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RITVA INKINEN

# Proteoglycans and Hyaluronan in the Intervertebral Disc and Cartilage Endplate

## Effects of Aging and Degeneration

Doctoral dissertation

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Department of Anatomy
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Professor Markku Tammi, M.D., Ph.D.
Department of Anatomy

**Authors Address:**
Department of Anatomy
University of Kuopio
Tel. +358 10 4294357
Orion Pharma, Espoo, Finland
e-mail: ritva.inkinen@orionpharma.com

**Supervisors:**
Professor Markku Tammi, M.D., Ph.D.
Department of Anatomy
University of Kuopio

Docent Mikko Lammi, Ph.D.
Department of Anatomy
University of Kuopio

Kaija Puustjärvi, M.D., Ph.D.
Department of Physical Medicine and Rehabilitation
University Hospital of Helsinki

**Reviewers:**
Sally Roberts, Ph.D.
Centre for Spinal Studies, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, UK

Associate Professor Mats Grönblad, M.D., Ph.D.
Department of Physical Medicine and Rehabilitation
University Hospital of Helsinki

**Opponent:**
Professor Bruce Caterson, Ph.D.
Connective Tissue Laboratory
School of Molecular and Medical Biosciences
Cardiff, UK

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## ABSTRACT

Spinal disorders form one of the most common causes of long-term disability in the middle-aged population of the western countries. Spinal disorders are an expanding health problem and although the economic impact on society is vast, the pathophysiology of the spinal disorders still has not been clarified in detail.

Proteoglycans (PGs) and hyaluronan (HA) are essential macromolecules for the function of the intervertebral disc and cartilage endplate. They also reflect tissue changes during aging and degeneration. PGs and HA were investigated in this study to find specific age- and degeneration-related alterations in the human, canine and porcine intervertebral disc and cartilage endplate. In addition, the effects of two widely used non-steroidal anti-inflammatory drugs (NSAIDs), tiaprofenic acid and indomethacin, were studied in an experimental porcine disc degeneration model.

In the human intervertebral disc, the content of small PGs increased, and the glycosylation of large PGs was altered with disc degeneration, while less change was associated with aging. The alterations in PG content and glycosylation in degeneration differed from those in aging or maturation.

In the animal experimental model, degenerative changes were seen mainly in the nucleus pulposus, although the process was induced by a scalpel wound in the annulus fibrosus. An injury in the annulus alters the biomechanics, nutrition and metabolism of the cells and changes matrix production and degradation. The PG alterations in a degenerating porcine disc were modified by administration of the NSAIDs. Tiaprofenic acid may afford protection to the PGs, at least during the early phases after disc injury.

Most HA was found in the inner parts of the disc. Particularly in human discs, cell-associated HA appeared to be masked, probably by aggrecan, whereas there was less masking of HA in the interterritorial matrix. In the canine cartilage endplate, HA was clearly cell-associated, even appearing inside the chondrocytes. Whether this is a sign of synthesis or uptake and degradation of HA remains unclear. In the outer annulus fibrosus, HA was located between the collagenous lamellae, perhaps serving as a lubricant when the lamellae slide along each other.

This study confirms the biochemical and biomechanical importance of PGs and HA in the extracellular matrix of spinal tissues. Large PGs and HA were more concentrated in the inner parts of the disc. The changes in large PG glycosylation and small PG concentration in degeneration is suggested to reflect an alteration in cellular differentiation, or to result from cleavage by proteinases, leading to inappropriate structures for the biomechanical environment of the disc. The animal models give valuable information about the normal structure and pathogenesis of the intervertebral disc and cartilage endplate. The more we know about the normal function of the disc, the more possibilities there are for more specific treatments of spinal diseases.

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Medical Subject Headings: aging; anti-inflammatory agents; non-steroidal; spine; spinal diseases; cartilage; articular; intervertebral disk; proteoglycans; hyaluronic acid; propionic acid; indomethacin; glycosylation; animal; human; extracellular matrix; osteoarthritis

*To Ypeli, Miisu and Pipsa*

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Espoo, November 2004

Ritva Inkinen

**ABBREVIATIONS**

| | |
|---|---|
| aa | Amino acid |
| bHABC | biotinylated hyaluronan binding complex |
| BSA | Bovine serum albumin |
| C | Cervical |
| CS | Chondroitin sulphate |
| CS-0 | Chondroitin 0-sulphate |
| CS-4 | Chondroitin 4-sulphate |
| CS-6 | Chondroitin 6-sulphate |
| DAB | 3,3`diaminobenzidine |
| Da | Dalton |
| DS | Dermatan sulphate |
| ECM | Extracellular matrix |
| EDTA | Ethylenediamino tetra-acetic acid |
| EGF | Epidermal growth factor |
| GAG | Glycosaminoglycan |
| GalNAc | N-acetyl-D-galactosamine |
| GlcNAc | N-acetyl-D-glucosamine |
| GuCl | Guanidinium chloride |
| HA | Hyaluronan, hyaluronic acid |
| HS | Heparan sulphate |
| HPLC | High performance liquid chromatography |
| IL | Interleukin |
| KS | Keratan sulphate |
| L | Lumbar |
| LP | Link protein |
| NSAID | Non steroidal anti-inflammatory drug |
| PAGE | Polyacrylamide gel electrophoresis |
| PG | Proteoglycan |
| PB | Phosphate buffer |
| PBS | Phosphate buffered saline |
| SDS | Sodium dodecyl sulphate |
| PVDF | Polyvinylidene difluoride |
| T | Thoracic |
| TGF-β | Transforming growth factor beta |
| TNF-α | Tumor necrosis factor alpha |
| ww | Tissue wet weight |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Inkinen RI, Lammi MJ, Lehmonen S, Puustjärvi K, Kääpä E, Tammi MI: Relative increase of biglycan and decorin and altered chondroitin sulfate epitopes in the degenerating human intervertebral disc. Journal of Rheumatology 25: 506-514, 1998

II Inkinen RI, Lammi MJ, Ågren U, Tammi R, Puustjärvi K, Tammi MI: Hyaluronan distribution in the human and canine intervertebral disc and vertebral endplate. Histochemical Journal 31: 579-588, 1999

III Kääpä E, Holm S, Inkinen R, Lammi M, Tammi M, Vanharanta H: Proteoglycan chemistry after experimental intervertebral disc injury. Journal of Spinal Disorders 7: 296-306, 1994

IV Karppinen J, Inkinen RI, Kääpä E, Lammi MJ, Tammi MI, Holm S, Vanharanta H: Effects of tiaprofenic acid and indomethacin on proteoglycans in the degenerating intervertebral disc. Spine 20: 1170-1177, 1995

CONTENTS

# 1. INTRODUCTION

Musculoskeletal disorders, the majority of which are spinal disorders, form the most common cause of long-term disability in the middle-aged population of the western countries (Badley et al., 1994; Speed, 2004). In Finland, it has been estimated that 80% of all inhabitants suffer from back pain at some time during their lifetime (Heliövaara, 1989). Spinal disease is an extensive health problem (together with general aging of the population), and the economic impact on society is vast (Zitting and Vanharanta, 1998).

Numerous studies, epidemiological, genetic, biochemical, biomechanical, and work-related, among others, have been conducted or are ongoing to find risk factors, and to prevent and treat these disorders, but the pathophysiology of spinal disorders is still not known in detail (Riihimäki, 1999; Viikari-Juntura and Riihimäki, 1999).

Degeneration of the intervertebral disc, commonly starting as early as during adolescence, is probably the main cause for spinal disorders, especially for low back pain (Miller et al., 1988; Zitting and Vanharanta, 1998; Luoma et al., 2000). Several factors affecting the degeneration of the intervertebral disc have been found, but no generally recognized starting point of the disease, nor clear cause-consequence relationship between different factors have been identified. The basic biochemistry and biomechanics of the spine is complex and knowledge is still insufficient to allow a full understanding of the multifactorial etiology of spinal disorders or to find efficient treatments.

Proteoglycans (PGs) and hyaluronan (HA) are essential macromolecules for the functioning of the intervertebral disc and cartilage endplate. These molecules are sensitive indicators of tissue changes during aging and degeneration, and probably also of the efficiency of treatments. PGs and HA were investigated in this study to characterize further the role of these molecules during aging and degeneration of the intervertebral disc and cartilage endplate. In addition, the effects of two widely used anti-inflammatory drugs on disc degeneration were studied.

# 2. REVIEW OF LITERATURE

## 2.1. Structure and function of the spine

The human spine consists of 24 vertebral bodies, connected by intervertebral discs and zygapophyseal joints. The vertebral articulations are supported by spinal ligaments and muscles. Neural structures (the spinal cord and nerves) are located in and protected by the posterior part of the vertebrae. The diverse biophysical properties of spinal tissues enable the flexibility of the spine and at the same time provide stability and shock absorbing properties. The complex structure of spinal tissues allows the spine to bear high loads such as compression (including body-weight effect, muscle-ligament tension, and intra-abdominal pressure), shear, bending, torsion, and their combinations (Dolan and Adams, 1995).

## 2.2. Structure and function of the intervertebral disc and the cartilage endplate

The number of intervertebral discs varies in mammalian species: for example in the human and porcine spine there are 23 discs, while the canine spine has 26, excluding sacral and coccygeal discs. However, the macroscopic structure of intervertebral discs is very similar in several animal species (Tominaga et al., 1995; Wilke et al., 1996; Elliott and Sarver, 2004). The intervertebral disc is composed of a highly hydrated nucleus pulposus in the middle, surrounded by a lamellar annulus fibrosus. Superior and inferior surfaces of the disc, next to the vertebral bodies, are covered with cartilaginous endplates (Fig.1).

The main components of the extracellular matrix (ECM) in the intervertebral disc and endplate are water, PGs and collagens, which comprise over 90% of the normal disc volume. The biomechanical properties of the disc depend largely on these macromolecules (Urban and McMullin, 1988). Other matrix glycoproteins, for example elastin, serum proteins and lipids have been found in the intervertebral disc, but the importance of these macromolecules is still poorly understood (Eyre, 1988; McDevitt, 1988; Yu, 2002).

The overall cell density of the disc is low, much lower than that of many other connective tissues (Maroudas, 1988). Cells constitute 1-5% of the total tissue volume (Oshima and Urban, 1992). In human articular cartilage, there are about 14 000 cells/mm$^3$, whereas in the human intervertebral disc the density is only 6 000 cells/mm$^3$ (Maroudas et al., 1975). The number of the cells is higher in many other vertebrates, for example the canine nucleus pulposus contains approximately 14 000 cells/mm$^3$ (Holm et al., 1981).

The main role of the intervertebral discs and the cartilage endplate is to serve as a shock-absorber and stabiliser during compression, bending, extension and rotation of the spine (Hukins, 1988). The structure and macromolecular composition of the spinal tissues are related to their function and the relative concentration of these components is different depending on the location inside the individual disc and along the spine.

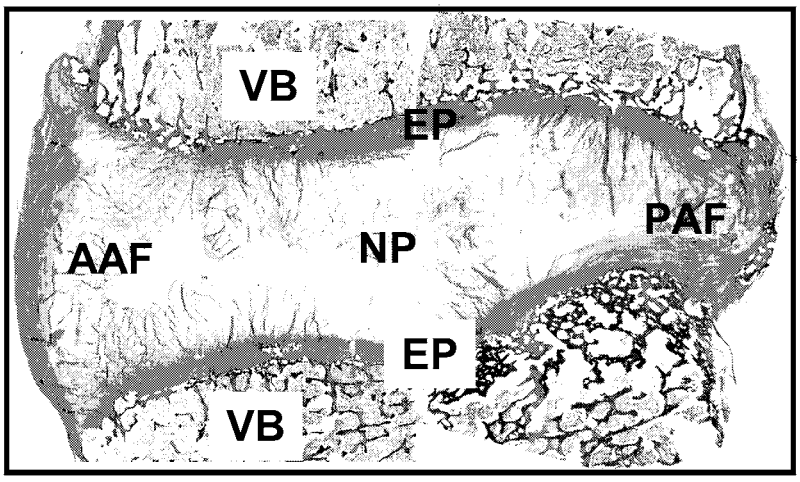


**Figure 1.** Structure of the human intervertebral disc, cartilage endplate and vertebral bone. Nucleus pulposus (NP), anterior annulus fibrosus (AAF), posterior annulus fibrosus (PAF), EP (cartilage endplate) and VB (vertebral bone).

### 2.2.1. Nucleus pulposus

In humans, the nucleus pulposus occupies 30-50% of the total volume of the disc (Ayad and Weiss, 1987) (Fig.1). However, the relative size of the nucleus pulposus changes during aging, and the boundary towards the annulus fibrosus becomes less apparent (Hukins, 1988; Humzah and Soames, 1988; Roberts, 2002; Hunter et al., 2003).

As a highly-hydrated tissue, the nucleus pulposus is capable of resisting compressive forces. The water content can exceed 80% in early life. With age the content of water decreases progressively (Adams and Muir, 1976; Bernick and Cailliet, 1982; Postacchini et al., 1984). The high water content is related to the high PG concentration, as the PGs are able to absorb water due to their negatively charged glycosaminoglycan (GAG) chains (Maroudas, 1988). Consequently, the PGs constitute about 50% of the nucleus pulposus, while collagens account for about 5% of the dry weight of the tissue (Ayad and Weiss, 1987). The collagen network is loose and irregular, and the individual fibrils are thin (Roberts et al., 1989).

The cells of the nucleus pulposus are mainly chondrocytes or chondrocyte-like cells (Chelberg et al., 1995; Oegema, 2002; Roberts, 2002). The rounded cells with processes reside in the matrix individually or in clusters, surrounded by a pericellular capsule (Maroudas et al., 1975; Roberts et al., 1991; Roberts et al., 1994; Errington et al., 1998; Roberts, 2002). Early in life notochordal cells are present in the nucleus pulposus, but they are replaced by chondrocytes soon after infancy in humans. In some animals, such as mice, the notochordal cells persist in the nucleus throughout life (Errington et al., 1998; Oegema, 2002; Roberts, 2002; Hunter et al., 2003).

