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CHENGJUAN QU

Articular cartilage proteoglycan biosynthesis and sulfation

Doctoral dissertation

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ABSTRACT

Glucosamine (GlcN) and glucosamine sulfate (GS) have been used to treat the patients with osteoarthritis (OA) as a disease-modifying agent. Previous *in vitro* studies have focused on the effects of GlcN or GS on cartilage metabolism, whereas *in vivo* studies have investigated their potential for the treatment of OA. Although these results have raised promises of the disease-modifying effects of GlcN or GS, the cellular mechanisms behind these proposed effects are not clear. In general, the effectiveness of GS in the treatment of OA as a symptomatic and as a disease-modifying agent is a matter of debate.

Loss of proteoglycans (PGs) in OA could be partly due to deficient water binding e.g., by undersulfation of glycosaminoglycans (GAGs). In this study, the molar ratios of chondroitin sulfate (CS) disaccharide isoforms were analyzed with fluorophore-assisted carbohydrate electrophoresis to investigate the hypothesis that sulfate deficiency is involved with the development of bovine and human OA. Our present results indicate that the molar ratio of non-sulfated CS disaccharide in human samples was much lower than that detected in bovine samples, and it did not increase in human OA samples. Conversely, this ratio significantly decreased in bovine OA samples.

Furthermore, the steady-state levels of aggrecan mRNA expression and sulfated GAG synthesis were analyzed by using Northern blot assay, quantitative real time reverse transcription polymerase chain reaction and [³⁵S]sulfate incorporation analyses in bovine primary chondrocyte cultures. Aggrecan which is a large CS-PG of cartilage provides osmotic resistance for the cartilage helping it to absorb the compressive loads. Loss of PGs is a major cause of joint dysfunction and disability in OA. However, our results from 25 individual animals showed that none of the different forms of hexosamines, nor the GS salt, could stimulate aggrecan mRNA expression or GAG synthesis in bovine primary chondrocytes.

Glucosamine is produced intracellularly from endogenous glucose, and is one of the basic sugar structures required for CS synthesis. It is converted to UDP-glucuronic acid (GlcA) and UDP-N-acetylgalactosamine (UDP-GalNAc) before use for the synthesis of CS polysaccharide chain. If exogenous GS is made available to the cultured cells, it can be directly incorporated into the CS synthesis by UDP-GalNAc via GlcN-6-phosphate bypassing fructose-6-phosphate. Thus, the levels of intracellular UDP-N-acetylhexosamines and UDP-GlcA were explored with reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry in bovine primary chondrocytes to analyze whether a physiologically attainable level of GS could stimulate CS synthesis by increasing intracellular UDP-sugar levels. Our present results with the cells from nine individual animals did not support this hypothesis.

In conclusion, bovine and human articular cartilage PGs were not undersulfated in the early stage of OA. Exogenous GS did not increase steady state levels of aggrecan mRNA expression, GAG synthesis or intracellular levels of nucleotide-activated precursors of GAG synthesis in bovine primary chondrocytes.

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To Yawu, Yang and Yilan

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Chengjuan Qu

ABBREVIATIONS

COL2A1	procollagen (α_1) II
CS	chondroitin sulfate
Di-6S	chondroitin 6-sulfate disaccharide
Di-4S	chondroitin 4-sulfate disaccharide
Di-2S	chondroitin 2-sulfate disaccharide
Di-0S	chondroitin 0-sulfate disaccharide
Di-SB	chondroitin 2,4-sulfate disaccharide
Di-SD	chondroitin 2,6-sulfate disaccharide
Di-SE	chondroitin 4,6-sulfate disaccharide
DMEM	Dulbecco's Modified Eagle's Medium
DS	dermatan sulfate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
FCS	fetal calf serum
FG	femoral groove
FLC	femoral lateral condyle
FMC	femoral medial condyle
GAG	glycosaminoglycan
GalN	galactosamine
GalN-6S	galactosamine 6-sulfate
GalNAc	N-acetylgalactosamine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glc	glucose
GlcA	glucuronic acid
GlcN	glucosamine
GlcN-3S	glucosamine 3-sulfate
GlcN-6S	glucosamine 6-sulfate
GlcN-6-P	glucosamine-6-phosphate
GlcNAc-6-P	N-acetylglucosamine-6-phosphate
GlcNAc	N-acetylglucosamine
GFAT	glutamine:fructose-6-phosphate aminotransferase
GNPNAT	glucosamine-6-phosphate N-acetyltransferase
GS	glucosamine sulfate
HA	hyaluronan
HAS	hyaluronan synthase
HAS2	hyaluronan synthase 2
HS	heparan sulfate
IdoA	iduronic acid
IGD	interglobular domain
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IL-1 α	interleukin-1 α

IL-1 β	interleukin-1 β
KS	keratan sulfate
ManN	mannosamine
MMP	matrix metalloproteinase
NASIDs	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
PAT	patella
PG	proteoglycan
PTR	proteoglycan tandem repeat
QRT-PCR	quantitative real time reverse transcription polymerase chain reaction
SLRPs	small leucine-rich PGs
TGF- β	transforming growth factor β
TLP	tibial lateral plateau
TMP	tibial medial plateau
TNF- α	tumor necrosis factor α
UA	uronic acid
UDP	uridine diphosphate
UDP-GlcA	UDP-glucuronic acid
UDP-Glc	UDP-glucose
UDP-Hex	UDP-hexose(s)
UDP-HexN	UDP-N-acetylhexosamine(s)
UDP-GlcNAc	UDP-N-acetylglucosamine
UDP-GalNAc	UDP-N-acetylgalactosamine
WOMAC	Western Ontario and McMaster University osteoarthritis index
28S rRNA	28S ribosomal RNA

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to by the corresponding Roman numerals:

- I** **Lammi MJ, Qu CJ, Laasanen MS, Saarakkala S, Rieppo J, Jurvelin JS, Töyräs J:** Undersulfated chondroitin sulfate does not increase in osteoarthritic cartilage. *J Rheumatol* 2004; 31: 2449-53.
- II** **Qu CJ, Rieppo J, Hyttinen MM, Lammi MJ, Kiviranta I, Kurkijärvi J, Jurvelin JS, Töyräs J.** Human articular cartilage proteoglycans are not undersulfated in osteoarthritis. *Connect Tissue Res* 2007; 48: 27-33.
- III** **Qu CJ, Karjalainen HM, Helminen HJ, Lammi MJ.** The lack of effect of glucosamine sulphate on aggrecan mRNA expression and ³⁵S-sulphate incorporation in bovine primary chondrocytes. *Biochim Biophys Acta.* 2006; 1762: 453-9.
- IV** **Qu CJ, Jauhiainen M, Auriola S, Helminen HJ, Lammi MJ.** Effects of glucosamine sulfate on intracellular UDP-hexosamine and UDP-glucuronic acid levels in bovine primary chondrocytes. *Osteoarthritis Cartilage* 2007; 15: 773-9.

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1. INTRODUCTION

Osteoarthritis (OA) is a degenerative musculoskeletal disease, mainly affecting articular cartilage but also involving pericartilage structures (Brandt 2000; Felson 2004). It usually causes pain and malfunction of the affected joint. The incidence of OA increases with aging. The etiology of this disease is poorly understood, although several risk factors are recognized (Panula et al. 1998; MacGregor et al. 2000; Sinkov and Cymet 2003; Felson 2004). There is no optimal cure for this disease at present. However, many treatments for OA have been proposed including nonpharmacological treatments, such as physical and surgical interventions, and pharmacological treatments, such as analgesic, nonsteroidal anti-inflammatory drugs (NSAIDs) and nutrient treatment. Glucosamine (GlcN) and glucosamine sulfate (GS) have been used as nutraceuticals to treat patients with OA due to their suggested analgesic and symptom-relieving properties as well as chondroprotective and disease-modifying effects (Reichelt et al. 1994; McAlindon et al. 2000; Reginster et al. 2001; Pavelka et al. 2002).

