniated disc is due to local production and that the increased immunopositivity for the TGF-β receptor will provide an increased number of binding sites for TGF-β, increasing the action of TGF-β on disc cells.

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ORIGINAL ARTICLE

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Oncoprotein c-Fos and c-Jun immunopositive cells and cell clusters in herniated intervertebral disc tissue

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Abstract The oncoproteins c-Fos and c-Jun create a transcriptional site early response activating protein (AP-1) mediating the regulation of gene expression in response to extracellular signalling by, for example, cytokines. These proteins are important in the signalling pathway from the cell membrane to the nucleus. Previously, oncoproteins have been located in articular synovium and in chondrocytes, participating in transcription. There is, however, no such study of intervertebral disc tissue. In disc degeneration and after herniation, cell proliferation markers have been demonstrated. In the present study we visualize the AP-1 transcriptional site factors c-Fos and c-Jun in 38 human herniated intervertebral disc tissue samples by immunohistochemical staining with monoclonal antibodies. No immunoreactivity could be observed in control disc tissue, indicating that after herniation, disc cells are entering from the resting stage to the cell cycle. Furthermore, c-Jun immunoreactivity was also observed in disc cell clusters, thus demonstrating them to be active transcriptional sites in disc tissue. c-Fos immunoreactivity was seen in 15/38 and c-Jun in 28/38 herniated discs (39% and 74% respectively). Immunopositive *groups* of disc cells were noted in 7/28 (25%) of the oncoprotein-immunopositive samples. We did not see any difference in immunoreactivity between female and male patients. Furthermore, we did not notice any statistical difference regarding the immunoreaction for proto-oncogenes c-Fos and c-Jun in extrusions, sequesters and protrusions. Nor did immunostaining show any significant relationship with preoperative pain duration. We concluded that, in herniated disc tissue, the oncoproteins c-Fos and c-Jun are activated in disc cells and cell clusters. In the future, learning more about this transcriptional signal pathway may result in new specific treatments for intervertebral disc pathology.

Keywords Oncogene proteins · c-Fos protein · c-Jun protein · Disc herniation

Introduction

Proto-oncogenes encode proteins with three main sites of action: the cell-surface membrane, the cytoplasm and the nucleus [2]. The Jun oncogene is expressed as a 65-kd protein [5]. It was first detected in cancer research [4], which explains why it is called an "oncogene". It is a member of the early activating protein (AP-1) family of transcription factors, which mediate the regulation of gene expression in response to extracellular signaling [3]. For example, stimulation of quiescent murine fibroblasts by growth factors and by phorbol esters results in a rapid and transient transcriptional activation of proto-oncogenes [7].

Furthermore, transient inhibition of protein synthesis induces expression of proto-oncogenes and stimulates resting cells to enter the cell cycle [18].

The proto-oncogene products c-Fos and c-Jun connect or heterodimerize through their leucine zippers to form the AP-1 transcription factor [15]. The transcriptional activity of the heterodimer is regulated by signal-dependent phosphorylation and dephosphorylation events.

In human chondrocytes, c-Fos and c-Jun genes are expressed both in normal and osteoarthritic articular cartilage [26]. Osaki et al. have demonstrated that in rat chondrocytes the cell growth inductive and the mitogenic effect of transforming growth factor β1 (TGF-β1) are mediated by the c-Fos gene [13]. In an experimental osteoarthritis (OA) model, oncoproteins were detected predominantly in the synovial lining cells [16], whereas in normal synovial lining cells such reactivity was low. Furthermore, in OA cartilage chondrocytes at the superficial and middle layers were found to participate in the synthesis of oncoproteins [16].

Overexpression of the c-Fos proto-oncogene has recently been shown to inhibit chondrocyte differentiation [21]. Interestingly, however, the rates of proliferation and apoptosis were unaffected. Nodule formation was inhibited if induction of c-Fos was only at early stages of differentiation [21].

The level of expression of c-Jun is lower in maturing or hypertrophic chondrocytes than in proliferating chondrocytes [9]. This suggests that the c-Jun family negatively regulates the maturation process of chondrocytes.

Activation of AP-1, a heterodimeric complex of Fos and Jun proteins, is required for chondrocyte matrix metalloproteinase production and cell proliferation [10]. Cytokines and growth factors induce reactive oxygen species production, which is a signal pathway for activation of AP-1. In herniated disc tissue, matrix metalloproteinase activity is prevalent [17], and there is a change in the balance between degradative enzymes and endogenous inhibitors.

Clusters of disc cells have been observed in degenerated intervertebral disc tissue [8]. Earlier studies have reported similar clusters of chondrocytes in osteoarthritic cartilage, where they have been linked to a possible production of extracellular matrix components involved in matrix repair [8]. In intervertebral disc tissue, such clusters could perhaps participate in the repair of tissue damage, but very little is so far known about such clusters of disc cells.

We analyzed the transcription promoters c-Fos and c-Jun in herniated disc tissue in order to locate potential cell transcription and cell activation, thereby identifying possible differences in oncoprotein expression between normal and herniated disc tissue. Previously there has been no research concerning oncoprotein expression in intervertebral disc tissue. These proteins are essential in the signal pathway from the cell membrane to the nucleus. Further knowledge regarding this signal pathway may bring forth new specific treatments for disc pathology.

Materials and methods

We analyzed tissue material from 38 discectomy operations. As a normal control we used nucleus pulposus material from a disc tissue bank (–70°C) of four organ donors, none of whom had any history of low back pain. The control tissue was obtained by an anterior approach. A detailed description of the clinical data of the disc tissue material from the herniated discs and normal controls is given in Table 1. The preoperative pain duration was reported by the patients in a routine low-back pain patient questionnaire, including items on preoperative radicular pain duration, a pain drawing, the Oswestry Disability Index and patient demographics.

After removal, tissue material was immediately frozen to –70°C in the operating theater, and 8-μ-thick cryostat sections were fixed in ice-cold acetone. Immunoreactions were detected using an avidin biotin complex- (ABC-) peroxidase staining kit (Vectastain Elite, Vector Laboratories, Burlingame, Calif., USA). Hematoxylin was used for counterstaining.

We used monoclonal c-Fos and c-Jun antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), both at the dilution 1:50. As a positive control we used rheumatoid arthritic synovia and human dermal skin samples. As a method control for monoclonal immunostaining, we also stained sections omitting the primary antibody. We also did immunostaining as above following preabsorption of the two monoclonal antibodies with the corresponding antigen at 1:10.

Three sections from each disc specimen were stained with both antibodies. The presence or absence of immunostaining was compared with clinical data (age, gender, preoperative pain duration and prolapse type).