### 2.2.2. Annulus fibrosus

The soft nucleus pulposus is surrounded and protected by the firm annulus fibrosus. Collagens are the main macromolecules in the ECM of the annulus fibrosus, constituting about 50-60% of the tissue dry weight (Ayad and Weiss, 1987). The amount of PGs and water is significantly lower than in the nucleus pulposus, although

there are considerable differences between the inner and outer annulus (Eyre and Muir, 1976; Bruehlmann et al., 2002). The inner annulus is more fibrocartilaginous-like tissue, rich in PGs, and the collagen network is fine. The structure of the outer annulus, particularly in the anterior side of the disc, is more similar to ligaments and tendons, with a highly organised, lamellar collagen network (Eyre, 1979; Urban and Roberts, 1996). These lamellae run parallel to each other at a 60-70° angle with respect to the spinal axis. This structure makes the tissue resistant to rotational and tensile forces, as well as to bending and twisting (Hukins, 1988; Humzah and Soames, 1988).

Innervation of the intervertebral disc extends to the outer annulus and ligaments (Bogduk, 1983; Palmgren et al., 1999), whereas nerves are not seen in the healthy nucleus pulposus and cartilage endplate (Roberts, 1995; Johnson et al., 2002). However, in degenerated discs nerve ingrowth has been reported (Harrington et al., 2000; Johnson et al., 2002; Melrose et al., 2002a).

The cells maintaining the annulus fibrosus ECM are mainly chondrocyte-like cells of mesenchymal (Hukins, 1988) or perinotochordal origin (Ralphs et al., 1994; Rufai et al., 1995; Hayes et al., 2001). The morphology of the cells seems to reflect the mechanical environment of the annulus fibrosus (Giori et al., 1993; Lotz et al., 1998). In the inner annulus the cells are more rounded in shape with a dense pericellular capsule, whereas in the outer annulus the cells are more elongated and almost fibroblast-like, aligned parallel to the lamellae (Roberts et al., 1991; Roberts, 1995; Errington et al., 1998). The annulus cells have cellular processes similar to those of cells in the nucleus pulposus (Errington et al., 1998; Bruehlmann et al., 2002).

### 2.2.3. Cartilage endplate

The roles of the cartilage endplate are related to the prevention of the nucleus pulposus bulging into the vertebral bone, and absorption of the hydrostatic pressure during compression and loading (Bernick and Cailliet, 1982; Broberg, 1983; Antoniou et al., 1996a).

In man, the cartilage endplate is on average 0.6 mm thick. However, the thickness ranges from 0.1 to 1.6 mm, depending on the location within the disc and the position of

the disc in the vertebral column (Roberts et al., 1989). The structure of the cartilaginous endplate resembles that of hyaline cartilage (Bernick and Cailliet, 1982; Roberts et al., 1989), although cell density and the appearance and distribution of the macromolecules vary according to the site within the endplate and the location of the motion segment (endplate, intervertebral disc and vertebra) (Broberg, 1983; Antoniou et al., 1996a). In general, there are more collagens close to the vertebral bone and the outer annulus fibrosus. The PGs and water predominate in the area nearest to the nucleus pulposus and the inner annulus fibrosus, and the cell density is higher there, too (Roberts et al., 1989; Bishop and Pearce, 1993).

The cells of the cartilage endplate are chondrocytes, mostly occurring as single cells but able to proliferate to form clusters in areas of abnormality (Roberts et al., 1989). Chondrocytes undergo lifelong changes in phenotype. During development, when the growth plate is present, most of the cells are proliferating and hypertrophic, whereas during adulthood and degeneration, resting and calcifying chondrocytes are prevalent (Maroudas et al., 1975; Bernick and Cailliet, 1982).

### 2.2.4. Nutrition of the intervertebral disc and cartilage endplate

The intervertebral disc is an avascular tissue, supported by adjacent tissues. Blood vessels in the vertebral bone and the longitudinal ligaments serve as routes for nutrients and waste products. The solutes move by diffusion through the cartilage endplate and the outer annulus fibrosus (Maroudas et al., 1975; Urban et al., 1977; Maroudas, 1988; Roberts et al., 1996). The effectiveness of the transport depends on the distance, macromolecular composition of the ECM, size and structure of the solute and other characteristics of the tissue (e.g. physiological state and age of the disc) (Maroudas et al., 1975; Urban et al., 1977; Urban et al., 1978; Maroudas, 1988).

The low oxygen concentration in the disc, mainly due to its avascularity, necessitates energy production via anaerobic glycolysis, particularly in the inner parts, which leads to a high concentration of lactate and low pH of the tissue (Holm et al., 1981). This specific tissue environment together with the low cell density results in a relatively low metabolic activity of the intervertebral disc, an example of which is the slow synthesis

rate of PGs, which in the worst case predisposes to degeneration (Holm et al., 1981; Oshima and Urban, 1992; Urban, 2002).

## 2.3. Macromolecules of the intervertebral disc and cartilage endplate

### 2.3.1. Proteoglycans

A PG molecule is composed of a core protein to which one or more (1 to ~200) GAG chains and oligosaccharides are covalently attached. GAGs are linear carbohydrate polymers that consist of repeating disaccharide units. The number of the disaccharide units in a GAG chain varies from few to one hundred or even more. Thus, the molecular mass of a PG ranges from ~50 000 to several millions of daltons (Heinegård and Sommarin, 1987). GAGs are polyanionic by nature and the high number of hydrated cations attracted by the negative charges leads to the high water binding capacity of the molecule. This accounts for the biomechanical role of ECM PGs, which includes exerting a swelling pressure that maintains fluid balance and resists compressive forces (Wight et al., 1991). The function of the oligosaccharides in PGs is not so well known, but a protective and covering role has been suggested (Yanagishita, 1993).

PGs have important functions in the matrix assembly and tissue architecture as well as in cell adhesion, differentiation and proliferation (Ruoslahti, 1988; Mizuguchi et al., 2003). These properties are achieved mainly by the interactions of PGs with other ECM molecules such as collagens, fibronectin, fibromodulin, and growth factors (Yamaguchi et al., 1990). PGs are found not only in ECM, but also on the cell surface and in the intracellular granules (Ruoslahti, 1988; Wight et al., 1991).

#### 2.3.1.1. Glycosaminoglycans

In the disc tissue, five different GAGs are present: chondroitin sulphate (CS), dermatan sulphate (DS), hyaluronan (HA, hyaluronic acid, hyaluronate), heparan sulphate (HS), and keratan sulphate (KS) (Emes and Pearce, 1975; Hascall, 1988; McDevitt, 1988; Ruoslahti, 1988; Taylor et al., 1992; Melrose et al., 1994; Urban and Roberts, 1996).

CS and DS have the same basic carbohydrate backbone of N-acetylgalactosamine (GalNAc) and glucuronic acid/iduronic acid (GlcA/IdoA), the former linked by β1-4 and the latter by β/α1-3 bonds. The average size of a CS chain is typically 20-30 kDa (Wight et al., 1991). CS is sulphated at carbon 4 (CS-4) or carbon 6 (CS-6) position of the GalNAc unit (Hascall, 1988; Ruoslahti, 1988; Hardingham and Fosang, 1992), and DS usually at carbon 4 of GalNAc and often at carbon 2 of IdoA (Rosenberg et al., 1985; Choi et al., 1989). Monosulphated disaccharides, sulphated at carbon 4 or 6 of GalNAc are the most common isoforms, however, non-, di- and trisulphated disaccharides are also found in tissue PGs (Cheng et al., 1994).

KS contains N-acetylglucosamine (GlcNAc) and galactose (Gal) linked via β1-4 and β1-3 bonds, respectively. KS type IIA, present in large PGs, is usually 10-20 kDa in size, and the chains are sulphated at position 6 of either or both sugars. A nonsulphated KS backbone (polylactosamine) is present in many other glycoproteins (Hascall, 1988; Wight et al., 1991). The water binding capacity of KS is lower than that of CS, due to the absence of the carboxylic group. KS type I is present in small PGs (fibromodulin and lumican) (Funderburgh, 2000). KS II is attached through an oligosaccharide structure linked O-glycosidically to the hydroxyl group of serine or threonine, while KS I binds N-glycosidically to an asparagine residue of the core protein (Choi and Meyer, 1975).

HS has the backbone structure of GlcNAc and GlcA or IdoA in various proportions with α1-4 and β1-3 or α1-3 linkages, respectively (Yanagishita, 1993). The molecular weight of the HS chain is usually below 50 kDa. A number of changes take place during or following the HS chain elongation, including GlcA -> IdoA isomerization, and several sulphation events. These add the negative charges and create structural variations that facilitate a number of specific protein interactions (Wight et al., 1991).

HA is a high molecular weight carbohydrate chain, the size being as much as 10 MDa (~25 000 disaccharide repeats), consisting of alternating GlcNAc(β1-4) and GlcA(β1-3) moieties. The molecule does not contain sulphate groups at all (Holmes et al., 1988; Fraser and Laurent, 1996). The open, randomly oriented structure with polyanionic characteristics makes it highly viscous, even gel-like, and ready to interact with water (Laurent and Fraser, 1992). HA has a distinct ability to bind to ECM proteins and PGs

(Heinegård et al., 1998), and cell surface receptors (Dowthwaite et al., 1998). In the ECM, large aggregating PGs, such as aggrecan (Heinegård and Hascall, 1974), versican (Shinomura et al., 1993) and neurocan (Rauch et al., 1992), bind to HA with noncovalent linkages. HA is able to form networks, e.g. in the vitreous humour (Fraser and Laurent, 1996). In general, HA is present in most vertebral tissues, particularly in the ECM, and in the pericellular space of skin and musculoskeletal connective tissues (Fraser and Laurent, 1996). It has an important role in embryonic development and in the repair and regeneration processes of tissues when cells differentiate, proliferate, migrate and form new matrices (Laurent, 1970; Toole, 1981; Hascall, 1988; Laurent and Fraser, 1992; Fenderson et al., 1993; Pitsillides et al., 1995; Tammi et al., 2002). HA is implicated in the pathophysiology of several diseases, such as rheumatoid arthritis (Asari et al., 1996; Kikuchi et al., 1996), and participates in wound healing, and is present during the growth of tumors and metastases (Toole et al., 2002). In addition to the roles mentioned above, HA serves as a space filler and lubricant, e.g. in synovial joints (Engström-Laurent and Hällgren, 1987).

#### 2.3.1.2. Large proteoglycans and hyalectans

Large PGs (defined as having a large core protein, several GAGs, and a high molecular weight) are called hyalectans when they are able to bind HA and link protein (LP) and form a PG aggregate (Iozzo, 1998). In cartilaginous tissues, the proportion of these aggregating PGs of the total PGs varies from 50 to 85%. Some large PGs are unable to form stable aggregates (Heinegård and Sommarin, 1987; Lohmander, 1988), probably as a consequence of partial cleavage of the core protein by several proteinases present in the tissue (Carney et al., 1985; Liu et al., 1991; Little et al., 2002), or because of the absence of LP (Tengblad et al., 1984).

Aggrecan is the major large PG in the ECM of cartilaginous tissues (Hascall, 1988; Heinegård and Oldberg, 1989), including the intervertebral disc and cartilage endplate (Emes and Pearce, 1975; Adams and Muir, 1976; Buckwalter et al., 1985; Roberts et al., 1994; Johnstone and Bayliss, 1995). The 220-kDa core protein of aggrecan (Doege et al., 1988) contains several domains. The globular domain, G1, short interglobular

domain (IGD) and G2 are located close to the $NH_2$-terminal end of the protein core. The G1 domain is responsible for interaction with HA and LP. The domain consists of three looped subdomains: A, B, and B' (Neame et al., 1989; Neame and Barry, 1993). Aggrecan binds to HA at the B-B' loop and to LP at the A loop (Matsumoto et al., 2003). The G3 domain, able to bind other ECM proteins via lectin repeats, is located in the carboxy-terminal end of the core protein. Alternative splicing in this domain has been noticed to modulate interactions and assembly of the ECM proteins (Day et al., 2004). The KS-binding region, CS-binding regions CS1 and CS2, and a number of binding sites for N-linked oligosaccharides are situated between G2 and G3 (Heinegård and Sommarin, 1987; Wight et al., 1991; Iozzo, 1998). The molecular weight of tissue aggrecan varies from 1 to 3.5 MDa with a core protein length of 100-300 nm (Heinegård and Sommarin, 1987).

Versican is another hyalectan type found in the ECM of spinal tissues (Shinomura et al., 1993; Melrose et al., 2001a; Sztrolovics et al., 2002a). Its expression is greatest during fetal development (Melrose et al., 2001a; Sztrolovics et al., 2002a). It has a structure partially homologous with aggrecan (Yang et al., 2003). However, the G2 domain is missing, and the CS/DS-binding region is different (Iozzo, 1998; Wight, 2002). In the G1 domain, versican binds to both HA and LP at the B-B' segment (Matsumoto et al., 2003). A lectin-like G3 domain in versican seems to mediate cellular adhesion and proliferation (Yang et al., 2003).

Perlecan is a HS/CS containing proteoglycan present in the basement membranes and pericellular matrices of the developing spine, mainly in the cartilage endplate and growth plate (Melrose et al., 2002b; Melrose et al., 2003). This molecule has a ~ 470-kDa core protein, three GAG chains, and five separate domains interacting with several basement membrane and ECM components, including collagen type IV, laminin and fibronectin (Iozzo et al., 1994; Melrose et al., 2003). Perlecan is present in normal development of the ECM in many cartilaginous tissues (Iozzo, 2001; Melrose et al., 2003), and in some pathological disorders, e.g. in the cardiovascular system (Schwartz and Domowicz, 2002).