The loss of the cartilage proteoglycans (PGs) from the extracellular matrix (ECM) is one of the early events in the pathogenesis of OA (Venn and Maroudas 1977; Grushko et al. 1989). Therefore, finding means to prevent PG loss and to stimulate PG synthesis has been considered to be one of the key issues in the design of new treatments for OA patients. It has been suggested that GlcN or GS could stimulate PG synthesis (Bassleer et al. 1988; Setnikar et al. 1991; Bassleer et al. 1998a), inhibit the cartilage degradation (Hua et al. 2002; Ilic et al. 2003), increase protein synthesis and protein kinase C activity of chondrocytes in a dose-dependent manner, and decrease cellular phospholipase 2 activity (Piperno et al. 2000). An increase in aggrecan mRNA expression with donor-dependent manner and an inhibition of the activity of matrix metalloproteinase-3 by GS has also been reported (Dodge and Jimenez 2003). Although these results have raised the promise of the disease-modifying effects of GlcN or GS on OA, the cellular mechanisms behind these proposed effects are not clear. On the other hand, some recent industry-independent clinical trials in the treatment of OA with GlcN and GS have generated negative results (Rindone et al. 2000; Hughes and Carr 2002; McAlindon 2003 and 2004; Cibere et al. 2004; Clegg et al. 2006). Furthermore, many

studies showing positive effects have been conducted with GlcN and GS concentrations that cannot be reached physiologically *in vivo* after the recommended dose of oral administration. It has recently been shown that after its oral administration the GlcN concentration reaches a very low level both in serum (Biggee et al. 2006) and synovial fluid (Lavery et al. 2005). This raises doubts about the mechanism of action of the proposed effect of GS. Also the sulfate moiety has been suggested to be a possible effector of GS (Hoffer et al. 2001). Indeed, it was previously shown that sulfate concentration could affect the sulfation level of chondroitin sulfate (CS) in cartilage explants (van der Kraan et al. 1988; Brand et al. 1989).

Glucosamine is one of the basic sugar structures utilized in CS biosynthesis. It is well-known that GlcN is converted intracellularly from glucose (Glc) via GlcN-6-phosphate into fructose-6-phosphate (Silbert and Sugumaran 2002), then rapidly further converted into uridine diphosphate-N-acetylgalactosamine (UDP-GalNAc) through UDP-N-acetylglucosamine (UDP-GlcNAc) (Silbert and Sugumaran 2002). Chondroitin sulfate is synthesized by adding UDP-activated monosaccharides of glucuronic acid (GlcA) and GalNAc consequently to one end of a growing, linear polysaccharide chain.

In the present study, CS disaccharide compositions of bovine and human articular cartilages were analyzed at various stages of OA using fluorophore-assisted carbohydrate electrophoresis. The effects of different hexosamines and GS salt on the steady-state levels of aggrecan and hyaluronan synthase (HAS) mRNA, and glycosaminoglycan (GAG) synthesis in bovine primary chondrocytes were analyzed by Northern blot assay, quantitative real time reverse transcription polymerase chain reaction (QRT-PCR) and [³⁵S]sulfate incorporation. The intracellular levels of UDP-N-acetylhexosamines (UDP-HexN) and UDP-GlcA in bovine primary chondrocytes after exposure to GlcN and GS were investigated by reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry.

2. REVIEW OF THE LITERATURE

2.1. Articular cartilage

Articular cartilage is a hyaline, avascular, aneural, alymphatic connective tissue that covers the bony surfaces of the joints. Its major functions are to act as a shock absorber during weight bearing, to protect the articulating bone ends and to provide smooth surfaces for the movement of articulating bones. It is comprised of 5% chondrocytes and 95% ECM, which is mainly composed of water (68-85%), PGs (5-10%) and collagens (10-20%). In addition, there is also a small amount of noncollagenous proteins and lipids present in cartilage. Articular cartilage is not a homogenous tissue. Instead, it is divided into four different zones: superficial, middle, deep and calcified zones (Fig. 1). The number, the size, the shape, and the metabolism of chondrocytes differ considerably in the different zones (Aydelotte et al. 1988; Aydelotte and Kuettner 1988). The superficial or tangential zone contains proportionally the highest collagen content, and a relatively low content of PGs. The relative amount of collagens decreases whereas that of PGs increases in each zone closer to the tidemark, the border of calcification. The heterogenous and depth- dependent composition and structure of articular cartilage account for much of its anisotropic and non-linear properties in compression and tension (Jurvelin et al. 2003). The compression modulus increases significantly with depth and thus the properties of articular cartilage zones greatly affect the biomechanical behavior of cartilage (Schinagl et al. 1997).

2.1.1. Chondrocyte

Chondrocyte is a specialized cell that produces the ECM of cartilage and organizes the collagens, PGs and noncollagenous proteins into a highly ordered structure. In the superficial zone, the chondrocytes are quite small, flattened and disc-shaped, and they lie parallel to the surface embedded within PGs and tangentially oriented collagen fibrils. The middle zone has round or oval chondrocytes, which are dispersed singly or in small groups within the matrix, while the deep zone possesses fairly large cells,

which are arranged in columns perpendicular to the cartilage surface. The course of the collagen fibrils in the deep zone follows the orientation of the chondrocyte columns. They have a role in the separation of the chondrocyte columns with the partitioning of interterritorial matrix rich in cartilage PGs, and the columns formed by the spheroidal chondrocytes are arranged along the axis of fibril orientation. The calcified zone is adjacent to the subchondral bone, is partly mineralized and acts as the transitional zone between the cartilage and the underlying subchondral bone. It contains few rounded chondrocytes, which are hypertrophic, and has a low content of PGs (Fig. 1).

Since articular cartilage consists mainly of water, it acts as a lubricated, wear-resistant, friction-reducing surface that is slightly compressible to ensure an even distribution of forces onto the bone end and the subchondral bone. Chondrocytes receive nutrition and oxygen via diffusion from synovial fluid through the cartilage matrix (Scott and Haigh 1988; Kuettner 1992). Due to this lack of a vascular supply to the cartilage, the chondrocytes are highly glycolytic cells and need a steady glucose supply to ensure their viability and ECM synthesis (Otte 1991; Lee and Urban 1997).

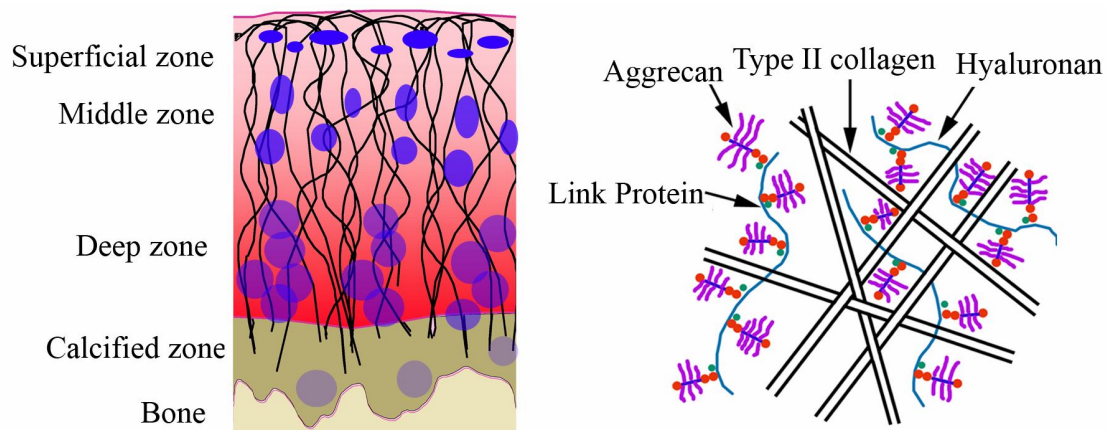


Figure 1. Schematic representation of the structure of articular cartilage.

2.1.2. Articular cartilage proteoglycans

Proteoglycans are macromolecules that consist of a protein core to which one or more GAG chains are covalently attached (Hascall and Sajdera 1969; Muir 1978; Kuettner

and Kimura 1985; Lohmander 1988). Aggrecan, the major large PG of cartilage, can aggregate with hyaluronan (HA) to form large supramolecular structures in the ECM that help to promote their retention within the collagen network (Hardingham and Muir 1972; Hardingham and Bayliss 1990; Hardingham and Fosang 1992). Proteoglycans have many key functions since they are involved in the structure, assembly, and breakdown of connective tissue matrix (Heinegård and Oldberg 1989; Knudson and Knudson 2001; Perrimon and Bernfield 2001). Proteoglycans can be found within the ECM, on the cell surface and in the intracellular granules (Ruoslahti 1988; Heinegård and Oldberg 1989; Knudson and Knudson 2001; Perrimon and Bernfield 2001).