Statistical analyses were done using the SOLO statistical software program (BMDP, Los Angeles, Calif., USA). Groups were compared using either chi-square analyses or Fisher's exact test, as applicable. Level of statistical significance was set at $P<0.05$.

Table 1 Clinical data of 38 herniated disc samples from discectomy operation and five normal controls (*DHT* disc herniation tissue, *DNT* normal disc control tissue, *S* sequester[a], *E* extrusion[b], *P* protrusion[c])

Tissue type	Sex(M/F)	Prolapse type	Radicular pain	Age (years)
DHT (*n*=38)	22/16	S=12, E=24, P=2	3wks–36mths; mean=6.9mths	20–74; mean 41.6
DNT (*n*=4)	3/1			31–53; mean 43

[a] *Protrusion* is an abnormal bulging of the annulus fibrosus, which remains continuous
[b] *Extrusion* is where tissue is exposed to the epidural space, but remains continuous
[c] *Sequester* is free disc tissue material in epidural space [20]

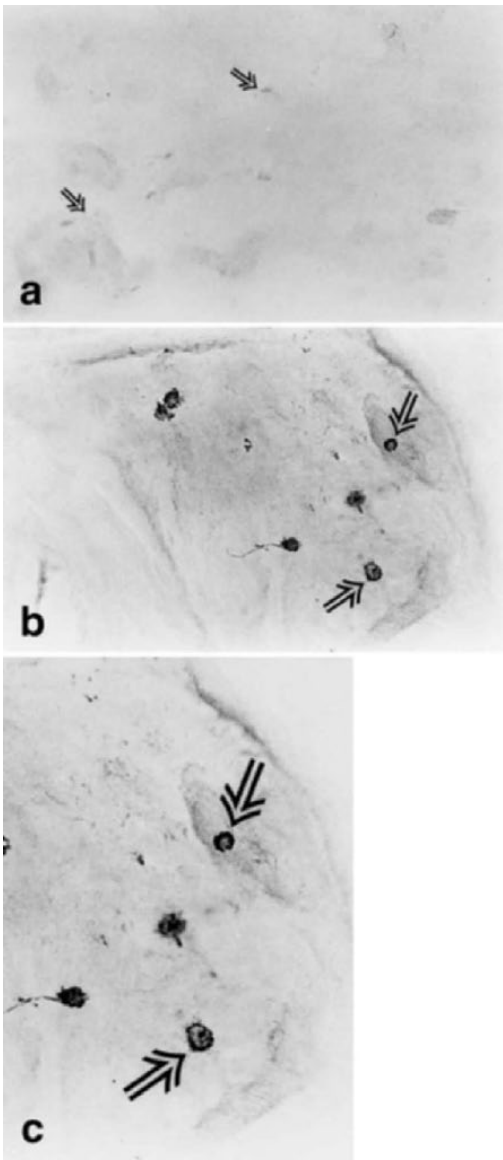

Fig. 1 **a** Control disc from a 13-year-old female organ donor. Monoclonal c-Fos oncoprotein antibody at dilution 1:50 using the avidin-biotin complex- (ABC-) immunostaining method, with hematoxylin counterstaining (original magnification ×370). Note the lack of immunoreactivity (*open arrows*) **b** Oncoprotein c-Fos immunopositive disc cells (*open arrows*) in an extruded disc herniation sample from a 39-year-old male patient (ABC-immunostaining, hematoxylin counterstaining, original magnification ×370). **c** Higher magnification of the oncoprotein c-Fos immunopositive cells (*open arrows*) seen in **b**. Observe the perinuclear location of the immunoreaction (ABC-immunostaining, hematoxylin counterstaining, original magnification ×550)

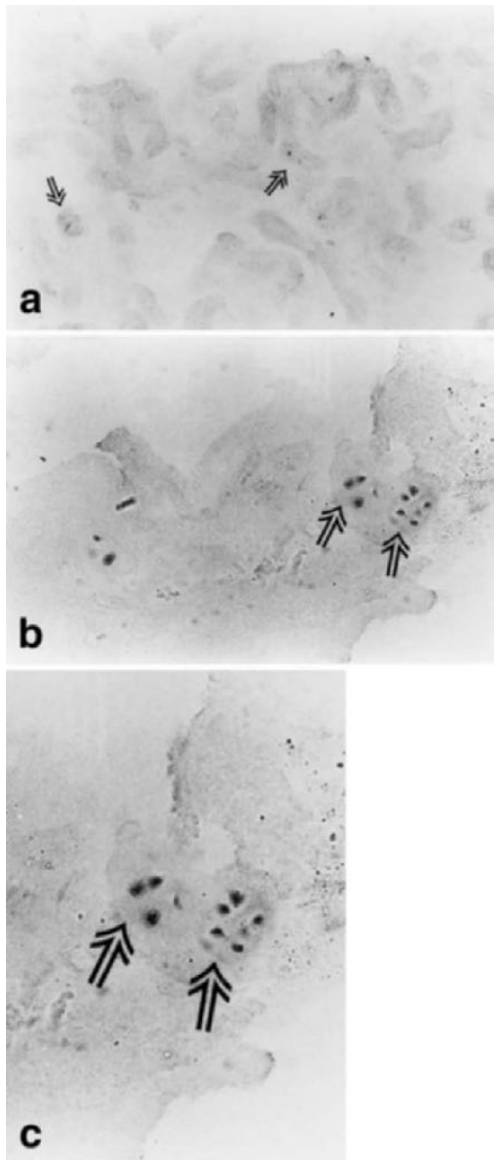

Fig. 2 **a** The same control disc as in Fig. 1a, now stained with oncoprotein c-Jun monoclonal antibody at dilution 1:50 (ABC-immunostaining method, hematoxylin counterstaining, original magnification ×250). Note the total lack of immunoreactivity. Pale hematoxylin counterstained nuclei can be seen (*open arrows*). **b** Oncoprotein c-Jun immunopositive disc cell groups (conglomerates) (*open arrows*) in an extruded disc herniation sample from a 45-year-old female patient (monoclonal antibody at dilution 1:50, ABC-immunostaining method, hematoxylin counterstaining, original magnification ×370). **c** Higher magnification of the c-Jun immunopositive cells (*open arrows*) seen in **b**. Observe the nuclear location of the immunoreaction (ABC-immunostaining, hematoxylin counterstaining, original magnification ×550)

Table 2 Clinical data of the patients and immunohistochemical staining results (ABC-peroxidase immunostaining method, monoclonal antibodies at dilution 1:50) for oncoproteins c-Fos and c-Jun. Samples are arranged according to prolapse type [*Co* Immunoreactivity in disc cell conglomerates (groups of cells)]