LP (~40 kDa) has a critical role in the structure and function of PG aggregates. It stabilizes the interaction between HA and the G1 domain of the PG core protein

(Watanabe and Yamada, 1999; Shi et al., 2004). LP has a high structural homology with the G1 domain of aggregating PGs, and it contains a binding site for HA (Heinegård and Hascall, 1974; Tengblad et al., 1984). The HA and PG binding LP gene family (HAPLN) consists of four members that are all located in the genome next to the core protein genes of aggrecan, versican, neurocan, and brevican (Spicer et al., 2003). The amount and heterogeneity of LP differs according to spine level and location within a single disc (Pearce et al., 1989). LP has an important role in the normal development and assembly of the ECM in the cartilaginous tissues (Pearce et al., 1989), and also in some pathophysiological events, such as in the initial stage of arthritis (Zhang et al., 1998).

#### 2.3.1.3. Small leucine-rich proteoglycans

The small leucine-rich (SLR) PG family consists of nine members forming three distinct subfamilies. All SLR-PGs have 9-12 leucine-rich motifs in the carboxy terminal end of the core protein (Neame et al., 1989; Matsushima et al., 2000). Biglycan (Fisher et al., 1989; Bianco et al., 1990), decorin (Krusius and Ruoslahti, 1986; Bianco et al., 1990), fibromodulin (Oldberg et al., 1989) and lumican (Blochberger et al., 1992) are present in the intervertebral disc and cartilage endplate as SLR-PGs (Melrose et al., 2001a).

Decorin and biglycan have a similar core protein containing one (decorin) or two (biglycan) CS/DS chains. The molecular mass of the core protein is ~ 45 kDa. The GAG chains are in general longer than those in large PGs, ~ 30-40 kDa, and these PGs contain also N- or O-linked oligosaccharides (Fisher et al., 1989). Biglycan has a molecular mass of 150-240 kDa, and decorin 90-140 kDa (Wight et al., 1991). The function of decorin is related to its ability to regulate collagen fibril formation (Vogel et al., 1984; Schönherr et al., 1995). Decorin is also involved in several biological functions such as cell attachment, migration and proliferation (Yamaguchi and Ruoslahti, 1988; Yamaguchi et al., 1990; De Luca et al., 1996; Merle et al., 1997; Merle et al., 1999). Biglycan is engaged, for example, in the collagen fiber assembly (Schönherr et al., 1995; Reinboth et al., 2002), elastic fiber formation (Reinboth et al.,

2002), and bone formation (Chen et al., 2003). Both decorin and biglycan are able to bind to TGFβ and inactivate this growth factor (Yamaguchi et al., 1990; Kresse et al., 1993; Hindebrand et al., 1994; Iozzo, 1997).

Fibromodulin and lumican have core proteins homologous to those of biglycan and decorin, but they contain KS chains instead of CS/DS chains. The molecular weight of these molecules is only ~ 60 kDa, ~ 40 kDa representing the core protein (Iozzo, 1997). The function of fibromodulin and lumican is not known exactly, but probably they are involved in collagen fibril formation (Säämänen et al., 2001) and network regulation during tissue organization (Blochberger et al., 1992; Hindebrand et al., 1994).

### 2.3.2. Collagens

In cartilaginous tissues, collagens form a fibrillar network entrapping negatively charged PGs within the fibrillar matrix. Generation of a high osmotic pressure by PGs leads to a tensional force on the collagen fibrils and enables the tissue to resist compressive forces (Heinegård and Sommarin, 1987; Eyre et al., 2002). In the outer parts of the disc, a strong, highly organized, lamellar collagen network protects the spine from great mechanical stresses (Eyre, 1988; Humzah and Soames, 1988; Bruehlmann et al., 2002). A less organized collagen network is present in the nucleus pulposus and the inner annulus fibrosus (Eyre, 1988; Roberts et al., 1991; Hunter et al., 2003). Since the ECM of the cartilage endplate resembles that of hyaline cartilage, the appearance and function of collagens are also similar to those in hyaline cartilage Tarkoitin sitä että nikaman päätelevyn "kollageenipatteri" muistuttaa hyaliiniruston vastaavaa (Roberts et al., 1991). In addition to their mechanical role, providing tensile strength for the tissue, collagens have several functions not only in normal matrix assembly and development, but also in pathological events, such as fibrosis (Prockop and Kivirikko, 1995; Beller et al., 2004; Bouzeghrane et al., 2004).

Collagen types I, II, III, V, VI, IX, X, XI, XII and XIV have been found in the intervertebral disc (Eyre, 1988; Aigner et al., 1998; Lammi et al., 1998; Eyre et al., 2002). The amount of collagen type I is small in the inner parts, increasing towards the outer parts of the disc and also towards the border between the cartilage endplate and

the calcified tissue next to the vertebral bone. Collagen type II is the most common in the inner parts of the intervertebral disc (Eyre and Muir, 1976; Roberts et al., 1989; Roberts et al., 1991). The main collagen of the cartilage endplate is of type II, although cartilage-specific collagen types VI, IX, X, XI, and XII have been found too (Cremer et al., 1998).

### 2.3.3. Other macromolecules

Elastic fibres form a highly organized network in spinal tissues although the maximal proportion of elastin is only 5% of the total dry weight of the disc (Yu, 2002). In the nucleus pulposus, the fibres are oriented radially and anchor into the cartilage endplate, while in the annulus fibrosus the fibres are located between the collagen lamellae and form bridges across the lamellae. The elastic fibres provide the tissue with elastic properties and they may have a role in disc cell metabolism by contributing to the movement of nutrients and waste products (Yu, 2002; Yu et al., 2002). In some pathophysiological situations, impaired formation of elastic fibres is associated with accumulation of CS and DS proteoglycans (Hinek et al., 2004). Also other ECM proteins such as fibronectin have been found in the intervertebral disc (Gotz et al., 1995; Hayes et al., 2001; Di-Lullo et al., 2002). Fibronectin may have a role in the pathogenesis of the intervertebral disc, since its gene expression was upregulated in a degeneration model of rabbits (Anderson et al., 2002).

### 2.3.4. Biosynthesis and metabolism of the macromolecules

PGs are synthesized by chondrocytes or chondrocyte-like cells. Transcripts of the core protein gene are processed into mRNAs, and further decoded into polypeptides, which are processed and transported through the rough endoplasmic reticulum (RER) to the Golgi apparatus. The biosynthesis of GAGs (excluding HA) on the core protein takes place in the Golgi apparatus and includes complex, sequential reactions catalyzed by several enzymes. Elongation, one monosaccharide at a time to the non-reducing end of a growing polysaccharide chain, is followed by sulphation of the chain. Finally, newly synthesized PGs are transported to the cell membrane, or secreted into the ECM.

Sometimes PGs remain in the intracellular space (Lohmander et al., 1986; Heinegård and Sommarin, 1987; Ruoslahti, 1988; Wight et al., 1991). Biosynthesis of HA takes place on the plasma membrane, and the newly synthesized molecule is pushed into the extracellular space through a pore in the plasma membrane (Tammi et al., 2002). The interaction between HA, PGs and LPs occurs in the ECM (Hascall, 1988).

The early events of collagen biosynthesis take place in the endoplasmic reticulum (ER). Modifications of the polypeptide occur both inside the cell and in the extracellular space. A folded pro-collagen form (e.g. type I and II collagens) of the molecule is secreted through the Golgi apparatus into the ECM, where the propeptides are removed, and fibrils and covalent cross-links are formed (Eyre, 1988; Prockop and Kivirikko, 1995).

Normal tissue turnover, a balance between synthesis and degradation of the ECM macromolecules, is regulated by cells. The cells respond to several factors, e.g., cytokines (several interleukins) and growth factors, which usually stimulate and restrain the activation of degradative enzymes, respectively. If the homeostasis of normal turnover of the ECM is disturbed for some reason, the enzymes present in the tissue are overactivated in process(es), which leads to tissue destruction (Rowan, 2001). Most of the enzymes present in the cartilaginous tissues belong to the family of metalloproteinases, MMPs (matrix metalloproteinases) and ADAMs (a disintegrin and a metalloprotease). MMPs, such as collagenases and gelatinases, are mainly responsible for collagen degradation, although these enzymes degrade also other ECM components, including PGs (Lark et al., 1997). ADAMs, like aggrecanases, fragment PGs (Sandy et al., 1995; Lark et al., 1997; Roberts et al., 2000; Mort and Billington, 2001; Rowan, 2001). Members of cysteine and aspartic proteases, and lysosomal enzymes (Mort and Billington, 2001), as well as several cytokines (Rowan, 2001), may also contribute to aggrecan breakdown. MMPs are controlled in several ways, including inhibition by endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Rowan, 2001).

## 2.4. Changes in the intervertebral disc and cartilage endplate with aging and degeneration

### 2.4.1. Structural changes

During development, and particularly during aging and degeneration, the intervertebral disc and cartilage endplate undergo extensive mechanical and biochemical changes (Humzah and Soames, 1988; Mundlos, 1994; Buckwalter, 1995; Gottschalk et al., 2001; Hayes et al., 2001; Boos et al., 2002). Alterations in aged and degenerated discs are often similar, although in the latter they are more prominent (Adams and Muir, 1976; Ayad and Weiss, 1987; Boos et al., 2002).

The morphological changes may be partly visible macroscopically. Early age-related changes are seen in the shape and color of the nucleus pulposus and annulus fibrosus. Thinning of the disc, formation of clefts and splits inside the nucleus pulposus, and the presence of intraosseus herniations and vertebral osteophytes are typical alterations in old age (Lipson and Muir, 1980; Vernon-Roberts, 1988).

In humans, the first signs of aging can be observed as early as at the end of the first decade of life, concurrently with the normal maturation of the musculoskeletal system. Together with growth and enlargement of the intervertebral disc, the vascularization and lymph vessels of the cartilage endplate and annulus fibrosus disappear, gradually followed by changes in the nutritional environment of the disc (Urban et al., 1977; Holm et al., 1981).

The lack of a blood supply, together with the extended diffusion distance of nutrients, reduces $O_2$ tension and pH of the tissue, and increases the production of lactate (Urban et al., 1977; Urban et al., 1978; Maroudas, 1988; Oshima and Urban, 1992; Horner and Urban, 2001). This leads to a decline in the number, activity and viability of disc cells (Horner and Urban, 2001; Rannou et al., 2004). Decreased matrix synthesis rates and increased activity of degradative enzymes leads to impaired ability of the cells to maintain an appropriate ECM (Guiot and Fessler, 2000; Urban et al., 2001; Boos et al., 2002; Sive et al., 2002). These events predispose the nucleus pulposus to breakdown during the second decade of life, whereas the annulus fibrosus is usually affected later in life (Boos et al., 2002).

Calcification of the cartilage endplate in later years further deteriorates the biomechanical integrity of the spine and the nutrition of the intervertebral disc (Roberts et al., 1996; Roberts, 2002). Increased sensory and sympathetic innervation, and proliferation of blood vessels in the cartilage endplate and underlying vertebral bodies are also characteristic of disc degeneration (Brown et al., 1997; Freemont et al., 1997; Johnson et al., 2001; Melrose et al., 2002a).

Although the initiation and progress of disc degeneration seems to be mainly nutrition-related (Urban et al., 1977; Holm et al., 1981; Horner and Urban, 2001; Boos et al., 2002), the ultimate causes for disc degeneration may have a biomechanical (Iatridis et al., 1999) or traumatic origin (Freemont et al., 2001). Occupational physical stress, smoking, taller height and obesity have also been considered as potential causes (Heliövaara, 1989; Marini, 2001). Moreover, several genes have been shown to predispose to disc degeneration (Kawaguchi et al., 1999; Sambrook et al., 1999; Guiot and Fessler, 2000; Marini, 2001; Paassilta et al., 2001; Ala-Kokko, 2002; Solovieva et al., 2002; Pluijm et al., 2004). Some other diseases or disorders, such as atherosclerosis, may also contribute to the development of disc degeneration (Kurunlahti et al., 1999). Therefore, the basis for the pathophysiology of disc degeneration must be multifactorial and is mostly unknown (Freemont et al., 2001).

### 2.4.2. Changes in the extracellular matrix

#### 2.4.2.1. Proteoglycans

During aging and degeneration of the intervertebral disc and cartilage endplate, the PG and water content in the ECM decrease, resulting in low fixed charge density and swelling pressure (Adams and Muir, 1976; Cole et al., 1986; Pearce et al., 1987; Urban and McMullin, 1988; Scott et al., 1994; Antoniou et al., 1996a; Antoniou et al., 1996b). This impairs the functional properties of the disc. These changes are most prominent in the nucleus pulposus, where water content may be reduced by about 20% of the tissue wet weight (Antoniou et al., 1996b). The water and GAG content decrease clearly also in the cartilage endplate, by 5-10 and 50%, respectively (Lipson and Muir, 1980; Roberts et al., 1989; Antoniou et al., 1996a). In contrast, in the annulus fibrosus

the GAG content remains relatively intact during aging (Postacchini et al., 1984; Antoniou et al., 1996b). Many of the changes in GAGs start to occur before maturity and continue with aging: the amount of HA and KS increases, whereas CS content decreases, correlating with the declining water content (Adams and Muir, 1976; Scott et al., 1994). Also the content of CS-6 and CS-4 has been shown to decrease during aging (Ayad and Weiss, 1987; Olczyk, 1993). At the same time, the ratios of KS/CS and CS-6/CS-4 appear to increase in the intervertebral disc (Pearce and Grimmer, 1976; Olczyk, 1993) and articular cartilage (Roughley and White, 1980).