Extracellular matrix proteoglycans

Aggrecan, the large PG of the cartilage, has numerous GAG chains bound to its core protein (Heinegård et al. 1987; Carney and Muir 1988; Heinegård and Oldberg 1989; Kiani et al. 2002). The size of an aggrecan monomer is $1-4 \times 10^6$ Da, while its protein backbone has a molecular size of 210-250 kDa. It is a well-characterized large aggregating PG predominantly found in the articular cartilage (Doege et al. 1991; Hardingham et al. 1994; Watanabe et al. 1998; Kiani et al. 2002). Approximately 90% of aggrecan is comprised of CS chains, but it also contains keratan sulfate (KS) chains, and both O- and N-linked oligosaccharides (Lohmander et al. 1980; Nilsson et al. 1982; Buckwalter et al. 1994; Kiani et al. 2002). Its critical function is to distribute the load in weight-bearing joints (Hardingham and Bayliss 1990; Watanabe et al. 1998). Aggrecan plays a key role in mediating both chondrocyte-chondrocyte and chondrocyte-matrix interactions via binding HA (Hardingham and Muir 1972; Kiani et al. 2002; Watanabe and Kimata 2006). Its core protein contains three globular domains, G1, G2 and G3, and the CS 1 and 2 domain located between G2 and G3 domains, and KS chains located in the initial part of core protein between G2 and G3. Keratan sulfate can be attached to the core protein also anywhere on G1 and G2 or the short interglobular domain (IGD) located between G1 and G2 domains. A number of O-linked and N-linked oligosaccharides are also attached to the protein core (Fig. 2) (Nilsson et al. 1982; Chandrasekaran and Tanzer 1992; Hardingham et al. 1994; Margolis and Margolis 1994; Watanabe et al. 1998; Knudson and Knudson 2001; Kiani et al. 2002).

The G1 domain consists of 3 looped subdomains, A, B and B'. Immunoglobulin (Ig)-type fold is present in A, while PG tandem repeat (PTR) units are formed from B and B' subdomains, which provide the binding site for aggrecan and HA (Knudson and Knudson 2001; Kiani et al. 2002). The G1 domain not only mediates the specific interactions with HA through the PTR domain, but it also interacts with the link protein via the Ig fold (Heinegård and Hascall 1974; Tang et al. 1979; Perin et al. 1987; Mow et al. 1989; Grover and Roughley 1994; Kiani et al. 2002).

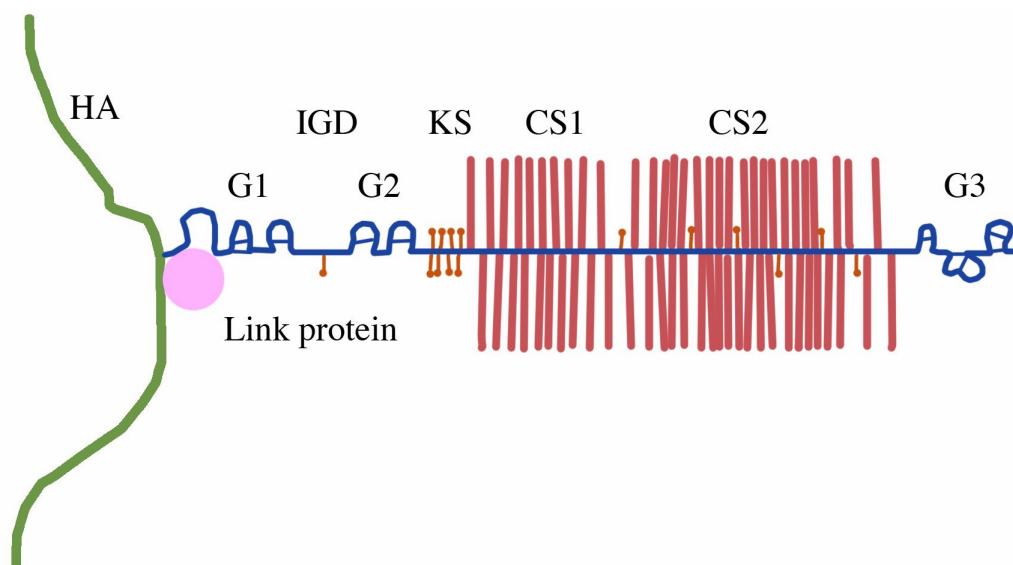


Figure 2. Molecular structure of aggrecan molecule showing its characteristic structural domains (HA: hyaluronan; IGD: interglobular domain; KS: keratan sulfate-binding region; CS1: chondroitin sulfate-binding region 1; CS2: chondroitin sulfate-binding region 2).

The IGD domain is a short extended region that separates G1 and G2 in the aggrecan molecule. It contains many different kinds of cleavage sites for proteinases, such as matrix metalloproteinases (MMPs), plasmin, leukocyte elastase and cathepsin B (Hardingham and Fosang 1995; Mort and Buttle 1997; Mort et al. 1998). Therefore, it has been suggested that MMPs and aggrecanases are involved in the turnover of aggrecan in normal and diseased cartilage (Lark et al. 1995; Singer et al. 1995; Lark et al. 1997; Kiani et al. 2002).

The G2 domain contains two PTR domains that are similar to the corresponding structure in the G1 domain and link protein, but the G2 domain does not interact with

HA and link protein (Kiani et al. 2002). Instead, the suggested function of the G2 domain is to inhibit aggrecan secretion (Kiani et al. 2001). It is known that the amino acid sequence of the G2 domain varies in different species (Antonsson et al. 1989; Doege et al. 1991).

Adjacent to the G2 there is a KS binding region containing 30-50 KS chains in mature aggrecan. The function of the KS binding domain is not clear, however, it might be involved in the tissue distribution of aggrecan (Kiani et al. 2001 and 2002).

The CS1/ CS2 domain is located between the G2 and G3 domains in the aggrecan molecule, and it is the largest domain in aggrecan molecule. It contains about 100 CS chains. Chondroitin sulfate chains are negatively charged, thus providing a hydrated, viscous gel that can absorb a compressive load (Watanabe et al. 1998; Kiani et al. 2001 and 2002).

In the C-terminal end, there is a globular G3 domain. The G3 domain is a complex region produced by alternative splicing of exons in post-transcriptional processing (Baldwin et al. 1989). It is claimed that the alternative splicing varies in different species (Kiani et al. 2002). The G3 domain contains three folded modules, the epidermal growth factor (EGF)-type module, a carbohydrate recognition domain, and a complement binding protein-type domain, as well as a short C-terminal tail (Kiani et al. 2002) with homology to some ligands, which can bind sugars like galactose and fucose (Halberg et al. 1988). The G3 domain appears to play a key role in GAG chain attachment and PG secretion (Domowicz et al. 2000). All of the above aggrecan domains play key roles in the maintenance of normal cartilage structure and function.

In OA, it is generally acknowledged that there is a net loss of PGs in articular cartilage. However, the OA induced changes in the molecular size of aggrecan are controversial with results in the literature showing either no change, a reduction, or an increase in the molecular size of aggrecan (Sweet et al. 1977; Vasani 1980; Brocklehurst et al. 1984). Furthermore, loss of PG aggregation in advanced OA has been reported (Palmski and Brandt 1976; Vasani 1980; Inerot et al. 1991). The chondroitin sulfate content and the rate of GAG synthesis have been observed to be increased in the early stages of OA when compared with changes in an age-matched control, but, the content

and synthesis rate are decreased following the progression of OA (Mankin and Lippiello 1971; Thompson Jr. and Oegema Jr. 1979).