No. (age, years)	Sex (M/F)	Pain duration (months)	Prolapse type	Operation level	Staining result c-Fos/c-Jun
1 (63)	F	4	P	L3/4	–/+
2 (40)	M	36	P	L4/5	–/+
3 (74)	M	3	E	L3/4	–/+
4 (39)	M	2.5	E	L3/4	+/+(Co)
5 (34)	M	1.75	E	L4/5	+/+
6 (55)	F	3	E	L2/3	–/–
7 (40)	M	2.5	E	L5/S1	+/+
8 (58)	M	2.5	E	L4/5	+/+
9 (61)	M	3	E	L5/S1	–/–
10 (20)	M	3	E	L5/S1	–/+
11 (44)	M	12	E	L4/5	–/+(Co)
12 (34)	M	30	E	L4/5	+/+
13 (36)	F	6	E	L5/S1	–/+(Co)
14 (45)	F	6	E	L5/S1	–/–
15 (49)	F	5	E	L4/5	–/+
16 (41)	F	3.5	E	L4/5	+/+
17 (43)	M	4	E	L4/5	+/+(Co)
18 (39)	F	9	E	L4/5	+/+
19 (34)	M	5	E	L4/5	+/+
20 (47)	F	3.5	E	L4/5	–/+(Co)
21 (33)	F	12	E	L5/S1	+/+
22 (29)	M	7	E	L3/4	–/+
23 (61)	M	4	E	L5/S1	–/–
24 (23)	F	6	E	L5/S1	–/–
25 (52)	M	3	S	L4/5	–/+(Co)
26 (33)	F	2	S	L5/S1	+/+
27 (33)	F	0.75	S	L4/5	–/–
28 (32)	M	2.5	S	L5/S1	+/+
29 (34)	F	3	S	L5/S1	–/+
30 (55)	M	2	S	L4/5	–/+
31 (34)	F	13	S	L4/5	–/–
32 (38)	M	24	S	L5/S1	+/+
33 (25)	M	3.5	S	L5/S1	–/–
34 (26)	M	5	S	L4/5	–/–
35 (43)	M	6	S	L4/5	–/–
36 (43)	M	6	S	L4/5	–/+(Co)

Results

The clinical data of herniated discs are shown in Table 1. Herniated disc tissue samples were classified by the operating surgeon as previously described [20, 22]. Control discs showed a normal morphology macroscopically; no signs of autolysis were observed, i.e. all disc cells looked intact. All control discs were immunonegative for both c-Fos and c-Jun (Fig. 1a, Fig. 2b). Rheumatoid arthritic synovia and dermal samples, studied as positive controls, showed c-Fos and c-Jun immunopositive cells. Sections stained omitting the primary antibody did not show any immunoreaction for either of the oncoproteins. Neither did sections preabsorbed with the corresponding antigen.

Oncoprotein c-Fos immunoreaction was noted in 15/38 herniated samples (39%) (Fig. 1b,c) and for c-Jun in 28/38 (74%) (Fig. 2b,c; Table 2). For each of these disc samples studied, when immunopositivity was detected, it was observed in all three sections cut through the respective specimen. When a disc sample was immunonegative, none of the three sections studied showed any immunoreactivity. Immunopositive clusters of disc cells (conglomerates, i.e., ≥3 cells) were seen in 25% of the total immunopositive samples (7/28) (Table 2). The immunoreaction in these clusters of disc cells was mainly of the c-Jun type (Fig. 2b,c).

None of the studied herniated discs showed immunoreactivity only to c-Fos. Immunoreactivity only to c-Jun was detected in 13 disc herniation samples and reactivity to both oncoproteins in 15 disc herniation samples (Table 2).

Twenty-three of the herniated samples were from men and 15 from women. The age of the patients varied from 20 to 74 years (mean 41.6 years). Twenty-four of the her-

Table 3 Immunohistochemical staining results for oncoproteins c-Fos and c-Jun in subgroups of herniated disc tissue samples (n=38)

Subgroups	Immunoreactivity*		
	c-Fos	c-Jun	Conglomerates[a]
Males (n=23)	10/23	18/23	5/18
Females (n=15)	5/15	10/15	2/10
Extrusions (n=24)	12/24	19/24	5/19
Sequesters (n=12)	3/12	7/12	2/7
Protrusions (n=2)	0/2	2/2	0/2

*Group differences nonsignificant (Fisher exact/Chi-square tests, as applicable)
[a] Number of samples with c-Jun immunoreactive disc cell *groups* of all samples with c-Jun immunoreactivity

Table 4 The relationship of immunoreactivity for oncoproteins c-Fos and c-Jun with pain duration before the disc herniation operation. In the acute/subacute group pain duration was ≤3 months, in the chronic group pain duration was >3 months

Groups	Immunoreactivity*			
	c-Fos	c-Jun	Conglomerates[a]	
Acute/subacute group (n=14)		6/14	11/14	2/11
Chronic group (n=24)	9/24	17/24	5/17	

*Group differences were nonsignificant (Fisher exact/Chi-square tests, as applicable)
[a] Number of samples with c-Jun immunoreactive disc cell *groups* of all c-Jun immunoreactive samples

niation samples were extruded, 12 sequestrated and 2 protruded. Detailed immunoreaction in different subgroups is shown in Table 3 and Table 4.

We did not note any gender-related differences in the immunoreactivity. Nor were there any statistically significant differences regarding proto-oncogene immunoreactions in extrusions compared to sequestrations (Table 3). The total number of protrusions (n=2) was too small to make any statistical analysis.

Comparing the pain duration before operation, there were no statistically significant differences between the acute/subacute and chronic group regarding the immunoreaction for the proto-oncogenes c-Fos and c-Jun (Table 4). Nor did the presence of immunoreaction in cell groups (conglomerates) show any statistical difference between these two groups (Table 4).

Discussion

Nuclear oncoproteins c-Fos and c-Jun (or its related proteins JunB or JunD) represent the transcription factor AP-1 (activating protein-1), and they may act as an intracellular messenger converting short-term signals generated by extracellular stimulators into long-term changes in cell phenotype. This is done by regulating the expression of downstream genes that possess an AP-1 binding site [1].

The importance of AP-1 transcription factors in chondrocyte differentiation has been highlighted in an in vitro study by Thomas et al. [21]. In particular, their study found that the induction of c-Fos resulted in a concomitant increase in the expression of fra-1 and c-Jun. Furthermore, the overexpression of c-Fos was found to directly inhibit chondrocyte differentiation.

It has previously been shown that the growth-stimulative effect of transforming growth factor β1 in chondrocytes is mediated by the activation of the oncoprotein c-fos gene [13]. This is mediated by protein kinase activation [25]. In the present study, we show the expression of the oncoproteins c-Fos and c-Jun in herniated disc tissue. We have previously reported expression of TGF-β and induction of TGF-β receptor in these same tissues [23]. This may suggest that the proliferative effect of TGF-β is at least partly mediated by these oncoproteins in herniated disc tissue as well.