In addition to the quantitative changes in PGs, several qualitative alterations are seen in the intervertebral disc and cartilaginous tissue during aging and degeneration. The size of large PGs and HA decreases (Adams and Muir, 1976; Pedrini-Mille et al., 1983; Phillips et al., 2002; Sztrolovics et al., 2002b), the heterogeneity of different PG populations increases (Melrose et al., 1991; Johnstone and Bayliss, 1995; Taylor et al., 2000), the ability of PGs to aggregate with HA declines (Bayliss et al., 2000), and the heterogeneity and fragmentation of LP increase (Pearce et al., 1989). These changes are caused by decreased or lacking PG synthesis (Venn and Mason, 1986; Bayliss et al., 1988; Bolton et al., 1999; De Groot et al., 1999; Guiot and Fessler, 2000; Taylor et al., 2000; Antoniou et al., 2001; Cs-Szabo et al., 2002; Sive et al., 2002) and/or increased catabolism of PGs by proteinases (Pearce et al., 1989; Nguyen et al., 1991; Melrose et al., 1992; Sztrolovics et al., 1997; Pratta et al., 2000; Rees et al., 2000; Roughley et al., 2002). The ECM and the cells of the nucleus pulposus and annulus fibrosus respond to degeneration differently, the former being more susceptible to such changes (Cs-Szabo et al., 2002; Horner et al., 2002).

The synthesis of large PGs decreases during aging and degeneration and the concentration and synthesis of small leucine-rich PGs increases in the intervertebral disc (Melrose et al., 1997b; Cs-Szabo et al., 2002), resembling the changes in articular cartilage (Roughley et al., 1993; Roughley et al., 1996). This increase in small PGs is proportional to other PGs rather than absolute. It has also been shown that the small leucine-rich PGs are more resistant to proteolytic enzymes, probably due to their location on the collagen fibrils (Sztrolovics et al., 1999b; Rees et al., 2000). Thus, the changes in the concentration and structure of small PGs depend largely on the stage and

grade of degeneration, which is different in the annulus fibrosus than in the nucleus pulposus (Cs-Szabo et al., 2002).

Recently, several studies have evaluated the influences of genetic background on extracellular matrix diseases. Many of the mutations and polymorphisms described in articular cartilage or intervertebral disc are closely associated with collagen genes (Aszodi et al., 1998). However, mutations in aggrecan (Doege et al., 1997; Kawaguchi et al., 1999; Watanabe and Yamada, 2002), link protein (Watanabe and Yamada, 2002) and perlecan (Schwartz and Domowicz, 2002) genes have also been reported to lead to defects in the tissue architecture, such as chondrodysplasia (Schwartz and Domowicz, 2002; Watanabe and Yamada, 2002), or to predispose to disc degeneration at an early age (Kawaguchi et al., 1999). Biglycan-deficient mice have been described to have delayed osteogenesis and age-related osteopenia and arthritis (Chen et al., 2003; Chen et al., 2004), while fibromodulin/biglycan knock-out mice have severely disturbed collagen fibril structures, which leads to premature osteoarthritis (Young et al., 2003).

#### 2.4.2.2. Other ECM molecules

The amount of collagens and non-collagenous proteins increases, at the expense of PGs, with aging and degeneration of the intervertebral disc and cartilage endplate (Adams and Muir, 1976; Lyons et al., 1981; Olczyk, 1992; Kääpä et al., 1994; Scott et al., 1994; Anderson et al., 2002), perhaps owing to changes in collagen crosslinking and biosynthesis (Bernick et al., 1991; Kääpä et al., 1994; Antoniou et al., 1996a; Antoniou et al., 1996b; Guiot and Fessler, 2000). The synthesis of type II procollagen decreases with aging, but type II collagen denaturation and type I procollagen synthesis seem to increase in later years and with degeneration (Antoniou et al., 1996a; Antoniou et al., 1996b). Disrupted collagen lamellar architecture, reduced amount of collagen and thinning of the fibres occur during aging and degeneration of the disc (Bernick et al., 1991; Aigner et al., 1998; Aigner and McKenna, 2002; Gruber and Hanley, 2002). Mutations and polymorphisms of collagen type I (Pluijm et al., 2004), type II (Sahlman et al., 2001) and type IX (Paassilta et al., 2001; Solovieva et al., 2002) genes have been identified as risk factors for disc degeneration.

An age and degeneration related increase in fibromodulin and lumican, together with structural alterations of these molecules, have been noticed in the human intervertebral disc (Sztrolovics et al., 1999a). In the articular cartilage, a higher concentration of fibromodulin has been found in association with calcification of the cartilage (Säämänen et al., 2001). Autosomal recessive mutant twy (tiptoe walking Yoshimura) mice have an abnormal expression of MGP (matrix Gla protein), which leads to hypertrophy of chondrocyte-like cells and pathologic calcification of the cartilaginous tissue also in the spine (Ohtsuki et al., 1998).

### 2.4.3. Animal and cell culture models

A variety of experimental cell and tissue culture and animal models have been used to study early changes in disc aging and degeneration, and to test suitable therapeutic approaches. Although there are many differences, e.g. in biomechanical characteristics and cell densities, between the spines of bipeds (humans) and quadrupeds (most mammals), the ECM structure of spinal tissues and the nutritional and metabolical cascades of the spine are quite similar.

There are several *in vivo* animal models mimicking human disc degeneration and disease. In the experimental annular tear model, a wound is made by a transverse stab or scalpel incision through or into the annulus fibrosus. This leads to distruption of the annulus fibrosus and degenerative changes inside the disc, or even gives rise to herniation of the nucleus pulposus. It can also lead to the formation of vertebral osteophytes, depending on the depth of the incision (Smith and Walmsley, 1951; Haimovici, 1970; Lipson and Muir, 1980; Lipson and Muir, 1981a; Lipson and Muir, 1981b; Osti et al., 1990; Melrose et al., 1997a; Melrose et al., 1997b; Anderson et al., 2002; Melrose et al., 2002a; Melrose et al., 2002c).

The fixation of spinal segments and excessive loading of the spine have been investigated as a model of disc degeneration, too. Immobilization of the spine by lumbar fusion causes changes that resemble disc degeneration (Phillips et al., 2002; Hutton et al., 2004). Also, high or static compressive loading of the disc seems to induce harmful responses in the ECM, similar to those found in degenerated tissue (Hutton et al., 1998; Lotz et al., 1998; Iatridis et al., 1999; Hutton et al., 2002). However, the response to

loading seems to vary, probably due to differences in the magnitude and frequency of the load (Puustjärvi et al., 1993b; Hutton et al., 2000).

Another approach to experimental degeneration has been to target ECM molecules, mainly PGs, of the intervertebral disc tissue. By injecting chondroitinase ABC into the disc, PG degradation initiates degenerative changes (Oegema et al., 1988; Ando et al., 1995; Lu et al., 1997; Norcross et al., 2003). Some animal species or breeds, dogs and mice in particular, have spontaneous degenerative changes in the spine, providing a valuable model for research (Goggin et al., 1970; Higuchi et al., 1983; Venn and Mason, 1986; Sampson and Davis, 1988; Ghosh et al., 1992). Also normal age-related alterations have been studied in several animal species (Silberberg, 1971; Cole et al., 1986; Taylor et al., 2000; Okuda et al., 2001).

During recent years, cellular and ECM mechanisms have been studied *in vitro* using cells from healthy (Ichimura et al., 1991; Maldonado and Oegema, 1992; Gruber et al., 1997a; Gruber et al., 1997b; Ishihara and Urban, 1999; Gan et al., 2000; Stern et al., 2000; Melrose et al., 2001b; Wang et al., 2001; Yung et al., 2001; Horner et al., 2002; Masuda et al., 2002; Alini et al., 2003) and degenerated (Desai et al., 1999; Roughley et al., 2002; Thonar et al., 2002; Stern et al., 2004) discs of several animal species. Also the effects of aging (Maeda and Kokubun, 2000; Okuda et al., 2001; Thonar et al., 2002), loading (Hutton et al., 1999; Hutton et al., 2001; Chen et al., 2002; Yamazaki et al., 2002) and therapeutic agents on disc (Johnson et al., 2002; Roughley et al., 2002; Yang et al., 2003) and cartilage endplate (Wehling et al., 1997) cells have been investigated *in vitro*.

### 2.5. Treatments of back pain with degenerative disc disorder

The majority of treatments (>90%) for spinal disorders (mainly given for the accompanying low back pain) are conservative - analgesic drugs and physiotherapy in order to relieve pain and improve functioning (Malmivaara and Airaksinen, 1999; Luoma et al., 2000; Guzman et al., 2001; Speed, 2004). Only in special cases, e.g. in case of compression of neural structures of the spine, is surgery clearly indicated, while the indications for fusion operations due to disc degeneration are less well defined. At

the moment, there are no clinical treatments for repairing, reversing or retarding disc degeneration (Alini et al., 2002; Roughley et al., 2002).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of osteoarthritis, rheumatoid arthritis and spinal disorders to alleviate pain and to reduce inflammation. The primary target of NSAIDs is to inhibit the activation of cyclo-oxygenases (COX) in inflamed tissues to reduce prostaglandin synthesis (Ghosh, 1988; Yunus, 1988; Ding, 2002; Moilanen and Vapaatalo, 2003; Mason et al., 2004). The efficacy and side-effects of NSAIDs are well understood in both spinal (Calabro, 1985; Buchanan and Kassam, 1986; Calabro, 1986; Yunus, 1988; Dimar et al., 1996) and cartilage disorders (Bahous, 1981; Vetter, 1987; David et al., 1992). More selective, second generation NSAIDs have been developed to target the cyclooxygenase isoenzyme COX-2, which is more important in inflamed tissue than COX-1 (Moilanen and Vapaatalo, 2003).

NSAIDs differ greatly in their effects on the matrix metabolism of the intervertebral disc and articular cartilage during long-term administration. However, the number of studies on the intervertebral disc is low. Pre-clinical studies carried out *in vitro* and *in vivo* with cartilaginous tissues suggest that NSAIDs influence the rates of PG and collagen synthesis (Carney, 1987; David et al., 1992), and PG breakdown by affecting the activities of matrix-degrading enzymes (Ratcliffe et al., 1993). Some NSAIDs, such as aspirin, seem to accelerate the degeneration and reduce the concentration of PGs in the cartilage matrix (Ding, 2002). "Chondroprotective" action, mainly against chondrocyte death, has been noticed *in vitro* with ibuprofen (Mukherjee et al., 2001) and indomethacin (Notoya et al., 2000). On the other hand, indomethacin has been shown to suppress PG synthesis in the chondrocytes of the canine intervertebral disc (Yoo et al., 1992). According to several studies, tiaprofenic acid does not exert detrimental effects on cartilage PG synthesis (Muir et al., 1988; Brandt et al., 1990; Howell et al., 1991; Meyer-Carrive and Ghosh, 1992). Clinical trials on the joint matrix reveal that indomethacin has a negative influence, whereas other NSAIDs, such as tiaprofenic acid, do not (Dingle, 1999; Ding, 2002).

A number of new ideas concerning the therapy of cartilage and intervertebral disc disorders have been tested. Mostly treatments targeting different agents in the

inflammation cascades in rheumatoid arthritis have been studied, and some treatments are currently in the clinical phase. Biological therapies (e.g. monoclonal antibodies) blocking the action of pivotal cytokines (several ILs, TNF-α) have been most effective (Blake and Swift, 2004; Cooper and Freemont, 2004). Several cytokine signalling pathways affecting certain kinases and gene products (MMPs) are also actively being investigated (Blake and Swift, 2004). Another approach has been to activate the degenerated disc and cartilage tissue by using growth factors (e.g. TGF-β and EGF) (Thompson et al., 1991; Walsh et al., 2004), gene therapy (Nishida et al., 1999; Evans et al., 2004; Sobajima et al., 2004; Trippel et al., 2004; Yoon and Boden, 2004), synthetic peptides (Mwale et al., 2003), and even stem cells (Gafni et al., 2004). Tissue engineering treatments, aiming at repairing or replacing the degenerated tissue by using authentic cells and matrix, has also been tested (Hunziker and Kapfinger, 1998; Gruber et al., 2002; Roughley et al., 2002).

## 3. AIMS OF THE STUDY

The aim of this work was to analyse PGs and HA in the healthy and diseased intervertebral disc and cartilage endplate, and to evaluate the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on the extracellular matrix in experimental disc degeneration.

The specific aims of the study were:

1. To characterize and quantitate the PGs in healthy and degenerated human intervertebral disc (I).
2. To investigate the concentration and spatial distribution of HA across the intervertebral disc and endplate (II).
3. To assay PGs in qualitative and quantitative terms in healthy and degenerated intervertebral disc in an experimental disease model (III).
4. To describe the effects of two nonsteroidal anti-inflammatory drugs, indomethacin and tiaprofenic acid, on the amount and quality of PGs in the degenerated intervertebral disc using an experimental disease model (IV).

# 4. MATERIALS AND METHODS

## 4.1. Study material

### 4.1.1. Human samples (I, II)

Intervertebral discs (L2-3 or L3-4) from six organ donors (aged 13, 31, 41, 45, 49 and 53 years) were used for biochemical measurements. The discs from the donors aged 13, 31, 41, 45 and 53 years were healthy (grades 1-2), whereas the disc of the 49-year-old donor showed signs of early degeneration (grades 2-3) (Thompson et al., 1990). Degenerated anterior annulus fibrosus samples (L4-5 or L5-S1) were collected from seven patients (aged 39-46 years) subjected to an anterior interbody fusion operation. All discs showed grade 4-5 degeneration (Adams et al., 1986; Guyer and Ohnmeiss, 1995). In study **II**, four L5-S1 discs (from 27-, 37-, and two 44-year-old donors) containing adjacent endplates were used for histochemical analysis. The discs were healthy according to macroscopic appearance, but no grading was performed. All samples for biochemical analyses were divided on the basis of appearance into outer anterior annulus fibrosus (OAF), middle annulus fibrosus (MAF), inner annulus fibrosus (IAF), nucleus pulposus, (NP) and posterior annulus fibrosus (PAF), and weighed (**I**, Fig. 1). In addition, the anterior annulus sites, OAF, MAF, and IAF of the healthy donors were pooled for comparison between healthy and degenerated annulus fibrosus.