Decorin and **biglycan** are the major small leucine-rich PGs (SLRPs) in the ECM, and decorin was originally named due to its binding on the surface of the collagen fibrils (Scott and Orford 1981). Decorin and biglycan usually contain one and two CS/dermatan sulfate (DS) chains (Roughley and Lee 1994). The sizes of their core proteins are approximately 40 kDa (Iozzo 1997). **Fibromodulin** and **lumican**, the other members of the SLRP family present in cartilage, contain KS-PG chains and have protein cores of molecular weights of 42 and 38 kDa, respectively (Plaas et al. 1990; Roughley and Lee 1994; Iozzo 1997). In decorin and biglycan, the GAG chains are O-linked to the core protein. However, KS chains in fibromodulin are N-linked ones. The core proteins of the SLRPs allow them to interact with fibrillar collagen and regulate the fibrillogenesis (Vogel et al. 1984; Hardingham and Fosang 1992; Hedbom and Heinegård 1993; Roughley and Lee 1994; Scott 1996; Roughley 2006). The binding of the SLRPs on the surface of collagens also limits the access of collagenases and, thus, they can protect the fibrils from proteolytic cleavage. Moreover, the four SLRPs have also been reported to interact with type VI, XII and XIV collagen, fibronectin and elastin, and several growth factors such as EGF, transforming growth factor β (TGF- β), and the cytokine tumor necrosis factor α (TNF α) (Roughley 2006). The SLRPs can also help modulate chondrocyte metabolism by regulating the interaction of growth factor with GAG chain (Roughley 2006). *Borrelia burgdorferi*, which causes Lyme borreliosis, adheres on decorin in the joints and skin (Brown et al. 2001). Decorin is also a ligand for the EGF receptor, activating this protein by binding to the receptor (Iozzo et al. 1999). It is internalized partly with the EGF receptor (Feugaing et al. 2007). However, there are many endocytotic pathways for decorin uptake, modulated by EGF receptor signaling (Feugaing et al. 2007). For biglycan internalization, the clathrin-mediated endocytosis appears to be a major route (Götte et al. 2004). Biglycan can modulate bone morphogenetic protein 4-induced osteoblast differentiation (Chen et al. 2004). Fibromodulin interacts with type I and II collagen through different peptides (Viola et al. 2007). In cornea, lumican can only exist in the PG form. In adult cartilage, it is present as a glycoprotein form lacking KS though in young cartilage, it is present as

KS-PG (Grover et al. 1995; Corpuz et al. 1996; Melching and Roughley 1999; Knudson and Knudson 2001).

Epiphycan is another small leucine-rich CS/DS-PG with a 35 kDa core protein (Iozzo 1997), and a primary structure similar to osteoglycin (Johnson et al. 1997). Epiphycan may participate in the chondrocyte differentiation and osteogenesis (Kurita et al. 1996; Knudson and Knudson 2001). **Versican** is another HA-binding PG found in cartilage at low levels, which has an N-terminal G1 and C-terminal G3 domain similar to that of aggrecan. It lacks the G2 domain, and its G1-domain binds HA and link protein in a different manner when compared with aggrecan (Matsumoto et al. 2003 and 2006). The G3 domain of the versican has two EGF-type repeats, one lectin-like sequence and one complement regulatory protein-type domain (Zimmermann and Ruoslahti 1989).

Cell surface proteoglycans

Syndecans and **betaglycan** are typical transmembrane CS / heparan sulfate (HS)-PGs characterized by a core protein which is composed of an extracellular domain, a single membrane-spanning domain and a short cytoplasmic domain (Cheifetz and Massague 1989; Bernfield and Sanderson 1990; Hardingham and Fosang 1992). The syndecan gene family contains four different PGs, [syndecan-1; syndecan-2 (fibroglycan); syndecan-3 (N-syndecan); and syndecan-4 (amphiglycan)], which have similar transmembrane and cytoplasmic domains, but different extracellular domains (Tkachenko et al. 2005). Syndecan regulates cell behavior by binding cells to ECM and by binding growth factors via their HS chains (Bernfield and Sanderson 1990; Bernfield et al. 1992). The expression, composition and function of each syndecan family member differ in different cells and tissues (Saunders et al. 1989; Mali et al. 1990; Kim et al. 1994; Knudson and Knudson 2001; Woods 2001). Betaglycan is a receptor in the TGF- β signaling pathway, which binds TGF- β through its core protein (Andres et al. 1992). Betaglycan has also been called the type III TGF- β receptor. The cytoplasmic domain of betaglycan is believed to have functional roles in regulating TGF- β signaling (Blobe et al. 2001). It was recently reported that betaglycan can suppress breast and prostate cancer progression (Dong et al. 2007; Turley et al. 2007).

Intracellular granule proteoglycans

Serglycin is an intracellular PG, found especially in the storage granules of connective tissue mast cells. It has several roles e.g. in packaging and regulation of the activity of proteolytic enzymes (Schick et al. 2001).

2.1.3. Glycosaminoglycans

Glycosaminoglycan (GAG) is a long, linear unbranched polysaccharide containing a repeating disaccharide unit, which consists generally of uronic acid (UA), that can be either GlcA or iduronic acid (IdoA), and hexosamine (GalNAc or GlcNAc) (Hardingham 1999). Chondroitin sulfate, DS, HS, heparin, KS and HA are the different forms of GAGs found in articular cartilage. The components of a disaccharide and the linkage to the core protein in the various GAG chains differ.

Chondroitin sulfate

Chondroitin sulfate is the predominant form of GAG in articular cartilage, where it constitutes about 80% of the total GAGs. It consists of repeating disaccharide units containing a GlcA and GalNAc (Fig. 3). Chondroitin sulfate is covalently attached to the core protein. It has several important functions; maintaining the structural integrity of the cartilage tissue, holding water and nutrients. Chondroitin sulfate chains are seldom larger than 100 kDa, when the chains are fully mature elongated. Specific enzymes add the sulfate esters onto the particular hydroxyl groups to form CS. Disaccharides are often sulfated in either positions 4 (Di-4S) or 6 (Di-6S) of GalNAc (Fig. 3). In CS chains, there can be disaccharides with a sulfate group attached to the carbon 2 of GalNAc (Di-2S), or non-sulfated disaccharides (Di-0S), which have no sulfate group.

The content of the CS in human articular cartilage differs with age. The Di-6S content of the cartilage increases with growth and development up to the age of 20 years, whereas the Di-4S content decreases after birth until the age of 20 years (Bayliss et al. 1999). In mature articular cartilage, the Di-6S content is much greater than that of Di-4S (Bayliss et al. 1999). During the years between 20-85 of human age, there are only minor changes in the Di-6S and the Di-4S contents, while the Di-0S content of the cartilage remains at a relatively constant low level throughout life (Bayliss et al. 1999).

It has been reported that the concentration of the Di-4S increases significantly in the hip joint of OA patients (Mankin and Lippiello 1971).

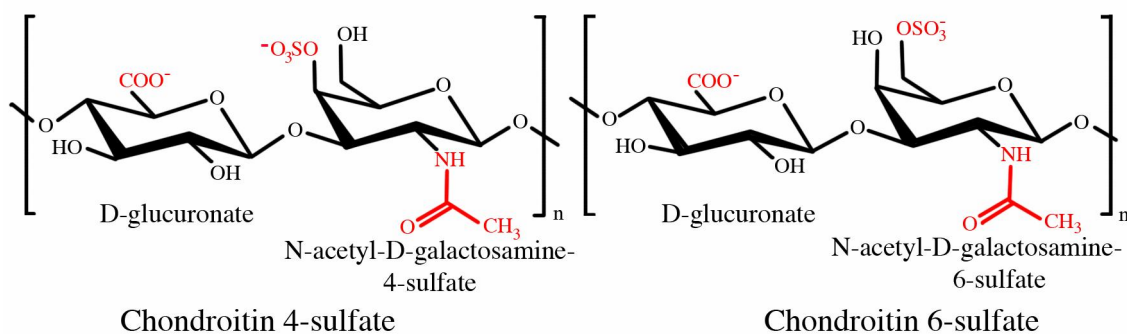


Figure 3. Molecular structure of chondroitin 4-sulfate and chondroitin 6-sulfate.

Previous studies have shown that similarly to GS, CS can be absorbed from the gastrointestinal tract in rats, dogs and also humans (Setnikar et al. 1986; Ronca et al. 1998). Furthermore, oral administration of CS has been shown to elevate PG synthesis in articular cartilage (Bassleer et al. 1998b; Uebelhart et al. 1998). A low sulfate concentration in culture media leads to the synthesis of undersulfated GAG in cartilage explants (Ito et al. 1982; Brand et al. 1989; van der Kraan et al. 1989). When the content of aggrecans decreases in OA cartilage, there is also a loss of CS (Thompson Jr. and Oegema Jr. 1979).

Keratan sulfate

Keratan sulfate consists of a repeating disaccharide unit of GlcNAc and galactose with alternating (1→3) β and (1→4) β bonds instead of GlcA. There are two different forms of KS, *i.e.* KS I and II in mammalian tissues. In the large PGs of the cartilage, KS II is present, whereas KS I is the main form in cornea (Seno et al. 1965; Funderburgh 2000). These two forms display major differences, *e.g.*, in the length of their carbohydrate chain, their linkage to the core protein, their association with the CS, their sensitivity to the alkali treatment, and the degree of sulfation (Seno et al. 1965; Hascall and Riolo 1972). Their molecular weight varies in the range of 4-20 kDa (Hascall and Riolo 1972). The content of KS increases in cartilage with aging (Inerot et al. 1978). Keratan sulfate content increases with depth (Venn and Maroudas 1977; Bayliss et al. 1983) that

may be due to low oxygen tension in the deep zone of cartilage (Scott and Haigh 1988). An increased KS content is also associated with the maturation of the articular cartilage (Säämänen et al. 1987). However, the KS content has been reported to be decreased in OA (Mankin and Lippiello 1971).