In rheumatoid cartilage, the matrix metalloproteinase-1 (MMP-1) promoter is demonstrated to be activated by Jun-related proteins as well as Fos/Jun-related protein heterocomplex [24]. Furthermore, c-Fos combined with any of the Jun-related proteins failed to stimulate the tissue inhibitor of metalloproteinases-1 (TIMP-1) promoter, although it was activated by Jun-related protein heterocomplexes [24]. In the present study, we did not see c-Fos immunopositivity alone (Table 2), whereas c-Jun immunoreactivity was noted as well as the immunoreactivity to both oncoproteins (Table 2). This may suggest that the proteolytic activity is controlled by the expression of the c-Fos and c-Jun oncoproteins in herniated disc tissue as well.

Interestingly, the oncoprotein expression in disc cell clusters was mainly of the c-Jun type. This may indicate that in disc cell clusters c-Jun may be more important than c-Fos. Turnover of the matrix components is established with an intricate balance between synthesis and degradation of the associated molecules, such as MMP and TIMP. c-Fos has been demonstrated to be degradative [24], whereas Jun-related proteins stimulate both MMP and TIMP [24]. This may indicate that disc cell clusters participate in the turnover of extracellular matrix components. Marked degradative enzyme activity in disc tissue after disc herniation has been noted previously [12]. Furthermore, disc tissue has a tendency to decrease in size after herniation [11, 19]. Matrix metalloproteinase activation in herniated disc tissue has previously been shown [17]. There was a change in the balance between degradative enzymes (MMPs) and endogenous inhibitors (TIMPs) [17]. Regulation of this balance could be partly mediated by AP-1 protein in disc tissue as well.

The duration of pain before operation had no significant association with the expression of oncoproteins. The results did not show any statistical difference between acute/subacute and chronic herniations.

Previously it has been shown by Paajanen and coworkers that the proliferation potential drops in recurrent herniations [14]. That study did not show any correlation between proliferative disc cells and the degree of disc degeneration on magnetic resonance imaging.

The formation of clusters of disc cells is associated with degenerative disc disease [8]. These clusters may produce certain extracellular matrix components and they may also function to repair damaged tissue. The pattern of proliferation cell nuclear antigen (PCNA) and proliferation-associated antigen Ki-67 positivity in degenerated disc tissue samples suggests that disc cell clusters arise through increased cell proliferation [8]. Furthermore, colony formation in vitro by annular intervertebral disc cells from patients with degenerative disc disease and young controls has previously been demonstrated [6].

Conclusions

Taken together, our findings of oncoprotein c-Fos and c-Jun expression in disc cells and cell clusters may indicate that disc cells respond to disc herniation. This might mean that disc cells participate in a reaction cascade, where they are actively in contact with the surrounding tissue. This also implies that more information about disc cells will be required before we can better understand mechanisms of disc degeneration and herniation, and the tissue remodelling that follows upon a disc herniation. Furthermore, in the future, we believe that disc cell research regarding the signal pathway from the cell membrane to the nucleus will provide more information on how disc cells function in different stages of the cell cycle, and in different pathological conditions. Pathological conditions of interest include disc degeneration and disc herniation. This may provide us with new types of treatment, e.g., gene therapy or perhaps blockage of the signal pathway.

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ORIGINAL ARTICLE

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Growth factor expression in degenerated intervertebral disc tissue
An immunohistochemical analysis of transforming growth factor beta, fibroblast growth factor and platelet-derived growth factor

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Abstract Degenerated intervertebral disc has lost its normal architecture, and there are changes both in the nuclear and annular parts of the disc. Changes in cell shape, especially in the annulus fibrosus, have been reported. During degeneration the cells become more rounded, chondrocyte-like, whereas in the normal condition annular cells are more spindle shaped. These chondrocyte-like cells, often forming clusters, affect extracellular matrix turnover. In previous studies transforming growth factor β (TGFβ) −1 and −2, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) have been highlighted in *herniated* intervertebral disc tissue. In the present study the same growth factors are analysed immunohistochemically in degenerated intervertebral disc tissue. Disc material was obtained from 16 discs operated for painful degenerative disc disease. Discs were classified according to the Dallas Discogram Description. Different disc regions were analysed in parallel. As normal control disc tissue material from eight organ donors was used. Polyclonal antibodies against different growth factors and TGFβ receptor type II were used, and the immunoreaction was detected by the avidin biotin complex method. All studied degenerated discs showed immunoreactivity for TGFβ receptor type II and bFGF. Fifteen of 16 discs were immunopositive for TGFβ-1 and −2, respectively, and none showed immunoreaction for PDGF. Immunopositivity was located in blood vessels and in disc cells. In the nucleus pulposus the immunoreaction was located almost exclusively in chondrocyte-like disc cells, whereas in the annular region this reaction was either in chondrocyte-like disc cells, often forming clusters, or in fibroblast-like disc cells. Chondrocyte-like disc cells were especially prevalent in the posterior disrupted area. In the anterior area of the annulus fibrosus the distribution was more even between these two cell types. bFGF was expressed in the anterior annulus fibrosus more often in chondrocyte-like disc cells than in fibroblast-like disc cells. Control discs showed cellular immunopositivity for only TGFβ-1 and −2 and TGFβ receptor type II . We suggest that growth factors create a cascade in intervertebral disc tissue, where they act and participate in cellular remodelling from the normal resting stage via disc degeneration to disc herniation.

Keywords Intervertebral disc · TGF-β · FGF · PDGF

Introduction

Intervertebral disc degeneration is a complex process characterised by biochemical and structural changes in both the nucleus pulposus and annulus fibrosus. The distinction between normal ageing and degeneration is important, but difficult to distinguish either morphologically or biomechanically. Different types of annular defects and tears, rim lesions, concentric tears and radiating clefts, can be observed in conjunction with the disc degeneration process [3].

To image degenerated discs, discography and magnetic resonance imaging (MRI) have been used. In some comparative studies MRI seemed to be more accurate [4, 21]. On the other hand MRI signal intensity depends strongly on the water content of the disc tissue sample [30] and thus the early stages of degeneration which do not affect the water content may not be detected by MRI [30].

Discography offers a sensitive evaluation of disc morphology and provides, compared to MRI, additional information about intradiscal pressure condition and pain provocation [3].

The relationship between intradiscal pressure and the morphological patterns in discography was first observed by Nachemson [11]. During discography typical pain reproduction has been noticed to associate with annular tears extending to the outer annulus [16]. None of the discograms showing normal morphology reproduced the patients' typical pain [16].