Human samples were taken with the permission of the National Board of Medical Affairs, and The Ethical Commissions of Kuopio University Hospital and the Orthopaedic Hospital of the Invalid Foundation, Helsinki.

### 4.1.2. Canine samples (II)

Intervertebral discs of six female purebred beagle dogs provided by the National Laboratory Animal Center (Kuopio, Finland) and Shamrock Ltd. (Hereford, UK), were used in the study. The dogs were given a standard diet and housed in standard cages. The dogs were killed at the age of 70 weeks according to a permission of the Animal Care and Use Committee of the University of Kuopio (Arokoski et al., 1991). The

intervertebral discs (T3-4) were removed and divided into three equal blocks for biochemical analyses: anterior annulus (AA), nucleus pulposus (NP) and posterior annulus (AP) blocks. The discs and adjacent endplates from L1-2 level were processed for histochemical analysis (Puustjärvi et al., 1993a).

### 4.1.3. Porcine samples (III, IV)

Intervertebral discs of domestic pigs (Sus scrofa, n=28), aged 8-18 months and weighing 75-150 kg were used in the experiments. Under anesthesia, the discs of 23 pigs were incised (L2-3 or L4-5) with a scalpel blade. The incision was 1.3 cm deep and situated in the middle of the anterior annulus fibrosus, parallel to the vertebral endplates (**III**, Fig. 1). After surgery, six animals received 300 mg of tiaprofenic acid (Roussel Pharmaceuticals, Paris, France) twice a day hidden in a meatball; another six (n = 6) received 75 mg of indomethacin (Orion Pharma, Espoo, Finland) twice a day, and the rest of the animals (n = 6) did not receive any medication (**IV**). Five non-operated age- and weight-matched animals served as a control group. All animals were housed in standard cages and fed a standard diet.

Three months postoperatively the animals were anesthetised, tracheostomy was performed, and the carotid artery and vena jugularis were catheterised. Two or three hours before killing the animals, [$^{35}$S]sulphate (3 mCi) diluted in Ringer's solution was injected intravenously. To assess the radioactivity of the plasma, blood samples were drawn through the arterial catheter at regular intervals (5, 10, 20, 30, 60, and 120 min), until the animals were killed with an overdose of anesthetics.

The spines were dissected free from soft tissues and frozen immediately. The intervertebral discs were separated from the vertebral bodies, and photographs (**III, IV**) or radiographs (**IV**) were taken for estimation of morphological changes. The discs were divided into 11 (**III**) or 5 (**IV**) equal rectangular blocks in the anterior-posterior direction and the blocks were weighed for wet and dry weight. In study **III**, blocks 1-4 represented the anterior annulus, 6-8 nucleus pulposus, 10-11 posterior annulus and 5 and 9 the transitional zone of the intervertebral disc (**III**, Fig. 2). In study **IV**, the discs were prepared and weighed as in study **I** (**I**, Fig. 1).

## 4.2. Biochemical and histochemical analyses

### 4.2.1. Quantitation of DNA

To evaluate cell numbers, the samples were assayed for DNA content after proteinase K (Sigma, St. Louis, USA) digestion in studies **III** and **IV,** using a fluorometric assay (Kim et al., 1988). Bisbenzimidazole (Hoechst dye 33258, 0.1%) in 0.05 M phosphate buffer (PB), pH 7.4, containing 2 M NaCl and 2 mM EDTA was used for the development of fluorescence. Calf thymus DNA (Sigma) served as a standard.

### 4.2.2. Quantitative and qualitative analysis of proteoglycans

*Extraction of proteoglycans and determination of uronic acid concentration*

The sample blocks were cut into small pieces, and PGs were extracted in 4 M GuCl (Fluka, Buchs, Switzerland) and 50 mM sodium acetate (pH 5.8) including inhibitors of proteases and bacterial growth for 20-48 h at 4°C in a shaker. The samples were centrifuged, the pellets washed, and the combined supernatants used for the characterisation of the extractable PGs. The cleared extracts were dialysed twice against 0.15 M NaCl (6 and 18 h) and distilled water (6 and 18 h) to remove interfering salts (**III**), or desalted on Sephadex G-25 columns (Pharmacia, Uppsala, Sweden) into deionized water (**I, II, IV**). The uronic acid content was then determined to evaluate the PG content of the samples (Blumenkrantz and Asboe-Hansen, 1973).

The non-extractable material was digested overnight at 60°C with 0.05% proteinase K in 10 mM EDTA and 0.1 M sodium phosphate (pH 6.5). The solubilized GAGs were precipitated with absolute ethanol containing 0.5% sodium acetate, washed with ethanol, and the uronic acid content was measured from the pellet dissolved in deionized water. The extractability of PGs in the sample was calculated as the percentage of the total uronic acid that was dissolved in the extract.

*SDS-agarose gel electrophoresis*

The relative amount and proportion of different PG subpopulations was studied with SDS-agarose gel electrophoresis. Samples containing equal amounts of PGs (1 μg uronic acid) were electrophoresed (GNA-200 Gel Electrophoresis Apparatus,

Pharmacia) in 0.75% SDS-agarose gels (Sea-Kem LE-agarose, FMC Bioproducts, Rockland, USA) for 2.5-3.5 hours with a constant current of 60 mA. The gels were fixed, stained with toluidine blue and dried on Gelbond film (FMC BioProducts) (Säämänen et al., 1989; Säämänen et al., 1990). The gels were scanned and the images analyzed with the image analysis software Image (by Wayne Rashband, NIH, Bethesda, USA). GAGs isolated from bovine nasal cartilage, and commercial CS (shark cartilage, Sigma) were used as standards in the electrophoresis (**I, III, IV**).

In study **III**, the presence of DS-containing small PGs was studied after specific cleavage of DS by chondroitinase B (*Flavobacterium* heparinum, Seikagaku Co., Tokyo, Japan). The PGs (7.5 µg of uronic acid) were incubated with the enzyme (1.5 mU) in 0.05 M Tris buffer (pH 8.0) overnight at 30°C, after which the samples were electrophoresed.

To further characterise the PGs (**I**), they were electroblotted after electrophoresis onto a PVDF membrane (Millipore, Molsheim, France) using 40 mM Tris-acetate buffer containing 1 mM sodium sulphate, 10% methanol, pH 6.8, in a semidry electroblotter (Pegasus, Mölln, Germany) at 0.8 mA/cm$^2$ for 60 min. PGs isolated from human articular cartilage were included as a positive control.

*SDS-PAGE*

To study the nonglycanated forms of the small PGs decorin and biglycan (**I**), 10% SDS-PAGE gels with 3% stacking gels were cast, and electrophoresis was performed in reducing conditions (Laemmli, 1970), followed by electroblotting onto PVDF membranes using 0.8 mA/cm$^2$ for 30 min. Another set of samples was pretreated with chondroitinase ABC (*Proteus vulgaris*, 12.5 mU/µg of uronic acid; Seikagaku) for 4 h at 37°C and then subjected to electrophoresis.

*Analysis of Western blots*

In study **I**, the expression of certain CS epitopes was investigated using the monoclonal antibodies 3B3 and 2B6, which recognise, respectively, a 6-sulfated N-acetylgalactosamine next to a terminal 4,5-unsaturated uronic acid, and a 4-sulfated N-acetylgalactosamine next to a 4,5-unsaturated uronic acid formed during chondroitinase

ABC treatment (Caterson et al., 1985; Caterson et al., 1992). In native CS chains, 3B3 is suggested to recognize a similar saturated structure (Caterson et al., 1992). In addition, the monoclonal antibody 7D4 was used (Caterson et al., 1985). The precise epitope of this antibody is not known, but it is probably a CS oligosaccharide close to the protein core, with 8-12 disaccharide units (Caterson et al., 1985; Caterson et al., 1992; Sorrell et al., 1993). The polyclonal antibodies LF15 and LF30 were used to detect both glycanated and non-glycanated forms of biglycan and decorin. These antibodies recognize peptide sequences near the N-termini of human biglycan (amino acids 11-24) and decorin (amino acids 5-17) (Fisher et al., 1987) (Figure 3, **I**).

In order to decrease the unspecific binding the membranes were blocked with 5% fat-free milk powder in PBS, pH 7.4, with 0.5% Tween 20® (Fluka AG, Buchs, Switzerland) (PBS-T), then treated with 10 mU/ml of chondroitinase ABC in 0.2 M Tris-acetate buffer containing 0.06 M sodium acetate, pH 8.0, at 37°C for 1 h, and washed in PBS-T. The membranes were incubated for 1 h with the primary antibodies (3B3, 7D4, 2B6, LF15 and LF30), all diluted 1:2000 in PBS-T containing 1% fat-free milk powder, and washed several times in PBS-T. CS epitopes recognized by 3B3 and 7D4 antibodies were analysed also from undigested membranes. The membranes were incubated with peroxidase-labeled secondary antibodies (1:500 dilution of swine anti-rabbit or goat anti-mouse secondary antibodies; Dakopatts, Glostrup, Denmark) for 1 h, and the membranes were washed in PBS-T. The bands were visualized by a chemiluminescence system using autoradiography films (Hyperfilm ECL, Amersham Int., Little Chalfont, UK) according to the manufacturer's instructions (Renaissance, DuPont, Boston, MA, USA). As negative controls, non-immune rabbit serum was used, or the primary antibody was totally omitted. A sample from human articular cartilage served as a positive control.

*Ratio of chondroitin sulfate to keratan sulfate*

The ratio of CS to KS was estimated in study **III** from the ratio of galactosamine to glucosamine in hydrolysates of extracted PGs. Hydrolysis was done in 2 M HCl for 8 h at 103°C, and the PGs were dried under an air stream. An internal standard (deoxy-glucose) and water were added and the monosaccharides were separated on a HPLC

system (Model 4000i; Dionex, Sunnyvale, USA) with an autosampler, computer interface, and software package. The chromatography was performed with 16 mM NaOH on a Carbopak PA1 column with an AS-6 guard column (Dionex) at a flow rate of 1.0 ml/min using pulsed amperometric (PAD) detection.

*Ratio of chondroitin 6-sulfate to chondroitin 4-sulfate*

The ratio of CS-6 to CS-4 was measured in studies **III** and **IV** from aliquots of the proteinase K digested samples after digestion in chondroitinase AC II (*Arthrobacter aurescens*; Seikagaku) (Säämänen and Tammi, 1984). The unsaturated, monosulphated isomers were chromatographed on a Carbopak PA1 column (Dionex) eluted at 0.5 ml/ min with 2 mol/l sodium acetate (pH 7.5), using ultraviolet detection at a wavelength of 232 nm.

*Sephacryl S-1000 Gel Filtration*

In study **IV**, the proportion of aggregating PGs at the different locations was analysed from pooled samples (individual samples in each group). The pools were lyophilised, and dissolved in 50 mM sodium acetate and 0.15 M NaCl, pH 6.5, including protease and microbial growth inhibitors, then incubated with 2% HA (Healon; Pharmacia) for 4h and frozen until further analysis. Aliquots were injected into a Sephacryl S-1000 column (1x30 cm; Pharmacia) immediately after thawing, eluted at a flow rate of 20 ml/h, and collected into 500 µl fractions. The PG content of the fractions was analyzed using Safranin O precipitation (Lammi and Tammi, 1988).

*In vivo radioactive tracer analysis*

The transport of solutes into the intervertebral disc and the rate of PG synthesis in the tissue were investigated by determining the amounts of free [$^{35}$S]sulphate precursor and [$^{35}$S]sulphate incorporated into the macromolecules after intravenous delivery of the radioactive precursor (Urban et al., 1978). The dried tissue slices were dialysed twice for 10 hours against 0.15 M NaCl to remove the free inorganic sulphate. The dialysate was concentrated and its radioactivity was counted with a liquid scintillator. After washing, the slices were digested overnight at 65°C with activated crude papain (20

mg/g ww; Sigma) in 0.1 M sodium acetate buffer (pH 5.5) containing 50 mM L-cysteine (**III**), or alternatively with 0.05% proteinase K in 10 mM EDTA and 0.1 M sodium phosphate buffer, pH 6.5 (**IV**). The radioactivity (counts/min/g ww) in each sample was normalised to the zero value of the plasma radioactivity of the respective animal.

### 4.2.3. Analysis of hyaluronan

*Hyaluronan concentration*

The content of HA in the human and canine samples was investigated in study **II**. First the samples were incubated with papain and GAGs purified with cetylpyridinium chloride and ethanol precipitations (**I**). The samples were dissolved in 40 mM ammonium acetate, pH 6.0, and incubated with 1 mU of *Streptococcus* hyaluronidase (Seikagaku) and 5 mU of chondroitinase ABC (Seikagaku) at 37°C for 3 h, and dried. Equal volumes of 100 mM 2-aminoacridone (Lambda Fluoreszenztechnologie GmbH, Graz, Austria) in a solution containing 3 volumes of acetic acid and 17 volumes of dimethylsulphoxide (Riedel de Haen AG, Seelze, Germany), and 1 M cyanoborohydride (Sigma) in distilled water were added and the tubes were incubated overnight at 37°C. Hyaluronan standards (Healon; Pharmacia) were processed in the same way and used for quantitation.

The fluorescent disaccharides were separated on 20% PAGE with 4% stacking gel in 0.1 M Tris-borate buffer, pH 8.3, using 15 mA for 70-90 min at 4°C (Mini-Protean II, Bio-Rad, Hercules, USA) (Jackson, 1994). The gel was recorded with a digital camera under UV-light (Biometra BioDoc II, Video Documentation System, Göttingen, Germany), and the bands were analysed with the NIH Image software.