Dermatan sulfate

Dermatan sulfate is the predominant GAG expressed in the skin, and it is also widely distributed in the ECM of blood vessel wall, tendon, sclera and other tissues (Fransson 1968b and 1968a; Fransson and Malmstrom 1971; Yanagishita et al. 1979; Cöster and Fransson 1981; Scott and Orford 1981; Sheehan et al. 1981; Damle et al. 1982). Dermatan sulfate consists of UA and GalNAc groups, such that the UA can be either GlcA or IdoA within the same GAG chain (Cöster and Fransson 1981). Iduronic acid can be sulfated at position C-2, and GalNAc at position C-4 or C-6 (Chatziioannidis et al. 1999). Dermatan sulfate is active in binding fibroblast growth factor-2 during wound repair and is also an important player in a variety of cellular events, such as wounding, infection, and tumorigenesis (Penc et al. 1998; Trowbridge and Gallo 2002).

Heparan sulfate

Heparan sulfate contains repeating disaccharide units of GlcNAc and GlcA/IdoA. The size of a chain is normally less than 50 kDa. Heparan sulfate is a sulfated polysaccharide covalently linked to the core protein, and it is present at the cell surface and also in ECM. Structurally, HS has been shown to display a greater number of possible variations than the other GAGs described above (Lindblom et al. 1991).

Hyaluronan

Hyaluronan, also called **hyaluronic acid**, is an unsulfated GAG component of connective tissue occurring in synovial fluid. Its function is to cushion and lubricate the synovial joint structures. Hyaluronan is also found throughout the body in plentiful amounts in many locations, such as heart valves, eyes, synovial fluid, cartilage, blood vessels, skin and the umbilical cord (Laurent and Gergely 1955; Goa and Benfield 1994).

Hyaluronan is a key component of cartilage, where it binds to other molecules, helping the cartilage to withstand the force of weight-bearing and movement of the joint. It has the simplest GAG structure, and consists of an alternating polymer of

GlcNAc and GlcA. Its molecular weight ranges from 300 kDa to 2000 kDa depending on the tissue. A number of molecules can bind to HA with noncovalent bond, including the link protein, which stabilizes the attachment of aggrecan to HA, and cell surface HA receptors, such as CD-44 (Ishida et al. 1997), which bind the chondrocyte to the HA.

2.2. Metabolism of articular cartilage proteoglycans

Proteoglycans are synthesized similarly to protein in general. Initially, a part of the nucleotide sequence of double-stranded DNA is transcribed into mRNA by the enzyme RNA polymerase. Then the core protein is synthesized from the mRNA transcript on rough endoplasmic reticulum (ER) and transferred to the Golgi complex. The glycosylation is initiated at ER or at the early Golgi by addition of xylitol to serine (Geetha-Habib et al. 1984; Vertel et al. 1993). The synthesis of PGs is completed in the Golgi by the addition of CS chains (Ratcliffe et al. 1985; Hirschberg and Snider 1987; Hirschberg et al. 1998) and sulfation (de Luca et al. 1973). After synthesis, PGs are transferred from the Golgi to the ECM, the cell surface or intracellular organelles. Hyaluronan is synthesized at the plasma membrane and transported immediately out of the cell (Prehm 1984; Prehm 2006).

It has been shown that interleukin 1- α (IL-1 α), interleukin 1- β (IL-1 β) and TNF- α can all inhibit PG biosynthesis and increase the rate of PG degradation in different tissues or chondrocyte cultures. In contrast, insulin-like growth factor 1 (IGF-1) and TGF- β have the opposite effect on PG metabolism (van de Loo et al. 1995). Matrix metalloproteinases and aggrecanases are the main enzymes involved in the degradation of aggrecan in cartilage (Tetlow et al. 2001; Nagase and Kashiwagi 2003; Visse and Nagase 2003; Struglics et al. 2006).

2.2.1. Metabolism of cartilage glycosaminoglycans

Glucose is a general precursor for cellular GAG biosynthesis. Chondrocytes take up Glc from the synovial fluid via Glc transporter family proteins. Inside the cell, glucose is converted into Glc 6-phosphate and fructose-6-phosphate. The conversion of fructose-6-phosphate to glucosamine-6-phosphate (GlcN-6-P) takes place by the enzyme

glutamine:fructose-6-phosphate aminotransferase (GFAT, Fig. 4). Glucosamine-6-P is rapidly converted into the N-acetylglucosamine-6-phosphate (GlcNAc-6-P) by acetyl-CoA:glucosamine-6-phosphate N-acetyltransferase (Acetyl-CoA: GNPAT, Fig. 4). Exogenous GlcN or GS supplemented to the cultured cells can enter this metabolic pathway by conversion into GlcN-6-P. N-Acetyl-glucosamine-6-P is further converted via N-acetylglucosamine-1-phosphate into UDP-GlcNAc, and by epimerase into UDP-GalNAc. These nucleotide-activated sugars, together with UDP-glucuronic acid (UDP-GlcA), are utilized in the assembly of GAG chains (Fig. 4). Chondroitin sulfate polysaccharide chain is composed of GlcA and GalNAc (Davidson and Meyer 1954), whereas keratan sulfate of galactose and GlcNAc (Seno et al. 1965), while HA consists of GlcA and GlcNAc (Meyer and Palmer 1934; Prehm 1983a and 1983b) (Fig. 4).

Hyaluronan is synthesized at plasma membranes (Prehm 1984), and directly released as a soluble product to the ECM (Prehm 1983a and 1983b). In the ECM, HA and aggrecan form large PG aggregates, that are stably connected with link protein. It has also been proposed that the inhibitors of HA export can prevent aggrecan loss from OA cartilage (Prehm 2005).

Normally, chondrocytes can regulate the balance of the processes of degradation and synthesis of the ECM in cartilage by secreting of a number of degradative enzymes and their inhibitors. In addition to MMPs (such as collagenases, stromelysin, and gelatinase), the degradative enzymes also include other proteinases, such as cysteine proteinases, cathepsins and serine proteinases. There are several anabolic growth factors e.g. IGF-1 and TGF- β . The enzymes secreted by the chondrocytes are released into the ECM where they begin to degrade the matrix structures. Interleukin-1 β and TNF- α can stimulate the synthesis and activation of proteolytic enzymes by chondrocytes, and both IL-1 β and TNF- α have been detected in the synovial fluid of the OA patients (Schlaak et al. 1996; Partsch et al. 1997; Horiuchi et al. 1999). The activation of these degradative enzymes is controlled by their inhibitors, such as tissue inhibitors of metalloproteinase (TIMPs) (Nagase and Brew 2003). These inhibitors function by forming complexes that inactivate the degradative enzymes. Chondrocytes are responsible for maintaining the balance between the degradative enzymes and their inhibitors. Aggrecanases that cleave the Glu373-Ala374 bond of the aggrecan core

protein play a key role in the early stages of cartilage destruction in rheumatoid arthritis and in OA (Nagase and Kashiwagi 2003). Recently, it was reported that blockade of aggrecanase cleavage in the aggrecan IGD could prevent cartilage erosion and promote cartilage repair (Little et al. 2007). In addition to be aggrecanases, MMP-3 is also thought to be an important enzyme involved in the cartilage degradation process, because it can degrade many constituents of ECM, including cartilage aggrecan, collagen II, IX, X and XI, laminin and fibronectin (Wu et al. 1991; Burrage et al. 2006). When the balance between these degradative enzymes and their inhibitors has become disturbed for any reason, such as seems to occur in OA, the enzymatic activity of MMPs can be elevated (Wang et al. 2004).