Type-I collagen has been abundantly located as a ring in the outer zone and in the outer lamellas of the inner zone of the annulus fibrosus [22]. Type-II collagen was present in the inner annulus, but not in the outer zone. In degenerated annulus fibrosus collagen biosynthesis has been noticed to be increased, but due to the faster turnover the total content of collagen remained unchanged [8]. The reinsertion of stimulated nucleus pulposus cells in an experimental animal model retarded disc degeneration [15]. It delayed the formation of cell clusters of chondrocyte-like cells, the destruction of disc architecture, and production of type-II collagen. Oegema et al have noted that in degenerated discs fibronectin content was elevated suggesting disc cell response to altered environment [14]. Fibronectin was frequently present as fragments capable of stimulating cells to produce metalloproteases and cytokines [14].

Growth factors are proteins regulating cell growth and the turnover of extracellular matrix components. We have previously demonstrated basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factorβ-1 and −2 and their receptor type II in human herniated intervertebral disc tissue [26–28]. In the present study we have focused on these same growth factors targeting disc degeneration in the absence of herniation.

Materials and methods

We obtained 16 discs from 12 patients operated for painful degenerative disc disease at the Texas Back Institute in Plano, TX, USA. The patients had undergone previous spine operations, mainly posterior fusions. The disc samples were convenience samples and the location of the sample within the disc prior to removal was judged clinically by the operating surgeon. Patients were operated by anterior fusion. Preoperative evaluation of degeneration, pain and possible annular tears were classified by the Dallas Discogram Description [20]. After removal, tissue representing different disc regions was immediately frozen in liquid nitrogen in the operating theatre. Anterior annulus fibrosus, nucleus pulposus and posterior annulus fibrosus were stored at −70°C in a deep-freeze. Eight micrometer-thick cryostat sections were fixed in ice-cold acetone. Eight normal control discs were obtained from five organ donors. Morphologically, all control discs lacked signs of degeneration, such as fissures and annulus fibrosus and nucleus pulposus could clearly be distinguished with well demarcated annular lamellae, corresponding to Thompson grades I and II [24]. The samples were collected 1 h post-mortem in various hospitals in Finland. In all cases the cause of death was unrelated to the spine. The control disc material was treated identically to the surgical samples. From frozen discs representative samples of various disc regions (anterior/posterior annulus fibrosus, nucleus pulposus) were cut with an electric saw. The control disc samples were then stored and processed further as described above.

All immunoreactions were detected using an avidin biotin complex-(ABC-) peroxidase staining method (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA). As chromogen substrate we used AEC (immunoreaction shown in red). Tissue sections were counterstained by haematoxylin.

Antibodies

Polyclonal anti-human TGF-β-1, TGF-β-2 and TGF-β receptor type II antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used at the dilutions 1:200,1:50 and 1:100 respectively. Polyclonal bovine bFGF antibody (R & D Systems Inc., Minneapolis, MN, USA) was used at the dilution 1:500 and polyclonal PDGF antibody (R & D Systems Inc) was used at the dilution 1:100. For all antibodies (TGFβ-1, −2,

TGFβ receptor type II, PDGF and bFGF) preincubation with the corresponding antigen (1:10) was done [26–28]. Sections were also stained omitting the primary antibody.

Samples were classified as being immunopositive if more than 20 immunopositive cells were noted. If only a few scattered immunopositive cells were found the total disc sample was classified as being immunonegative, as well as if there was a total lack of immunoreaction. All samples were independently examined blinded to their origin by two observers.

Statistical analysis

Statistical analysis was done using the Sigma Stat Version 1.0 (Jandel Scientific GmbH, Erlerath, Germany) software program. Groups were compared using the Fisher exact test. The level of statistical significance was set at $P < 0.05$.

Results

Clinical data of the patients are described in Table 1. The age of the patients varied from 29 years to 63 years (mean 44.8 years). According to the Dallas Discogram Description the degree of degeneration was 3 in five discs and 2 in seven discs. In one disc the degree of degeneration was 1. Discography data were missing in three discs. The age of the control donors varied from 28 years to 53 years (mean 43 years; Table 2).

Immunopositivity of TGFβ −1 and −2 was detected in 15 of 16 degenerated discs, whereas TGFβ receptor type-II immunopositivity was noted in all degenerated discs. All degenerated discs showed positive immunoreaction for bFGF, but somewhat surprisingly none of the degenerated discs were PDGF immunopositive (Fig. 1a).

Immunoreaction was present either in fibroblast-like disc cells, mainly seen in the annular area of the discs, or in chondrocyte-like disc cells (Fig. 1b, c), scattered more evenly throughout the discs. Chondrocyte-like disc cells were especially present in the nucleus pulposus. Fibroblast-like disc cells were spindle-shaped, often forming lines, whereas chondrocyte-like disc cells were more rounded, often forming cell clusters (Fig. 1c). Immunopositive (TGFβ-1 and −2, and TGFβ receptor type II and bFGF) blood vessels were also detected.

Detailed results of the studied degenerative disc samples are presented in Tables 3 and 4. Control discs appeared normal according to conventional histological staining. Furthermore, control discs did not show any immunoreactivity for PDGF or bFGF. TGFβ-I, -II and TGFβ receptor type II immunoreactivity was, however, noted .

Sections that were stained omitting the primary antibody did not show any immunopositivity. Antigen-preabsorbed immunoreactions were also totally negative.

As can be deduced from Table 4, in the anterior annulus the prevalence of bFGF immunopositivity in

Table 2 Clinical data of control disc patients (organ donors)

Patient	Gender	Age	Disc level
1.	F	53	L3–4
2.	M	45	L1–2
3.	M	45	L3–4
4.	F	41	L3–4
5.	F	31	L2–3

Table 1 Clinical data of the patients operated for degenerative disc disease

Patient	Gender	Age	DDD grade	Level	Discography result
1.	M	40	Deg 2Leak 4Pain R	L4–5	Posterior rupture
2.	F	29	Deg 3Leak 3Pain R	L5-S1	Both anterior and posterior rupture
3.	M	63	-	L2–3	Data missing
4.	M	44	Deg 2Leak 2Pain D	L4–5	Anterior rupture
5.	F	31	Deg 2Leak 3Pain R	L5-S1	Posterior rupture
			Deg 2Leak 3Pain S	L4–5	Posterior rupture
6.	M	60	Deg 2Leak 4Pain S	L5-S1	Posterior rupture
			Deg 2Leak 3Pain D	L4-L5	Posterior rupture
7.	M	42	Deg 3Leak 3Pain	L5-S1	Data missing
			Deg 2Leak 3Pain	L4-L5	Posterior rupture
8.	F	47	Deg 3Leak 3Pain	L5-S1	Posterior rupture
9.	M	44	–	L4-L5	Posterolateral rupture
10.	F	64	–	L3-L4	Data missing
11.	F	33	Deg 1Leak 4Pain S	L4-L5	Posterior rupture
12	M	41	Deg 3Leak 3Pain S	L3–4	Posterior rupture
			Deg 3Leak 3Pain S	L4-L5	Totally fissured