*Hyaluronan localization*

A biotinylated hyaluronan binding complex (bHABC) was used as a specific probe to localize HA in the intervertebral disc and endplate. The probe was prepared from the articular cartilage of bovine knee by extracting PGs and HA. The HA binding region and link protein were cleaved off by trypsin digestion and purified using hydroxylapatite chromatography and Sephacryl S-1000 gel filtration (Pharmacia). After

biotinylation, HA and unbound biotin were separated from the probe by Sephacryl S-400 gel filtration (Pharmacia) under dissociative conditions (Tammi et al., 1994).

The human and canine discs and the adjacent vertebral bodies were fixed in 10% formaldehyde, decalcified in 0.1 M PB, pH 7.4, with 10 % EDTA and 4% formaldehyde, and finally postfixed in 10% formaldehyde. Midsagittal sections of the samples were cut and embedded in paraffin (Paraplast plus; Sherwood Medical Co, St. Louis, USA), and 5 µm thick sections were cut on glass slides pretreated with 2% 3-aminopropyltriethoxysilane (Sigma).

The sections were deparaffinised and treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. After washing the slides with PB, pH 7.4, the nonspecific binding was blocked by incubating with 1% BSA (Sigma) in PB for 30 min at 37°C. Incubation with bHABC probe (3-5 µg/ml in PB containing 1% BSA) was performed overnight at 4°C. The slides were washed with PB, and the avidin-biotin-peroxidase complex (ABC-kit, Vector Laboratories, Burlingame, USA) was added to the sections for 1 h. The slides were washed and incubated in 0.05% DAB (Sigma) and 0.03% hydrogen peroxide in PB for 2-3 min followed by washing with deionised water, dehydration, clearing in xylene and mounting in D.P.X. Mounting Medium (BDH, Poole, England).

Some of the sections were digested with 0.25% trypsin (Type III, Sigma) in PB for 10 min at 37°C, or with chondroitinase ABC (460 mU/ml; Seikagaku) in 0.1 M Tris-acetate, pH 8.0, for 30 min at 37°C, before the bHABC staining to reduce the masking effect of proteins and PGs, respectively (Tammi et al., 1994; Parkkinen et al., 1996).

To control the staining specificity some of the sections were incubated for 5 h with and without (buffer only) *Streptomyces* hyaluronidase (100 TRU/ml) in 50 mM sodium buffer, pH 5.0, containing protease inhibitors (Ripellino et al., 1985), or stained with the probe preincubated for 4 h with hyaluronan oligosaccharides (12-14 monosaccharides long).

### 4.4. Statistical analyses

The significances of the differences between the groups were analyzed using the two-tailed Mann-Whitney U-test (**IV**).

# 5. RESULTS

## 5.1. Effects of aging and degeneration on the proteoglycans of the human intervertebral disc (I)

### 5.1.1. Uronic acid content and extractability

In the healthy human discs, the concentration of uronic acid varied between donors and sample sites analysed (from 1.4 to 8.1 μg/mg ww), although in all donors it increased towards the center of the disc. Uronic acid concentration was at its highest in the inner annulus of 5 of the 6 donors (mean 5.5 μg/mg). In the disc of the youngest donor, aged 13, the extractability of uronic acid was notably higher than in the discs of the other donors (**I**, Table 1). In the annulus fibrosus of the degenerated discs, the concentration of uronic acid (on average 1.7 μg/mg) and its extractability (75%) were lower than in the normal discs of similar age (4.1 μg/mg and 77%, respectively) (**I**, Table 2).

### 5.1.2. Distribution of large and small proteoglycans

*Aging and proteoglycan heterogeneity*

In toluidine blue stained agarose gels, the heterogenous low mobility bands representing native large PGs, and the less intense high mobility band showing the small interstitial PGs were seen in all samples (**I**, Fig. 3A). The large (aggrecan/versican) and the small SLR-PGs migrated apart from each other in the outer annulus fibrosus, but in the inner parts of the disc the larger PGs appeared more heterogenous and smaller in size than those in the outer region. The relatively prominent band of small PGs in the annulus was greatly reduced or absent in the nucleus pulposus.

*Aging and small proteoglycans*

Immunodetection of the blots showed that biglycan (**I**, Fig. 3B) and decorin (Fig. 3C) migrated in the same position as the toluidine blue stained high mobility band. The

amount of these small PGs was highest in the outer parts of the discs, particularly OAF. The relative amount of biglycan and decorin was highest in the disc of the youngest donor, while the samples from the 31- and 53-year-old donors had similar, but generally less intense staining patterns. In spite of the increased toluidine blue staining intensity in the small PG area, the blots of nucleus did not contain biglycan, but a small amount of decorin was present (**I**, Fig. 3A-C). The relative increase of small PGs in the nucleus pulposus of the older donors did not come from decorin or biglycan, which were visible only faintly in Western blotting. Therefore, it seems probable that proteolytic fragments of large PGs are responsible for the increase in the low molecular weight toluidine blue stained band.

Chondroitinase ABC treatment before agarose electrophoresis reduced the mobility of both decorin and biglycan compared with the native PGs (**I**, Fig. 3). As judged by their mobility, the small PGs were predominantly in their glycanated forms.

*Degeneration and proteoglycan heterogeneity*

In degeneration, a higher mobility of large PGs was seen in toluidine blue stained gels (**I**, Fig. 4A), suggesting that enhanced proteolysis is involved in the structural changes of the large PGs that occur in degeneration.

*Degeneration and small proteoglycans*

When an equal amount of uronic acid was electrophoresed on agarose gels, the signal from biglycan and decorin core proteins on Western blots was more intense in the degenerated intervertebral discs than in the healthy ones (**I**, Figs. 4A-B and 5). The bands with a molecular weight of 85-120 kDa in Fig. 5A and B represent glycanated small PGs. Additional, slower migrating bands of biglycan and decorin became visible after a longer exposure, likely representing nonglycanated forms of biglycan and decorin (**I**, Fig. 4B and C).

On SDS-PAGE, nonglycanated biglycan core proteins were present at 36 kDa in native samples (**I**, Fig. 5A). In degenerated tissues the relative amount of nonglycanated forms of biglycan was clearly higher. Glycanated biglycan was found at 85-110 kDa. A probable monoglycan form (just one CS/DS chain) was most clearly present in the

youngest donor (**I**, Fig. 5A). The size of the nonglycanated forms of decorin was about 45 and that of glycanated forms 85-120 kDa (**I**, Fig. 5B). The relative amounts of both nonglycanated and glycanated forms of decorin were higher in the degenerated annuli. Chondroitinase ABC treatment before electrophoresis resulted in biglycan and decorin mobility around 42 kDa (**I**, Fig. 5C, D). Glycanated forms of small PGs disappeared after digestion, but at the same time the total amounts of biglycan and decorin were obviously higher in the degenerated annulus fibrosus (**I**, Fig. 5C, D).

### 5.1.3. Changes of chondroitin sulphate epitopes on proteoglycans

The antibody 7D4(-) recognized mainly large PGs in agarose gels in all human samples (**I**, Fig. 6A). Predigestion of the blots with chondroitinase ABC enhanced the staining (**I**, Fig. 6B). The enhancement was striking in the small PG area in the degenerated samples; this was in contrast to the healthy tissues showing no signal in the small PG area, except for the sample of the 13-year-old donor.

The monoclonal antibody 2B6, binding to 4-sulphated stubs of the CS chains, recognized the small PGs in the annuli of both healthy and degenerated discs. The large PGs of the healthy annuli were all positive for 2B6, while the signal was totally lost in 4 of the 7 degenerated annuli, and clearly reduced in 3 (**I**, Fig. 6C). A weak staining was found for the native, undigested CS-6 chain terminal epitope 3B3(-) in the large PGs of both degenerated and healthy samples (**I**, Fig. 6D). After chondroitinase ABC digestion the 3B3(+) signal was strong in all samples, concentrating on the large PG area (**I**, Fig. 6E).

The heterogeneity of the PGs as judged by the width and variability of the toluidine blue stained bands was higher in the degenerated samples (**I**, Fig. 4). The higher toluidine blue intensity in the fast mobility end of the large PGs did not correlate with a similar strong signal in the immunolocalisations for CS epitopes (**I**, Fig. 6). This may be due to the enrichment of KS-containing fragments binding toluidine blue, but not CS antibodies. Nevertheless, even in the degenerated samples there were invariably also very large PGs, perhaps newly synthesized, in the Western blots for CS epitopes (**I**, Fig. 6).

## 5.2. Hyaluronan distribution in the human and canine intervertebral disc and endplate (II)

### 5.2.1. Hyaluronan concentration in the human intervertebral disc

The variation between individuals in the concentration (from 0.09 to 0.64 μg/mg ww) and proportion (from 3 to 32%) of HA of extractable uronic acid was considerable. There was a tendency for higher concentration of HA in the inner parts of the human disc, but the proportion of HA of total uronic acid seemed slightly higher in the annulus (OAF and PAF sample sites) than in the nucleus pulposus, representing 10-16% of the extractable uronic acid in various parts of the disc (**II**, Table 1).

### 5.2.2. Hyaluronan concentration in the canine intervertebral disc

As in human discs, the concentration of HA in the canine disc appeared higher in the nucleus pulposus (mean 0.80 μg/mg ww) than in the anterior (0.51) or posterior (0.32) annulus fibrosus. The HA content in the canine anterior annulus and nucleus was almost two times higher than in the corresponding human sites. The percentage of HA of the total uronic acid was highest in the annuli (10-12%), while the lowest values (mean 7 %) were found in the nucleus (**II**, Table 2).

### 5.2.3. Hyaluronan distribution in the human intervertebral disc and endplate

bHABC staining revealed that HA was present in all human vertebral tissues (**II**, Fig. 1). A high staining intensity was observed particularly in the inner parts of the discs (**II**, Fig. 1C-D).

The ECM of the nucleus pulposus was intensely stained with bHABC (**II**, Fig. 1E). Limited digestion with chondroitinase ABC (and trypsin) decreased the extracellular staining of the nucleus, but enhanced the high-contrast, cell-associated signal present in some, but not in all nucleus pulposus cells (**II**, Fig. 1F).

In the outer parts of the anterior annulus, the apparent staining intensity of the adjacent lamellae was influenced by the orientation of the lamellae (**II**, Fig. 1G). Extracellular staining was stronger in the inner parts of the annulus structure (**II**, Fig. 1C-D). Chondroitinase digestion highlighted the pericellular staining of the annulus cells (**II**, Fig. 1H).

In the cartilage endplate, the probe bound mainly to the interterritorial matrix (**II**, Fig. 1I), but chondroitinase digestion of the sections showed the presence of HA in the territorial and pericellular area (**II**, Fig. 1J). In the vertebral bone, there was a weak but definite staining for HA, mainly associated with osteocyte lacunae (**II**, Fig. 1I-J).

### 5.2.4. Hyaluronan distribution in the canine intervertebral disc and endplate

In the canine intervertebral disc, HA was also widely present, and the highest local signal intensity was seen in the cartilage endplate, adjacent to the nucleus (**II**, Fig. 2B). While HA staining in the nucleus varied from quite pale to intensities close to those in the other parts of the disc, trypsin and chondroitinase ABC digestions generally increased the extracellular matrix staining.

A strong pericellular, and occasionally apparently intracellular HA signal was found in a number of canine nucleus cells (**II**, Fig. 2C, G). This cell-associated signal was enhanced by the chondroitinase ABC and trypsin treatments (**II**, Fig. 2D, H). The ECM staining of HA seemed to reflect the orientation of the lamellae in the anterior annulus fibrosus. The staining signal was often very intense between the lamellae and what appeared to be bundles of collagen fibres (**II**, Fig. 3A, B). Digestion with chondroitinase and trypsin increased the pericellular staining of the annulus cells (**II**, Fig. 2F).

In the cartilage endplate, a strong cell-associated HA signal was observed (**II**, Fig. 2G), with some variation between individual animals, but always with the highest intensity adjacent to the nucleus (**II**, Fig. 2G, H). There was a gradual reduction in the number of HA-positive cells in the cartilage endplate towards the outer parts of the disc (**II**, Fig. 2G, H). At a higher magnification, HA appeared to be located intracellularly in addition to the pericellular location in the canine endplate (**II**, Fig. 3C). A comparable location for HA was present also in the human nucleus cells (**II**, Fig. 3D).

The transition area from nucleus to annulus was often quite abrupt in the canine discs, a feature consistent with the elevated staining of HA in the annulus (**II**, Fig. 3E). In one dog, this border appeared to contain a streak of HA-positive cells, resembling those in the endplate and apparently originating from the endplate (**II**, Fig. 3F).

### 5.3. Effects of disc degeneration, and treatments with tiaprofenic acid and indomethacin on proteoglycans of the porcine intervertebral disc in experimental disc injury model (III, IV)

#### 5.3.1. DNA content

The concentration of DNA, reflecting cell density, was highest in the posterior parts of the annulus fibrosus (0.7-0.8 μg/mg) and lowest in the nucleus pulposus (about 0.2 μg/mg) of the porcine intervertebral disc (**III**, Fig. 6). In the injured discs, the content of DNA was increased, mainly in the anterior annulus (up to 3-fold) and in the nucleus (2-fold). The drugs tended to decrease the concentration of DNA in the injured area of the anterior annulus (55% reduction in OAF by indomethacin) (**IV**, Table 1).

#### 5.3.2. Water content

The concentration of water increased slightly in the area of the injury (from 65 to 70%), but decreased considerably in the nucleus (from 85-90 to 75-80%), and remained at the level of unoperated controls in the posterior side of the discs (60-65%) (**III**, Fig. 4B). In study **IV**, the water content was highest in the indomethacin-treated animals in all sample sites, particularly in the posterior annulus, compared to samples from the nonmedicated animals. A comparison of the tiaprofenic acid and indomethacin treatments showed that the water content was significantly higher in the indomethacin group in the MAF (76 vs. 71%; $P<0.01$) and PAF sample sites (77 vs. 68%; $P<0.01$) (**IV**, Table 1).