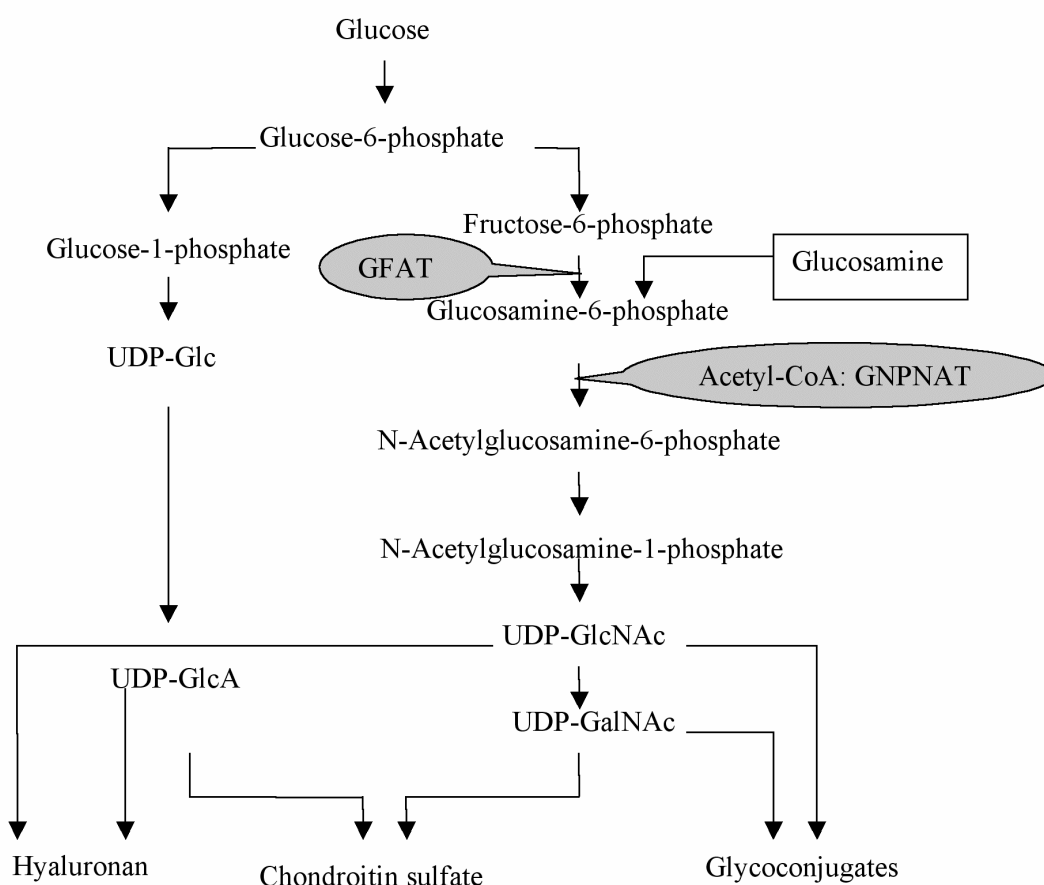


Figure 4. The biosynthetic pathway of chondroitin sulfate from glucose or glucosamine.

2.3. Changes in articular cartilage proteoglycans with aging and degeneration

Articular cartilage is subject to structural, compositional and mechanical changes with aging. Normally, the content of hexosamine does not change very much with age, although the concentration of UA has been reported to gradually decline, especially in the old cartilage (Buckwalter et al. 2005). Also, the content of hexose may increase from the birth up to the age of 35 years, from that age onwards there seems to be a gradual decline (Kosiagin 1986). The size of PG aggregates decreases due to the degradation of PGs in the ECM, and also due to the compositional alterations in aggrecan (Buckwalter et al. 1985; Thonar et al. 1986; Buckwalter et al. 1994) and link protein synthesis (Buckwalter and Rosenberg 1988; Buckwalter et al. 1994; Tang et al. 1996). With increasing age, the length of the CS-rich region of aggregating PG monomers decreases, the variability in aggrecan length increases, the content of aggrecan KS chains increases, the number of monomers per aggregate decreases, and there is a decline in the proportion of monomers that can aggregate (Buckwalter and Rosenberg 1988; Buckwalter et al. 1994). The changes are, however, different from those in human OA cartilage (Grushko et al. 1989). In OA cartilage, the content of GAGs generally decreases (Kosiagin 1986). Decorin and biglycan are molecules normally concentrated in the superficial layers of cartilage, while in OA cartilage there is a trend towards their loss from that site (Poole et al. 1996). Proteoglycan depletion from the ECM has been suggested to be a crucial component of OA (Grushko et al. 1989; Lohmander 1994). In canine experimental OA, the water content of the superficial cartilage increased by 13% with an apparent 37% decrease in the PG content and a 36% decrease in collagen content per wet weight (Guilak et al. 1994). However, no compositional changes were seen on a dry weight basis. Therefore, it was suggested that structural changes in the superficial zone of articular cartilage play a more important role in the determination of the mechanical properties than the biochemical composition (Guilak et al. 1994).

2.4. Osteoarthritis

Osteoarthritis (OA) is a group of overlapping and distinct degenerative musculoskeletal diseases that are believed to have many different etiologies. When the disease develops, it affects not only the articular cartilage, but also involves the periarticular structures of the affected joint. Ultimately, the articular cartilage degenerates with fibrillation, fissures, ulceration and full thickness loss of the whole joint surface (Brandt 2000; Felson 2004) and this usually causes pain and malfunction of the affected joint. The incidence of the disease increases with aging (Martin and Buckwalter 2002). It may affect any joint of the body, but especially the weight-bearing joints, such as hips, knees and ankles, are more susceptible to OA. The etiology of degenerative joint disease is poorly understood, although several risk factors are known, including age, obesity, and joint injury (Felson 2004).

2.4.1. Treatment of osteoarthritis

Even though there is no real cure for OA at the moment, there are many potential treatments available for the disease. A number of approaches are used for this purpose, e.g., non-pharmacological treatments including physical therapy and surgical intervention. Exercise and weight control are always the first recommendations provided to the patients. Orthopaedic surgery is one way to treat the patients with OA when the disease is particularly severe and unresponsive to the conservative treatments. More attention is being paid to the pharmacological treatment to OA, such as acetaminophen (or paracetamol) and nonsteroidal anti-inflammatory drugs (NSAIDs), which are often used to relieve the symptoms of OA (Jordan et al. 2003). NSAIDs block the cyclooxygenase enzymes and reduce the synthesis of prostaglandins. However, they can cause many different side effects including nausea, abdominal pain, diarrhea, or even gastrointestinal bleeding, liver and kidney damage, and high blood pressure. There are also other extremely serious side effects, e.g., gastrointestinal ulceration, perforation, and hemorrhage via inhibition of mucosal prostaglandin synthesis and platelet aggregation (Blower 1996). They can also cause hypertension and renal damage through inhibiting renal prostaglandin production.

Intra-articular injections of corticosteroids and HA have also been used to relieve pain and reduce inflammation in OA joints. Intra-articular corticosteroid injection can achieve benefits in reducing pain and relieving inflammation of the patients with OA, but the duration of the benefit is short-lived (Bellamy et al. 2006a). Hyaluronan has been shown to regulate many processes occurring in the synovial fluid and cartilage via an effect on matrix metabolism, chondrocyte growth and metabolism, and the regulation of the expression and activity of chondrodegradative enzymes. In addition to its symptom-modifying actions (Goldberg and Buckwalter 2005), HA could protect against PG depletion (Larsen et al. 1992; Kato et al. 1995; Bellamy et al. 2006b) and combat against the increase of IL-1 β , TNF- α , and IGF-1 mRNA expression (Noble et al. 1993). High molecular weight HA has been shown to have a structure-modifying and anti-inflammatory effect by down-regulating aggrecanase-2, TNF- α , IL-8, and inducible nitric oxide synthase in fibroblast-like synoviocytes of early stage OA patients (Wang et al. 2006).