Dallas Discogram Description [20] *Deg* Degree of disc degeneration (scale: 0–3), *Leak* degree of disc rupture (scale: 0–4), *Pain* provocation of pain (*D* dissimilar pain, *S* similar pain, *R* pain reproduction)

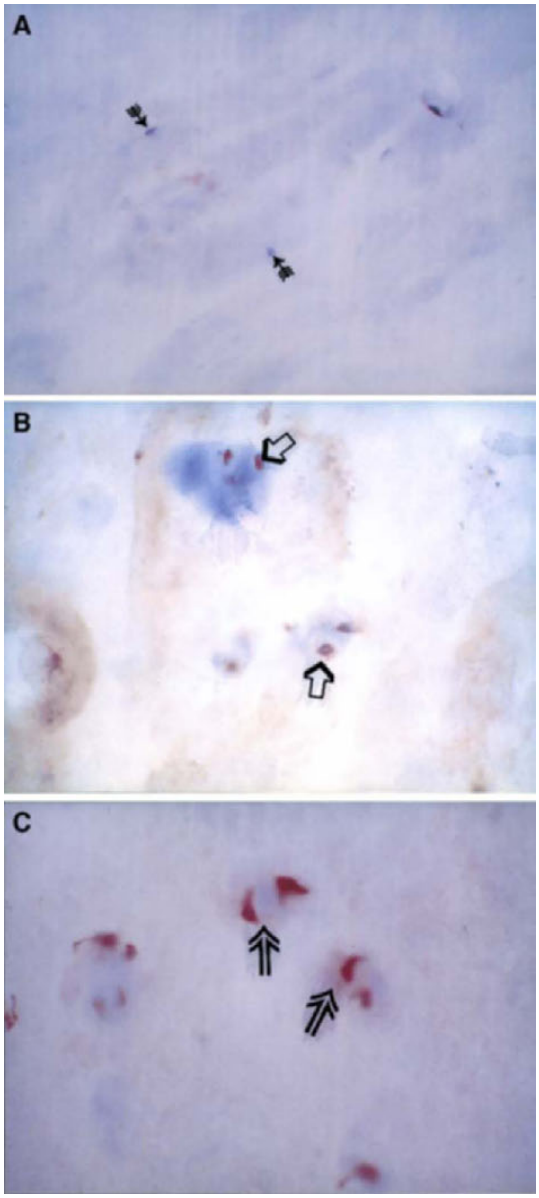

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Fig. 1 In all figures the ABC-peroxidase immunostaining method was used. The used AEC chromogen shows the specific immunoreaction in *red*. All the used antibodies were of polyclonal type and as counterstain we used hematoxylin.**a** Platelet-derived growth factor (PDGF) immunostaining in posterior annulus fibrosus from a 40 year old male patient. Note the total lack of positive immunoreaction. *Arrows* mark pale nuclei of disc cells. Original magnification×370. **b** Transforming growth factor (TGFβ-1) immunopositive chondrocyte-like disc cells (*open arrows*) in anterior annulus fibrosus. The surgical sample was obtained from a 42-year-old male patient operated for painful degenerative disc disease. The operation level was L5-S1. Original magnification×370. **c** The TGFβ-receptor type II immunopositivity (*open arrows*) in cluster of chondrocyte-like posterior annulus fibrosus disc cells from a 40-year-old male patient. The operation level was L4–5. Original magnification×370

chondrocyte-like disc cells was particularly high (present in 85,7% of samples). In the posterior annulus fibrosus and nucleus pulposus all growth factors, with the exception of PDGF which was totally absent from all degenerated discs, were highly prevalent in chondrocyte-like disc cells (present in 53.3–100% of samples). The prevalence of growth factor immunopositivity in fibroblast-like disc cells was lower (present in 0–50% of samples), with the highest prevalence in anterior annulus (present in 31.5–50% of samples).

Statistical differences in immunoreactivity with respect to disc region and disc cell type are shown in Table 5. In the nucleus pulposus immunopositivity was almost exclusively located in chondrocyte-like disc cells. In the posterior annulus fibrosus, which was often disrupted, statistically significant immunopositivity in chondrocyte-like disc cells was noted for bFGF, TGFβ −2 and TGFβ receptor type II ($P=0.0169$, $P=0.0025$ and $P=0.0183$ respectively). Furthermore, bFGF and TGFβ-2 immunopositivity was more often located in chondrocyte-like disc cells than in fibroblast-like disc cells ($P=0.0001$ and $P=0.0092$ respectively). In the anterior annulus fibrosus only for growth factor bFGF ($P=0.0063$) immunopositivity in chondrocyte-like disc cells predominated. Furthermore, only in the anterior annulus fibrosus bFGF immunopositivy in chondrocyte-like disc cells was detected more often than such immunoreactivity for TGFβ-1 and −2. In addition, only in the anterior annulus fibrosus TGFβ receptor type II was located both in chondrocyte-like and fibroblast-like disc cells (Table 5).

Discussion

Degenerated disc has lost its normal architecture. The shape of the annulus cells changes markedly with degeneration: a healthy disc contains spindle-shaped cells, whereas in degenerated discs cells are more rounded and are surrounded by unusual accumulations of extracellular matrix components [6, 18]. In the present study growth factor immunopositivity was noted in spindle shaped (fibroblast-like) as well as rounded (chondrocyte-like) disc cells. With respect to different areas of the disc, immunopositivity in chondrocyte-like disc cells was highly prevalent for most growth factors in the posterior annulus, and particularly in the nucleus pulposus. Compared with the other

Table 3 Immunostaining results for transforming growth factor (TGF) β-1 and −2, TGFβ receptor type II, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) in degenerated intervertebral discs studied