### 5.3.3. Uronic acid content and extractability

In general, the concentration of uronic acid was lowest in the outer layers of the unoperated intervertebral disc (below 1 μg/mg ww), increasing towards the nucleus pulposus (about 10 μg/mg). The injury caused a considerable decrease in the uronic acid content in the nucleus, to ~7 μg/mg, while the other parts of the disc remained close to the control level (**III**, Fig. 4A). Tiaprofenic acid significantly increased the uronic acid content in the OAF (mean 3 μg/mg ww) and NP (12 μg/mg) compared with the nonmedicated group (about 1 and 6 μg/mg, respectively), whereas indomethacin treatment had only a slight effect in the nucleus. When normalized to tissue dry weight instead of wet weight, the significance in the NP disappeared but the mean values remained still slightly higher with both NSAIDs compared with the values in nonmedicated animals (**IV**, Fig. 2).

In the healthy control discs, the extractability of PGs was lowest in the outer parts of the anterior annulus (about 23%), and highest in the nucleus pulposus (about 95%). After the injury, the extractability was found to be higher than in controls in the outer parts of the disc, but lower in the nucleus (**III**, Fig. 4C). The drug treatments increased the extractability of PGs, particularly in the anterior annulus (**IV**, Table 1).

### 5.3.4. Proteoglycan subpopulations

Toluidine blue staining of the electrophoresis gels demonstrated that PGs were heterogeneous, consisting of at least two distinct subpopulations of large PGs (mobilities 0.40-0.70 relative to CS), and in the annulus fibrosus of a third, fast-migrating band (relative mobility ~0.82). In the nucleus pulposus the fast mobility band was undetectable. The electrophoretic distributions of PGs from the control and injured discs showed no marked differences, although the proportion of the high-mobility band was higher in the outer part of the anterior annulus in two of the five operated discs. In the injured discs, this band was susceptible to chondroitinase B, indicating that at least some of this PG subpopulation contained DS (**III**, Fig. 8).

The injury did not influence the size of the large low mobility PGs. In discs treated with tiaprofenic acid, the proportion of large PGs tended to be higher in the OAF (66%) and the MAF (74%) than in discs from the other two groups (no treatment, 50 and 61%; indomethacin treatment, 54 and 63%, respectively) (**IV**, Table 3, Fig. 4).

### 5.3.5. Ratio of CS to KS and ratio of CS-6 to CS-4

The ratio of CS to KS, measured as the GalNAc/GlcNAc ratio by HPLC, varied widely (range 0.8-6.5) across the discs of healthy animals. The injury did not significantly change the mean ratio in any part of the disc (**III**, 7A).

The ratio of CS-6 to CS-4 in the outer parts of the discs was quite constant in all discs studied. In healthy controls the ratio was about two times higher in the nucleus pulposus than in the annulus (**III**, Fig. 7B). The injury produced a clear decrease in the CS-6 to CS-4 ratio in the nucleus pulposus. Tiaprofenic acid treatment increased the ratio almost to the level of healthy animals. Indomethacin had no marked effect on the ratio in the nucleus, but in the inner annulus there was a significant decrease ($P<0.01$) (**IV**, Fig. 3).

### 5.3.6. Proteoglycan aggregation

The elution profiles after Sephacryl S-1000 gel filtration showed that the percentage of aggregating PGs in the outer anterior annulus was markedly higher in the animals treated with tiaprofenic acid (36%) and indomethacin (29%), than in the untreated animals (15%) (**IV**, Table 3).

### 5.3.7. Proteoglycan synthesis

In the control porcine discs, the concentration of free, inorganic $Na_2SO_4$, reflecting the rate of solute transport, was highest in the anterior annulus (~0.5), decreased towards the nucleus (lowest value 0.25), and increased again towards the posterior annulus (~0.4). A disc injury caused clearly lower concentrations of inorganic sulphate across the whole disc (**III**, Fig. 5A). Treatment with tiaprofenic acid or indomethacin tended to

result in higher concentrations of inorganic sulphate in the outer part of the anterior annulus, whereas transport to the other parts of the disc was similar in all groups. The higher concentration of inorganic sulphate in the OAF was significant only with tiaprofenic acid when normalised to wet weight (0.28; $P<0.05$) (**IV**, Table 2).

The concentration of incorporated sulphate, indicating newly synthesized PGs, was highest in the outer parts of both the anterior and the posterior annulus. After disc injury the concentration of incorporated sulphate was slightly lower in most parts of the disc (**III**, Fig. 5B).

In study **IV**, the ratio of incorporated sulphate to the free tracer was highest in the NP in each group. Compared with the nonmedicated animals, those treated with tiaprofenic acid tended to have a higher ratio in the NP (from 0.24 to 0.30), but a lower one in the OAF and IAF (from 0.16-0.17 to 0.10; $P< 0.05$). Indomethacin had virtually no influence on the ratio, unlike in the nonmedicated group. The effects of the two medications on the ratio differed significantly in the PAF (tiaprofenic acid 0.10 and indomethacin 0.14; $P<0.05$) (**IV**, Table 2).

# 6. DISCUSSION

In this study, the concentration, quality and location of ECM PGs and HA was investigated in the healthy and degenerated intervertebral disc and cartilage endplate tissue of three different species.

The study confirmed the biochemical and biomechanical roles of PGs and HA in the ECM of spinal tissues. Large PGs and HA were more concentrated in the inner parts of the disc. The observed changes in large PG glycosylation and content and small PG content in degeneration is suggested to either reflect an alteration in cellular differentiation, or result from cleavage by proteinases, leading to inappropriate structures for the biomechanical environment of the disc. Furthermore, the animal models gave valuable information about the normal structure and pathogenesis of the intervertebral disc and cartilage endplate. Thus, the study provides basic information and indicators for more specific treatments of spinal diseases.

## 6.1. Effects of aging and degeneration on proteoglycans of the human intervertebral disc

### Proteoglycan content

The study supports numerous previous findings of a decreased total concentration (Adams and Muir, 1976; Lipson and Muir, 1981a; Lyons et al., 1981; Pedrini-Mille et al., 1983; Cole et al., 1986; Venn and Mason, 1986; Pearce et al., 1987; Bayliss et al., 1988; Urban and McMullin, 1988; Melrose et al., 1991; Olczyk, 1993; Scott et al., 1994; Johnstone and Bayliss, 1995) and increased heterogeneity of PGs (Jahnke and McDevitt, 1988; Taylor et al., 2000) with age and degeneration in the intervertebral disc.

### Chondroitin sulphate epitopes

Disc degeneration induced a change in the sulphation pattern of PGs. The chondroitinase ABC generated 4-sulphated CS stub gave a strong signal on large PGs in all healthy samples, while just a slight signal or none at all was apparent in the degenerated discs. An analysis showed a changed sulphation pattern close to the

reducing end of the GAG chains. A reduction in the total 4-sulfated CS in aging, and more so in degenerated human discs, was also previously observed by chromatographic separation of intact GAG chains (Olczyk, 1993). This raises the interesting question whether there is a certain population of large chondroitin 4-sulphate-rich PGs that is reduced during aging and specifically lost in degeneration, and whether these PGs are produced by one of the distinct cell types known to exist in the intervertebral disc (Postacchini et al., 1984; Maldonado and Oegema, 1992; Johnstone and Bayliss, 1995; Sive et al., 2002). The shift toward 6-sulphation is in sharp contrast to the shift observed in degenerating osteoarthritic and rheumatoid articular cartilages, which start to synthesize chondroitin 4-sulphate-rich, immature-type aggrecan (Cs-Szabo et al., 1995).

Immunohistochemistry of 3B3(-) and 7D4 epitopes has shown an association with the degree of disc degeneration (Roberts et al., 1994), and with arthritic articular cartilage (Slater et al., 1995). The present data (**I**) did not show a consistent difference in signal intensities. Thus, the total content of the native 7D4(-) and 3B3(-) epitopes seems not to be associated with disc degeneration.

When the Western blots were treated with chondroitinase ABC before staining with 3B3, strong signals emerged from both small and large PGs in all samples, indicating the widespread occurrence of the 6-sulphated stubs of CS chains. Interestingly, the 7D4 staining after chondroitinase digestion presented a novel, clear distinction between small PGs in the degenerated and normal adult discs, the former being positive, the latter negative. An increase in the signal for 7D4 after chondroitinase digestion has been described, although a prolonged digestion abolished the signal (Sorrell et al., 1993). The only healthy sample testing positive in the present study was that of the youngest donor (13 yrs). It may be speculated that digestion of the blotted, membrane-bound PGs causes partial GAG chain degradation, unmasking epitopes otherwise hidden. Although the identity of this epitope remains open (Caterson et al., 1992), the 7D4(+) reactivity may offer one more indicator of the pathologic processes in the degenerating disc.

**Small proteoglycans**

The glycanated, toluidine blue stained small PGs in agarose gels represented a higher proportion of total PGs in the 13-year-old healthy donor compared to the older healthy donors. Whether this is related to development, maturation, or aging is not clear. Western blotting of polyacrylamide gels indicated that with age, the proportions of both the glycanated and nonglycanated forms of biglycan were lower relative to all PGs. A lower proportion of the glycanated forms of the tissue small PGs has been reported in studies on aging human articular cartilage (Roughley et al., 1993) and intervertebral disc (Johnstone et al., 1993).

In intervertebral disc degeneration, the proportion of biglycan (both the glycanated and nonglycanated forms) of the total PGs increased compared with the proportion observed in healthy discs from donors of the same age. The data indicated that the decreased uronic acid content observed in disc degeneration is specific for the large PGs, while the small PGs are not reduced or may increase in the degenerated disc tissue. A proportional increase in nonglycanated small PGs has been described in articular cartilages obtained from patients with rheumatoid arthritis and osteoarthritis (Cs-Szabo et al., 1995), and to occur with age (Roughley et al., 1993). The association of increased biglycan and decorin in disc degeneration is supported by the fact that it is not a part of normal aging, where an opposite, decreasing trend has been found (Roughley et al., 1993). Here, no clear conclusions can be drawn about the possible role of these PGs in the early pathogenesis of human disc degeneration. However, in a canine model (Puustjärvi et al., 1994), it was found that the proportion of fast mobility PGs (representing most likely decorin and biglycan) was clearly higher in a disc predisposed to degeneration after strenuous running exercise. Also, in an ovine model of experimental disc degeneration, the content of small PGs was higher due to the elevated synthesis of decorin and biglycan (Melrose et al., 1997b).

A part of the nonglycanated forms of decorin appear as a result of proteolytic cleavage close to its N-terminus (Johnstone et al., 1993). Less is known about the possible proteolysis of biglycan, but biglycan may be synthesized with only one GAG chain (Järveläinen et al., 1991; Kresse et al., 2001), or perhaps none at all. Amino acid sequencing of bovine articular cartilage biglycan has also suggested that some of the

potential N-terminal, GAG attaching serines are unsubstituted (Neame et al., 1989). The binding of decorin and biglycan to collagen does not depend on the GAGs. Therefore, both the glycanated and nonglycanated forms of decorin and biglycan may compete for sites on collagen fibrils. The suggested role of decorin as a spacer of collagen fibrils requires a DS chain (Scott, 1988), as does its ability to bind cytokines and growth factors such as TGF-β (Roughley et al., 1994). For instance, collagen fibril spacing may be deranged by the lack of CS/DS chains on decorin, resulting in reduced plasticity and poor biomechanical properties. Hence, the relative content of native, glycanated decorin and its nonglycanated forms may be of utmost importance in the regulation of connective tissue structure and metabolism.

## 6.2. Hyaluronan in the human and canine intervertebral disc and cartilage endplate

### Hyaluronan in the nucleus pulposus

A strong extracellular matrix staining for HA in the human nucleus pulposus, reduced after chondroitinase ABC and trypsin digestions, is compatible with a relatively loose matrix structure rich in HA, not masked by PGs. The masking effect of aggrecan and other matrix proteins in the ECM of the nucleus pulposus and inner annulus fibrosus, relieved by the enzyme treatments, appeared more pronounced in the canine than in human discs. This is probably due to the larger average size of the resident aggrecan molecules in the dog (Franzen et al., 1981; Bayliss et al., 1983; Säämänen et al., 1989; Puustjärvi et al., 1994), reducing the exposed HA available to the probe. In contrast, relatively low-molecular-weight, presumably partly degraded, PGs are typical for the human nucleus pulposus (**I**).

In both species, the strong pericellular HA staining after enzyme treatments suggests an abundance of CS-rich aggrecan at this site. This observation may be explained by a process in which the pericellular area contains a high concentration of newly-synthesized aggrecan that later diffuses into the interterritorial matrix, where it becomes diluted and proteolytically cleaved. The variation in staining intensities between cells probably reflects the heterogeneity in the origin of the nucleus pulposus cells (Chelberg et al., 1995; Horner et al., 2002), or differences in their metabolic activity.

**Hyaluronan in the annulus fibrosus**

In the annulus fibrosus, histochemical staining and concentration of HA, like that of large PGs (**I**) (Puustjärvi et al., 1994), increased towards the center of the disc in man and dog. However, the HA to uronic acid ratio increased towards the outer parts of the disc, suggesting that HA is less masked by PGs in the outer annulus fibrosus. Based on the location of the HA signal in the annulus fibrosus, it seems reasonable to hypothesise that HA alone or in aggregates with large PGs resides on the surface of the collagen fibrils and may serve as a lubricant between the lamellae sliding along each other, as in skeletal muscles (Laurent and Fraser, 1992). This type of function for HA may well be crucial for the health and maintenance of a normal annulus fibrosus structure.