2.4.2. Glucosamine or glucosamine sulfate treatment of osteoarthritis

The use of GlcN to treat patients with OA was proposed for more than 20 years ago since it is a natural substance and "a building block" of PGs in the joint tissues (D'Ambrosio et al. 1981). Subsequently, many studies have investigated the effects of GlcN or its sulfate salt, GS, on OA in clinical trials (Lopes Vaz 1982; Muller-Fassbender et al. 1994; Bassleer et al. 1998a; Fenton et al. 2000; Rindone et al. 2000; Reginster et al. 2001; Gouze et al. 2002; Pavelka et al. 2002; Ruane and Griffiths 2002; Bruyere et al. 2004; McAlindon et al. 2004; Persiani et al. 2007) and the mechanism of action in *in vitro* experiments (Bassleer et al. 1988 and 1998a; Sandy et al. 1998; Piperno et al. 2000; Shikhman et al. 2001; Dodge and Jimenez 2003; Largo et al. 2003; Mroz and Silbert 2003; Mroz and Silbert 2004; Poustie et al. 2004; Persiani et al. 2005; Derfoul et al. 2007)

Several possible molecular mechanisms of GlcN and its derivatives on OA treatment have been proposed (Bassleer et al. 1988 and 1998a; Piperno et al. 2000; Dodge and Jimenez 2003; Tiku et al. 2007). Glucosamine sulfate has been reported to increase PG synthesis in human OA chondrocyte cultures (Bassleer et al. 1988 and

1998a), and to increase protein synthesis and protein kinase C activity in a dose-dependent manner at a 50 μ M concentration or higher, while decreasing cellular phospholipase 2 activity (Piperno et al. 2000). Glucosamine sulfate was shown to increase mRNA levels of aggrecan while at the same time it inhibited the activity of MMP-3 in cultured human OA articular chondrocytes in a donor-dependent manner (Dodge and Jimenez 2003). Glucosamine was shown to inhibit the aggrecanase-dependent cleavage induced by interleukin-1 β or retinoic acid in rat chondrosarcoma cells and bovine cartilage explants but only at a concentration above 2 mM (Sandy et al. 1998). Anti-inflammatory effects have also been reported in the form of suppressed IL-1 β and nitric oxide production in human articular chondrocytes (Shikhman et al. 2001). A recent *in vitro* study suggested that millimolar concentrations of GlcN could prevent collagen degradation in chondrocytes by inhibiting advanced lipoxidation reaction and protein oxidation (Tiku et al. 2007). Concentration of 2 mM GlcN was reported to enhance mRNA levels of aggrecan, type II collagen and TGF- β 1 in a dose-dependent manner in cultured bovine chondrocytes (Varghese et al. 2007). Uitterlinden et al demonstrated that 5 mM GlcN and GS could reduce the anabolic and catabolic processes of bovine chondrocytes by decreasing total GAG content and these compounds could also provide protection against IL-1 β mediated ECM breakdown (Uitterlinden et al. 2007). Glucosamine at 5 mM concentration down-regulated aggrecanase-1 and MMP3, and 5 mM GS additionally down-regulated aggrecanase-2 and the expression of the tissue inhibitor of MMP gene in human OA explants (Uitterlinden et al. 2006). It is important to notice that many above-mentioned studies could show effects only at concentrations, which cannot be reached by the recommended oral doses of GS.

Two large industry-sponsored clinical trials using the joint space width and improvement of the symptoms as evaluation criteria, indicated, that GS could retard the progression of knee OA development (Reginster et al. 2001; Pavelka et al. 2002). However, the precise measurement of the radioanatomic joint space width might be affected by the presence of pain. Since pain can impair a patient's ability to fully extend the knee joint for radiography (Odding et al. 1998; Adams et al. 1999). A magnetic resonance imaging study also showed that mild to moderate joint space narrowing could

be due to the meniscal extrusion rather than hyaline cartilage erosion (Adams et al. 1999). A recent industry-sponsored clinical trial showed that 1.5 grams of GS administered once daily was more effective than placebo and improved the Lequesne score (Herrero-Beaumont et al. 2007). However, some industry-independent clinical trials have doubted the effectiveness of GS in the treatment of OA (Rindone et al. 2000; Clegg et al. 2006). Glucosamine and CS alone or in combination did not reduce pain effectively in the total group of patients with knee OA. Exploratory analyses suggested that the combination of GlcN and CS might be effective in the subgroup of patients with moderate-to-severe knee pain (Clegg et al. 2006).

Some investigations have determined the serum GlcN concentration after oral administration of 1.5 grams of commercial GS and observed that the level of GlcN was very low, approximately 10 μ M (Persiani et al. 2005; Biggee et al. 2006), and the synovial fluid concentrations were at least 500-fold lower than those reported to modify chondrocyte metabolism in tissues (Lavery et al. 2005). It was shown that exogenous GlcN did not stimulate CS synthesis, and high concentrations of GlcN could actually inhibit the synthesis of CS in mouse and human chondrocytes (Mroz and Silbert 2003 and 2004). A recent clinical study on GS/CS combined with exercise showed that the GS/CS group did not fare better than the placebo group in function, pain, or when measured by Western Ontario and McMaster University Osteoarthritis Index (WOMAC) function and pain (Messier et al. 2007). Because of poor bioavailability of GlcN after oral administration, the mechanism of action has been questioned, and it has been suggested that the elevated levels of sulfate in plasma and synovial fluid which occur after oral administration of GS may in fact mediate the main effects of GS treatment on OA patients (Hoffer et al. 2001). A Cochrane review by Towheed et al concluded that the results from the studies using the products other than Rottapharm preparations or adequate allocation concealment did not show benefit in pain and WOMAC function, while those studies evaluating the Rottapharm preparation showed that GlcN was superior to placebo (Towheed et al. 2005). Contradictory results from different clinical trials have been also obtained, depending on the different GlcN preparations, various study designs and industry involvement, giving rise to an intense debate in the literature (Reginster 2007; Reginster et al. 2007; Vlad et al. 2007).

3. AIMS OF THE STUDY

Glucosamine is a basic structural unit of CS and HA of cartilage PG aggregates. Therefore, oral administration of GlcN, GS or CS, either alone or in combination have been widely used as nutraceuticals to treat OA, and they are also prescribed as drugs in some European countries. It has been suggested that in addition to having a symptom-modifying effect they are also structure-modifying agents, even though a number of recent clinical trials have not convincingly shown that either GlcN or GS would be more effective than placebo in the treatment of OA.

The aims of the study were:

1. To determine the sulfation degree of bovine and human articular cartilage CS in normal and early OA cartilage by electrophoretic separation of the CS disaccharides.
2. To analyze the effect of GlcN and GS on the GAG biosynthesis, and on the aggrecan and HAS mRNA levels by [³⁵S]sulfate incorporation, Northern blotting analysis and quantitative real time reverse transcription polymerase chain reaction (QRT-PCR), respectively.
3. To investigate the intracellular UDP-hexoses (UDP-Hex), UDP-N-acetylhexosamines (UDP-HexN) and UDP-glucuronic acid (UDP-GlcA) levels after GlcN and GS treatments by reversed-phase high-performance liquid-chromatography and ionization-electrospray mass spectrometry measurements.

4. MATERIALS AND METHODS

4.1. Study materials

4.1.1. Human samples

Full-depth human articular cartilage specimens (diameter 19 mm; age of donors 26-78 years, mean age 55 years) from femoral lateral and medial condyle (FLC, n=13; FMC, n=12), tibial lateral and medial plateau (TLP, n=12; TMP, n=13), femoral groove (FG, n=13), and patella (PAT, n=11) of cadaver joints were used for biochemical and histological analyses. The healthy and degenerated cartilage samples were scored according to the Mankin scoring method (Mankin et al. 1971). Human samples were taken with the permission of the Finnish National Authority for Medicolegal Affairs (TEO, 1781132/32/200/01).

4.1.2. Bovine samples

Full-depth healthy and degenerated articular cartilage specimens (diameter 19 mm, n=32) from the lateral facet of bovine patellae (age of animals 1-3 years) were used for biochemical and histological studies. Primary chondrocytes were isolated from the femoral condyles of 13 to 22 month-old healthy cows (n=25). Bovine joints were provided by the local abattoir (Atria, corp.)

4.2. Cell culture

Primary chondrocytes were isolated from the articular cartilage of bovine femoral condyles. Cartilage pieces were incubated with 0.5 mg/ml of hyaluronidase in serum-free Dulbecco's Modified Eagle Medium (DMEM), supplemented with 250 µg/ml fungizone, and 10 mg/ml gentamycin in an incubator at 37°C with 5% CO₂ for 30 minutes. Digestion was then continued overnight with 0.3 mg/ml of collagenase and 0.2 mg/ml of DNase in DMEM supplemented with 1% fetal calf serum, 250 µg/ml

fungizone, 0.5 µg/ml of ascorbic acid, and 10 mg/ml gentamycin. Next morning, the isolated chondrocytes were washed twice with phosphate-buffered saline (PBS), counted and plated in monolayer cultures at 1×10^6 or 0.5×10^6 chondrocytes per well of a 6-well plate or a 6 cm diameter Petri dish. Cultures were maintained in DMEM culture medium with high- (25 mM) or low- Glc (5.5 mM) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine, and maintained in a humidified incubator at 37°C with 5% CO₂ until the cells reached confluence.