Patient	Area	TGFβ −1	TGFβ −2	TGFβ receptor type II	BFGF	PDGF
1	Ant ann	–	–	DC	DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	DC	DC	DC	DC	–
2	Ant ann	F	DC	DC	..	..
	Nucleus	F,DC	F,DC	F,DC	..	..
	Post ann	F	F	..	..	..
3	Ant ann	F	F	F	–	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	DC	DC	F	–	–
4	Ant ann	–	–	–	DC	–
	Nucleus	–	–	–	DC	–
	Post ann	–	–	F	DC	–
5	Ant ann	–	DC	–	DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	DC	DC	DC	DC	–
	Ant ann	–	DC	DC	DC	–
	Nucleus	F	DC	DC	DC	–
	Post ann	F	DC	DC	DC	–
6	Ant ann	F	F	F	F,DC	–
	Nucleus	F	F	F	F,DC	–
	Post ann	–	DC	–	–	–
	Ant ann	F	F	F	F,DC	–
	Nucleus	DC	DC	DC	DC	–
Patient	Area	TGFβ-I	TGFβ-II	TGFβ receptor type II	BFGF	PDGF
6	Post ann	DC	F	F	DC	–
7	Ant ann	–	DC	DC	–	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	DC	DC	DC	..	–
	Ant ann	F	F	F,DC	F,DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	F,DC	F,DC	F,DC	DC	–
8.	Ant ann	F	F	F,DC	F,DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	–	DC	DC	–	–
9	Ant ann	–	F	DC	DC	–
	Nucleus	DC	DC	DC	DC	–
10.	Ant ann	DC	–	F	..	..
	Nucleus	F,DC	–	DC	..	..
	Post ann	DC	DC	DC	DC	–
11	Ant ann	DC	DC	F	DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	..	DC	–	DC	–
12.	Ant ann	DC	DC	DC	DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	DC	DC	DC	DC	–
	Ant ann	F	F	F	DC	–
	Nucleus	DC	DC	DC	DC	–
	Post ann	–	–	DC	DC	–

DC chondrocyte-like disc cell immunopositivity, *F* fibroblast-like disc cell immunopositivity, – no immunoreaction, .. data not available, *Ant ann* anterior annulus fibrosus, Post ann posterior annulus fibrosus, Nucleus nucleus pulposus

growth factors, bFGF immunopositivity was highly prevalent in chondrocyte-like disc cells particularly in the anterior annulus. Interestingly, PDGF immunopositivity was, however, absent from all disc regions. Thus in degenerated discs, there may be some regional differences with respect to the expression of growth factors. Growth factor expression is particularly prevalent in the rounded chondrocyte-like disc cells. In the anterior annulus the prevalence of growth factor immunopositivity is somewhat more even between chondrocyte-like and fibrocyte-like disc cells. But also in this region immunopositivity for bFGF was more prevalent in the chondrocyte-like disc cells. The observed growth factor immunoreactivity in disc cells suggests that these cells may be actively regulating extracellular matrix component turnover.

Discography showed disruption of the intervertebral discs, especially in the posterior region. The immunoreactivity for growth factors was detected throughout the discs, not only in the disrupted areas. Furthermore, the fact that immunopositivity was observed more often in chondrocyte-like, than fibroblast-like, disc cells may suggest that the former disc cell type is more metabolically active participating in the cellular remodelling of disc degeneration. We have in previous studies located these same growth factors in herniated intervertebral disc tissue [26–28]. In herniated disc tissue the predominant immunopositive disc cell type was the chondrocyte-like disc cell [26–28]. Fibroblast-like disc cell immunopositivity was rare in herniations. These findings may suggest a step-by-step change in cell type from normal disc tissue to pathological processes; i.e. disc degeneration and disc herniation.

In this study and in our earlier studies on herniated disc tissue [26–28] TGFβ-1, −2 and TGFβ receptor type II were the only growth factors observed in control discs. Some studies have suggested that this growth factor is absent in control disc tissue [7, 23]. However, in a recent biochemical tissue culture study on growth factors and inflammatory mediators in patients undergoing surgery for scoliosis, lumbar radiculopathy and discogenic pain, production of TGFβ-1 was demonstrated both in control (scoliosis) and degenerate human disc tissues [2]. In the same study Burke et al. [2] demonstrated the production of bFGF in control (scoliotic) as well as degenerated human nucleus pulposus in vitro. However, in the present immunohistochemical study and in earlier study by us on herniated disc tissue [26] bFGF could not be demonstrated in control discs. In a rat animal model on disc degeneration Nagano et al. [12] could not observe bFGF in control discs. When comparing the injured annulus fibrosus of merinos to intact ones, bFGF, TGFβ and osteonectin were strongly localized in blood vessels and cells in the vicinity of annular lesion [9] The immunohistochemical expression was maximal 12 month after the operation, and diminished by 26 months after the operation. In control discs, the expression of bFGF and TGFβ was localized to sparsely distributed cells in the annulus fibrosus.

Table 4 Summary of immunostaining results for TGF β-1 and −2, TGFβ receptor type II and bFGF in degenerated intervertebral disc tissue samples obtained from various regions of the disc

Disc area	Growth	Location of immunoreaction		
		DC	F	No immunoreactivity
Anterior annulus	TGFβ-1	3/16	7/16	6/16
Posterior annulus		8/15	3/15	4/15
Nucleus		13/16	4/16	1/16
Anterior annulus	TGFβ-2	6/16	7/16	3/16
Posterior annulus		11/15	2/15	2/15
Nucleus		13/16	2/16	2/16
Anterior annulus	TGFβ receptor type II	8/16	8/16	1/16
Posterior annulus		9/14	4/14	2/14
Nucleus		14/16	2/16	1/16
Anterior annulus	bFGF	12/14	4/14	2/14
Posterior annulus		10/13	0/13	3/13
Nucleus		14/14	1/14	0/14

DC chondrocyte-like disc cell, *F* fibroblast-like disc cell

Table 5 Statistically significant differences in TGF β-1, −2 and TGF βreceptor type II, and bFGF immunopositivity in different disc regions and disc cell types. (Fisher Exact Test, the level of statistical significance $p < 0.05$)

Disc region	Statistically significant difference in immunoreactivity		
Anterior annulus fibrosus	Cell type		
	DC	bFGF versus TGFβ −1	$P = 0.0007$
	DC	bFGF versus TGFβ −2	$P = 0.0106$
	DC	bFGF versus no immunoreaction	$P = 0.0004$
	DC	TGFβrec versus no immunoreaction	$P = 0.0155$
	F	TGFβrec versus no immunoreaction	$P = 0.0155$
	Antibody	Localization of immunoreaction	
	bFGF	DC versus F	$P = 0.0063$
Posterior annulus fibrosus	Cell type		
	DC	bFGF versus no immunoreaction	$P = 0.0169$
	DC	TGFβ-2 versus no immunoreaction	$P = 0.0025$
	DC	TGFβrec versus no immunoreaction	$P = 0.0183$
	Antibody	Localization of immunoreaction	
	bFGF	DC versus F	$P = 0.0001$
	TGFβ-2	DC versus F	$P = 0.0092$
Nucleus pulposus	Cell type		
	DC	bFGF versus no immunoreaction	$P < 0.0001$
	DC	TGFβ-1 versus no immunoreaction	$P < 0.0001$
	DC	TGFβ-2 versus no immunoreaction	$P = 0.0002$
	DC	TGFβrec versus no immunoreaction	$P < 0.0001$
	Antibody	Localization of immunoreaction	
	bFGF	DC versus F	$P < 0.0001$
	TGFβ-1	DC versus F	$P = 0.0038$
	TGFβ-2	DC versus F	$P < 0.0001$
	TGFβrec	DC versus F	$P < 0.0001$