**Hyaluronan in the cartilage endplate**

In the human cartilage endplate, HA was present as PG aggregates in the territorial and pericellular matrix, while interterritorial HA appeared in a more open matrix, as judged by the enzyme susceptibility data. The most striking result in canine cartilage endplate was the strong signal observed in the pericellular rim (lacuna) and probably also the intracellular compartment. While it is known that some intracellular HA resides in cultured epidermal keratinocytes (Tammi et al., 1998), and accumulates in the most aggressive colon carcinoma cells (Ropponen et al., 1998), the apparent abundance of intracellular HA in the endplate cells exceeds the content found in any cell type examined so far. HA is synthesized, and exogenous HA is taken up and degraded by cultured chondrocytes (Hua et al., 1993), but endogenous intracellular HA has not been directly assayed. Therefore, data on the regulation of HA uptake and degradation are lacking, and hence no mechanisms for its possible intracellular accumulation in the endplate cells have been established.

The staining intensity of HA in the cartilage endplate was generally stronger in the dog than in man. The low HA signal in man may be associated with the well-known age-related changes in endplate structure. At the age of 20-40 years, the human cartilage endplate undergoes gradual mineralization, later resorption of the calcified cartilage and replacement by bony tissue (Bernick and Cailliet, 1982). Specific differences between the canine and human cartilage endplates have been noticed also in the expression of

type X collagen (Boos et al., 1997; Lammi et al., 1998). The metabolism of HA in the intervertebral disc is thus altered with aging and degeneration in the human cartilage endplate, and by changed biosynthesis, enhanced denaturation of extracellular matrix proteins, and deterioration of the transport properties of the endplate (Bernick and Cailliet, 1982; Bishop and Pearce, 1993; Antoniou et al., 1996a; Roberts et al., 1996).

## 6.3. Effects of experimental degeneration on proteoglycans of the porcine intervertebral disc

### Cell density

The cell density in the injured discs was considerably higher in the anterior annulus and the nucleus pulposus than in the non-injured discs. Previous histological studies have shown that the cells proliferating after disc injury were fibroblasts (Smith and Walmsley, 1951). A disc injury has been found to activate the synthesis of collagen types I and III in the nucleus pulposus, further confirming that these new cells were more likely fibroblasts than chondrocytes (Kääpä et al., 1994). Possibly the nuclear cells switch their phenotype after injury and thereafter produce an abnormal ECM.

### Proteoglycan content and synthesis

The lower concentration of free, inorganic sulphate in the injured disc suggests a decline in the transport rate of solutes into the disc compared to the rate in control discs. Nutrients are transported into the disc mainly by passive diffusion, either through the endplate or through the peripheral parts of the annulus fibrosus (Brodin, 1955). In the case of large molecules, an active "pumping effect" is also of importance (Maroudas, 1988). The decrease in the transport rate of solutes may be due to partial blockage of nutritional routes into the disc. Chondrosis, ossification of end-plates, and formation of osteophytes have been observed after experimental disc injury and in human disc degeneration and aging (Bernick and Cailliet, 1982; Vernon-Roberts, 1988; Osti et al., 1990). Decreased elasticity of the nucleus evidently deteriorates the hydrodynamic properties of the disc and affects the active pumping of solutes. The reduced PG content of the nucleus also decreases the osmotic pressure in the disc and further decreases the

volume of the hydrated space and diffusion of solutes into and within the disc (Maroudas, 1988).

The annular injury decreased the total PG content of the nucleus pulposus. Loss of PGs from the nucleus pulposus in human disc degeneration is a clear and well-known phenomenon (Gower and Pedrini, 1969; Adams and Muir, 1976; Lyons et al., 1981; Pearce et al., 1987). PG synthesis has been noticed to vary widely in pathological human discs (Cs-Szabo et al., 2002), but in spondylolisthesis the synthesis rate was consistently diminished in the anterior annulus (Bayliss et al., 1988). In an experimental study on rabbit discs, synthesis of aggregating PGs increased in the early phase after disc injury, but later began to decrease progressively (Lipson and Muir, 1981b). In the articular cartilage in osteoarthritis, PG synthesis is activated in the early phase of the degenerative process (Carney et al., 1984), but in the advanced stages of degeneration the rate of sulphate incorporation is decreased (Brocklehurst et al., 1984). The present data (**III**) indicates a slight decrease in the amount of incorporated [$^{35}$S]sulphate across the whole disc three months after disc injury.

**Chondroitin and keratan sulphate ratio**

The ratio of CS to KS varied widely across the disc and was not influenced by the injury. The water-binding capacity varies with the composition of GAG molecules because CS has twice as many negatively charged ions in its molecular structure as does KS (Holm and Urban, 1987). The ratio of CS to KS in PGs varies between species, and with the position, age, and state of health of the tissue (Szirmai, 1970; Hardingham and Adams, 1976; Olczyk, 1993). The present results showing the radial distribution of these GAGs are in accordance with earlier results in pigs (Szirmai, 1970), and man (Hardingham and Adams, 1976).

**Chondroitin sulphate isomers**

CS consists mainly of monosulphated isomers of chondroitin disaccharides, the sulphate group being attached at position 4 or 6 of the galactosamine residue. The importance of this heterogeneity of sulphation is not fully understood, but it has been suggested that CS-6 may interact more strongly with collagen and other components of

the tissue than does CS-4 (Muir, 1980). An increase in the ratio of CS-6 to CS-4 with aging has been observed in the human intervertebral disc (Pearce and Grimmer, 1976; Olczyk, 1993). The CS-6 isomer seems to dominate also in the porcine disc, particularly in the nucleus pulposus. The annular injury produced a significant decrease in the ratio of CS-6 to CS-4 in the nucleus pulposus, perhaps as a result of the synthesis of immature PGs, as observed in canine articular cartilage after immobilization of the knee joint (Säämänen et al., 1989; Säämänen et al., 1990), and in bovine growth plates during the proliferative and maturation stages of chondrocytes (Deutsch et al., 1995).

**Small proteoglycans**

The annular injury increased the amount of small PGs in the outer layers of the anterior annulus in two experimental discs, which would partly explain the increase in PG extractability in this injured area. Small PGs are abundant in many fibrocartilaginous tissues. They bind specifically to collagen and elastic fibres, and influence collagen fibrillogenesis (Scott, 1984; Scott, 1991; Schönherr et al., 1995; Reinboth et al., 2002). Decorin regulates the growth of collagen fibres, while biglycan is also involved in cell regulation (Bianco et al., 1990). The present findings and previous observations on degenerated human discs (Davidson and Woodhall, 1959; Cs-Szabo et al., 2002) suggest that the appearance of small PGs in the disc may be associated with wound healing (Border et al., 1992), or the degeneration process (Cs-Szabo et al., 2002).

## 6.4. Effects of anti-inflammatory drugs on proteoglycans in the experimental degeneration of porcine intervertebral disc

**Cell density and water content**

Both NSAIDs, tiaprofenic acid and indomethacin, tended to decrease the DNA content in the injured area, probably because of decreased infiltration of leukocytes into the wound area during the injury-induced inflammation process. Compared with the water contents found in the non-treated control group, the water content was higher in all sites in the discs of the indomethacin group and consistently lower in the tiaprofenic

acid group. This was puzzling considering the mostly parallel changes caused by the two drugs in other parameters. Probably scar formation and fibrosis, with changes in collagen content (Kääpä et al., 1994) play an important role in hydration. Whether the increased water content is associated with just the inflammation process, or whether the matrix properties were altered for other reasons, is unclear.

**Proteoglycan content and aggregation**

The extractability of PGs increased slightly in the area of injury in the NSAID-treated groups compared with the nonmedicated group. There was a parallel increase in the proportion of aggregating PGs in the injured area with both medications. Possibly PGs that were able to aggregate with HA were leaking from the nucleus pulposus and were captured by the HA at the site of the injury, increasing the tissue content of uronic acid.

The tiaprofenic acid treatment increased the content of PGs, up to the levels found in normal porcine discs (**III**). Significant increases were observed in the nucleus pulposus and outer annulus fibrosus per wet weight in the tiaprofenic acid-treated group compared with the nonmedicated group. Per dry weight, both medications had a similar trend to maintain the amount of uronic acid in the nucleus pulposus, therefore having apparently protective effects on PGs during the early phases of disc degeneration.

**Proteoglycan synthesis**

In the nucleus pulposus, there was a slight but not statistically significant increase in newly synthesized PGs in the tiaprofenic acid-treated animals compared with the nonmedicated ones. In contrast, less PGs were synthesized in the anterior annulus in the animals treated with tiaprofenic acid than in the other groups, although uronic acid levels were elevated. This may indicate that a metabolic balance in PG concentration was reached in the injured area, and no stimulation of synthesis was necessary. A comparable inverse relationship between the rate of synthesis of PGs and the concentration of uronic acid has been noticed in the canine intervertebral disc after strenuous running exercise (Puustjärvi et al., 1994).

The effects of tiaprofenic acid and indomethacin have been studied in patients with osteoarthritis of the knee (Dingle, 1999; Ding, 2002). Many studies carried out on

chondrocytes derived from articular cartilage (Palmoski and Brandt, 1985; Muir et al., 1988; Fujii et al., 1989) or from intervertebral disc (Yoo et al., 1992) have shown that indomethacin suppresses PG synthesis. This *in vivo* study did not reveal any adverse effects of indomethacin treatment after disc injury.

**Chondroitin sulphate isomers**

The ratio of CS-6 to CS-4 was slightly higher in the drug-treated groups, especially in the nucleus pulposus. Probably this was not due to the synthesis of new PGs, but maybe due to the effects of the drugs (inactivation) on the degenerative enzymes present in the disc. There was a trend by the tiaprofenic acid treatment to shift the ratio closer to that found in unoperated animals in study **III**, an effect that is qualitatively similar to that caused by tiaprofenic acid on the content of PGs. In the outer part of an injured disc, the proportion of large PGs was higher in animals treated with tiaprofenic acid than in nonmedicated or indomethacin-treated animals. This suggests that discs from the tiaprofenic acid-treated animals are more resilient and better adapted to compressive-type forces. It would be interesting to know which PG species were specifically affected in the outer annulus during this kind of injury. What we do know is that altered biomechanics (e.g. scoliosis or trauma) may initiate changes in cell density (Urban et al., 2001), or switch the phenotype of cells, leading to changes in PGs that may contribute to disc degeneration (Sive et al., 2002; Palmer and Lotz, 2004).

## 7. SUMMARY AND CONCLUSIONS

In the human intervertebral disc, the content of small PGs was higher and the glycosylation of large PGs was altered with disc degeneration, while these changes were smaller with aging. The shift in PG glycosylation as well as the increase in small PGs in degeneration differed from that observed in aging or maturation. This is suggested to reflect an alteration in cellular differentiation, or cell type distribution in the diseased intervertebral disc (Horner and Urban, 2001; Urban et al., 2001; Johnson and Roberts, 2003), or to result from cleavage by aggrecanases (Pratta et al., 2000), resulting in inappropriate structures for the biomechanical environment of the disc.

The degenerative structural changes in the injured porcine intervertebral disc were similar to those found in experimental models on rabbits and sheep (Smith and Walmsley, 1951; Haimovici, 1970; Lipson and Muir, 1981b; Osti et al., 1990). The annular injury caused structural and biochemical changes, particularly in the nucleus pulposus where the phenomena were regarded as degenerative, whereas the reactions in the area of injury were considered as reparative. The results of the present and previous studies of experimental disc injury suggest that the integrity of the annular structures is essential for the normal cellular function of the whole disc. It is reasonable to suppose that an injury to the annulus alters the biomechanics and nutrition of the disc and that these in turn affect the cells and lead to changes in matrix production and degradation. This confirms the hypothesis of previous investigations that a traumatic annular tear can be a precursor of disc degeneration (Lipson and Muir, 1981b; Osti et al., 1990), although many other factors may also be involved in the initiation of degenerative disc disorders (Speed, 2004). Despite the fact that the present model of disc degeneration is not natural, it seems to be useful for studying the pathogenesis of disc degeneration during various disease stages.

The course of PG alterations in degenerating porcine discs was modified by administration of two NSAIDs. The results suggest that tiaprofenic acid may afford protection to PGs, at least during the early phases of the disc injury examined in this study. These observations are very similar to those obtained in studies on an experimental model of osteoarthritis (de Vries et al., 1988; Brandt et al., 1990; Howell

et al., 1991; Pelletier and Martel-Pelletier, 1991; Meyer-Carrive and Ghosh, 1992). The mechanisms behind this protection against PG loss need clarification (Ding, 2002), and the influence of long-term administration of NSAIDs also remains to be described (Cooper and Jordan, 2004).

Most HA was found in the inner parts of the canine and human discs. In human discs particularly, cell-associated HA was in tight contact with aggrecan, whereas less aggrecan masking the HA was apparently present in the interterritorial matrix. In the canine cartilage endplate, HA was clearly cell-associated, it was present even inside the chondrocytes. Whether this is a sign of synthesis or uptake and degradation of HA remains unclear. In the outer annulus fibrosus, HA was located between the collagenous lamellae, perhaps serving as a lubricant when the lamellae slide along each other as in skeletal muscles (Laurent and Fraser, 1992).

There were certain similarities between the species in the reactions of PGs and HA in aging and degeneration. During degeneration, the glycosylation of large PGs, mainly aggrecan, was altered in human and porcine discs. Also, a clear increase in small PGs, decorin and biglycan, was seen both in degenerated human discs and in the discs of the experimental degeneration model of pigs. The distribution of HA in human and canine spinal tissues differed clearly only in the cartilage endplate. The animal models provided valuable information about the normal structure and the pathogenesis of degeneration of the intervertebral disc and cartilage endplate. This knowledge should contribute to a better understanding of how the disc tissue functions and be useful in the formulation of new treatments for spinal diseases.

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