DC chondrocyte-like disc cell, *F* fibroblast-like disc cell, *bFGF* basic fibroblast growth factor, *TGFβ-1* transforming growth factor β-1, *TGFβ-2* transforming growth factor β-2, *TGFβrec* transforming growth factor β receptor type II

In an experimental animal model the degeneration process was delayed with the reinsertion of autogenous activated nucleus pulposus [15]. This activation was produced by coculturing nucleus pulposus cells with annulus fibrosus cells. During the coculture both cell types were proliferating. Furthermore, reinsertion of the nucleus pulposus delayed especially the formation of clusters of chondrocyte-like disc cells [15]. Such clusters were noted in the present study both in the nucleus pulposus and in annular areas of the degenerated discs. Furthermore, injection of intact nucleus pulposus has been demonstrated to be far more effective in delaying degeneration rather than injection of only nucleus pulposus cells [13], thus highlighting the importance of the extracellular matrix. In chondrocyte-like disc cell clusters marked matrix metalloproteinase activity has been demonstrated [19]. Interestingly, such enzyme activity was particularly intense in herniated discs [19]. The presence of growth factors, matrix metalloproteinases and oncoproteins [29] suggests that these particular disc cells have been activated in pathological conditions and regulate the turnover of extracellular matrix components. In rat degenerated intervertebral discs bFGF and its receptor were localized in chondrocyte-like

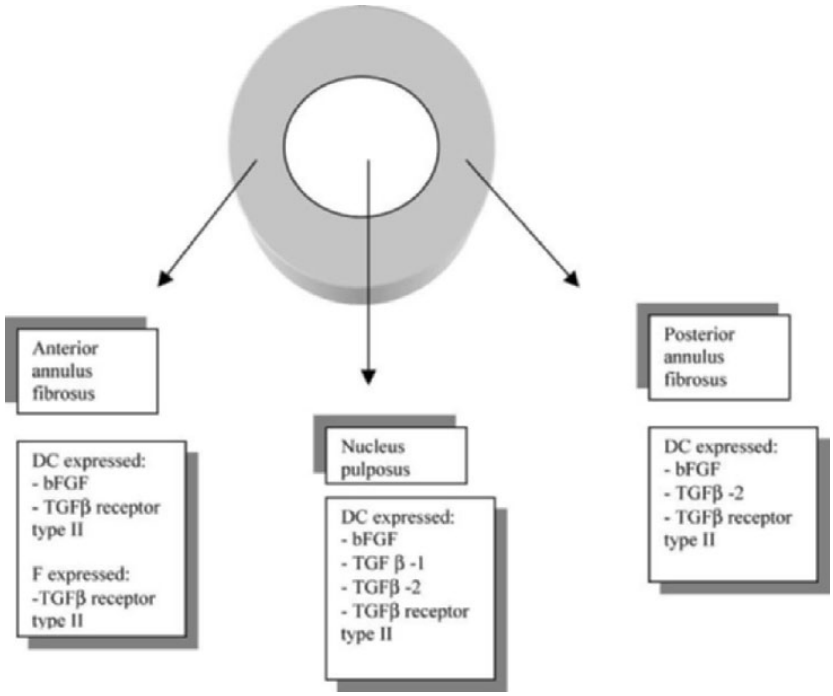

Fig. 2 Expression of growth factors (bFGF, TGFβ-1, −2 and TGFβ receptor type II) in different disc regions. (*DC* chondrocyte-like disc cell, *F* fibroblast-like disc cell, *bFGF* basic fibroblast growth factor, *TGF*β transforming growth factor β)

rounded cells, and the proliferation capacity of these cells exceeded that of normal annular spindle-shaped cells [12].

Experimental animal models suggest important functions for the above growth factors in intervertebral disc physiology and pathophysiology. Minamide et al. [10] have shown that epidural application of bFGF in the rabbit facilitates the resorption of the sequestrated intervertebral disc fragment, and stimulates neovascularization and proliferation of inflammatory cells. This effect was dose-dependent. In cell cultures, TGFβ and FGF have been demonstrated to be potent cellular proliferation stimulators [25]. Cells from the nucleus pulposus and the transition zone reacted more than annulus fibrosus cells. Furthermore, TGFβ was far more potent than FGF as a stimulator of cellular proliferation. In addition, TGFβ-1 decreased the level of active matrix metalloproteinase-2 (MMP-2) in nucleus pulposus cells [17]. Furthermore, the cell surface levels of metalloproteinase inhibitors also decreased. In another cell culture study, the presence of TGFβ first enhanced cellular proliferation, later on the mitogenic response decreased [5]. In addition, TGFβ-1 is a potent stimulator for proteoglycan production by disc cells [1].

Since there was a time gap between discography and the operation, possible local irritation by the discography procedure was not present at the time of operation when the tissue samples were taken for analysis. Of note, the growth factor immunoreactivity was detected throughout the discs, not only in the disrupted posterior areas.

Earlier we noted marked PDGF immunoreactivity in herniated disc tissue [27]. This immunoreactivity was located in blood vessels and cells, both chondrocyte-like disc cells and fibroblast-like disc cells. In the present study such disc cell-associated immunoreactivity was not observed. Furthermore, normal control discs were totally PDGF immunonegative. This may suggest that PDGF is latent until the disc becomes herniated. Characterising the nature of the PDGF activator, whether another growth factor or some other substance in the nerve root area, will require further research. We expect that more information on disc cell remodelling, production of different proteins that affect the extracellular matrix and cell proliferation, may be obtained by focusing future research on signal transfer mechanisms in disc cells and the gene activation process in these cells.

Conclusions

Our results show that growth factors are expressed in degenerated discs, in a different pattern than in control discs. Different types of expression were observed in the various disc areas (Fig. 2). In degenerated intervertebral disc tissue chondrocyte-like disc cells in the nucleus pulposus were immunopositive to all other growth factors except PDGF. In the anterior annulus fibrosus the most prevalent growth factor present in chondrocyte-like disc cells was bFGF. TGFβ receptor type II was expressed in both chondrocyte-like and fibroblast-like disc cells, whereas in the posterior annulus fibrosus the most prevalent growth factors expressed in chondrocyte-like disc cells were bFGF and TGFβ-2.

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