4.2.1. Treatment of chondrocytes with different sugars

The cells were treated with 1 mM of GlcN, galactosamine (GalN), mannosamine (ManN), GlcN 3-sulfate, GlcN 6-sulfate or GalN 6-sulfate for 0, 4, 8 and 24 h when the cells reached confluence after an 8-day-long cell culture.

To examine the effect of GS salt on PG synthesis, the chondrocytes were treated with 0, 10, 100 µM and 1 mM of Glc or GS salt on 6 well-plate for 24 h following 8-day-long cell culture. Glucosamine sulfate salt solution was prepared by adding equal molar concentrations of GlcN and sodium sulfate to the DMEM medium including the supplements described above.

The chondrocytes were also treated with different concentrations (10, 100 µM and 1 mM) of Glc, GlcN or GS for 10, 20, 30, 60 and 120 min following 2- or 8-day-long cell culture to analyze the intracellular UDP-sugar levels.

4.3. Histological analyses

The human (n=74) or bovine (n=32) samples were fixed in 4% (w/v) formaldehyde in 0.07 M sodium phosphate buffer, pH 7.0, for 48 hours at 4°C. After decalcification with 10% EDTA in 4% (w/v) phosphate-buffered formaldehyde for 12 days, microscopic sections (3-µm-thick) were prepared and cartilage degeneration was histologically graded using the Mankin scoring method (Mankin et al. 1971). Degeneration was independently quantified by three investigators from blind-coded samples, and the final Mankin score was calculated as the mean of those three scores. For some statistical

analyses, the samples were divided into three groups according to their Mankin score (Human: Group I, score 0 - 1; Group II, score 2 - 3; Group III, score 3 - 10; Bovine: Group I, score 0; Group II, score 1 - 3; Group III, score 3 - 10).

4.4. Biochemical Analyses

4.4.1. Quantitative and qualitative analyses of proteoglycans

The cartilage tissue was detached from the bone, immersed in PBS, and the wet weight was measured. Subsequently, the specimens were extensively freeze-dried to determine the dry weight of the tissue, and the water content was calculated from this information. The dried bovine cartilage samples were moisturized, cut into small pieces, and PGs were extracted at 4°C for 30 hours with guanidinium hydrochloride in 50 mM sodium acetate, pH 5.8, containing 10 mM EDTA, 100 mM ϵ -amino-n-caproic acid, and 5 mM benzamidine-HCl (Sajdera and Hascall 1969). The extract of each sample was collected, and the residual material was washed with PBS. The non-extractable fraction of the cartilage was digested for 24 hours at 60°C with 0.05% proteinase K in 10 mM EDTA and 100 mM sodium phosphate buffer (pH 7.4). As the PGs in human articular cartilage are poorly extractable compared with most other species, the dried samples were directly solubilized by digestion with 1 mg/ml papain in 150 mM sodium acetate, 50 mM Cys-HCl and 5 mM EDTA, pH 6.5, for 24 hours at 60°C. Uronic acid contents of the digests were quantitated from the ethanol-precipitated samples (Blumenkrantz and Asboe-Hansen 1973), and used to give an estimate of the PG content. The sum of uronic acid contents of the extract and residual tissue was used in calculations on bovine samples.

To evaluate the effect of different forms of hexosamines, GS salt and Glc on aggrecan mRNA expression, the treated samples were collected with Eurozol. Total RNA was extracted with chloroform and precipitated from the aqueous phase with isopropanol. The concentration of RNA was determined by spectrophotometric measurement at 260 nm.

4.4.2. Agarose gel electrophoresis (I)

Safranin O assay (Lammi and Tammi 1988) was used to confirm whether the precipitation resulted in an equal yield of the PGs in the precipitate. The extracted PGs (5 µg of UA) were precipitated in 75% ethanol overnight at 4°C, and dissolved in 100 mM Tris-sodium phosphate buffer. The PGs were electrophoresed in 1.2% agarose gel (Säämänen et al. 1988), and the gels were stained with toluidine blue.

4.4.3. Fluorophore-assisted carbohydrate electrophoresis (I, II)

The extracted PGs (5-10 µg UA, bovine samples) or papain digests (human samples) were precipitated in absolute ethanol. The samples were incubated with chondroitinase ABC (1 mU/µl) at 37°C overnight, the formed disaccharides and standards were fluorescently-labelled with 2-aminoacridone and separated on 30% vertical polyacrylamide gel with 1.5% stacking gel in 0.1 M Tris-borate running buffer, pH 8.9. The running time was 50-60 minutes at 700 V at room temperature (Inkinen et al. 1999). Different disaccharides generated during the enzymatic digestion are equivalently labeled during derivatization, and the linear behaviour of analysis has been demonstrated previously (Calabro et al. 2000). The gel was photographed under ultraviolet light, and the optical densities of digitized lanes were analysed to obtain an estimate of the molar proportions of the CS disaccharide isoforms with the ImageJ software (NIH).

4.4.4. Northern blot analysis (III)

Total RNA (\approx 20 µg) was separated on a 1.0% agarose/formaldehyde gel, and stained with ethidium bromide to confirm RNA integrity. RNA was transferred to a nylon membrane by standard blotting techniques (Nemeth et al. 1989). [α -³²P] dCTP-labeled cDNA probes for human aggrecan (Glumoff et al. 1994), procollagen(α_1)II (Aigner et al. 1992), bovine HAS-2 (Usui et al. 2000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al. 1985) and 28S ribosomal RNA (Iruela-Arispe et al. 1991) were hybridized with the membranes overnight at 42°C. After hybridisation, the membranes were washed twice at low stringency (2x SSC, 0.1% SDS for 5 min) and twice at high stringency (0.1x SSC, 0.1% SDS for 15 min at 42°C). Expressions of the

specific genes were normalized to the reference mRNA expression of 28S ribosomal RNA, because the expression of GAPDH as an enzyme acting on carbohydrate metabolism might be altered in response to the added sugars. The experiments were repeated in samples from 13 animals.

4.4.5. Quantitative real time reverse transcription polymerase chain reaction (IV)

Total RNA (10 µg) was treated with recombinant DNase I. The purity and integrity of RNA was determined by spectrophotometry, and by gel electrophoresis before reverse transcription (RT). For 20 µl of RT reaction, 0.5 µg of DNA-free total RNA from each group, random primers, and AbsoluteTM MAX QRTase Blend reverse transcriptase were used. The RT reaction was incubated for 60 min at 42°C, for 10 min at 75°C, and for 2 min at 4°C in a MJ Research PTC-200 device (Waltham, Massachusetts, USA). The cDNA was stored at -20°C prior to use.

The levels of mRNA encoding for bovine aggrecan and GAPDH were quantified with QRT-PCR employing Mx3000PTM Real-Time PCR System (Stratagene, La Jolla, CA, USA). The 25 µl of RT-PCR reaction contained 4 µl of cDNA, 12.5 µl of AbsoluteTM QPCR SYBR Green Mix, 0.5 µl of ROX reference dye, and 300 nM (GAPDH) or 100 nM (aggrecan) forward and reverse primers. The conditions of real time RT-PCR were: a single cycle of enzyme activation for 15 min at 95°C, followed by 40 amplification cycles for 30 s at 95°C denaturation, 1 min at 60°C annealing, and 30 s at 72°C extension. The sequences of the primers used in QRT-PCR were as follows: GAPDH forward primer: 5' TTC AAC GGC ACA GTC AAG G 3', reverse primer: 5'ACA TAC TCA GCA CCA GCA TCA C 3'; aggrecan forward primer: 5' CAC TGT TAC CGC CAC TTC CC 3', reverse primer: 5'GAC ATC GTT CCA CTC GCC CT 3'. The experiments were repeated in samples from 12 animals.

4.4.6. [³⁵S]sulfate incorporation analysis (III, IV)

Analysis of sulfate incorporation rate was used to investigate the effects of GS and glucose on GAG synthesis. When the cells reached confluency, the medium was changed with fresh media containing different concentrations (100 µM and 1 mM) of glucose or GS salt. [³⁵S]sulfate (5 µCi/ml) was added into each of the 6-well plates, and

the cultures were incubated in a humidified incubator at 37°C with 5% CO₂ for 24 and 72 h. The supernatant was collected, and the incorporated and the free [³⁵S]sulfate were analyzed after gel filtration separation on Sephadex G-25 (PD-10 columns). The experiments on the effect of GS on GAG synthesis were repeated 25 times from the cells of 25 animals. To test whether Glc concentration of the regular medium would affect the capacity of different hexosamines to increase [³⁵S]sulfate incorporation, the experiments with GlcN, GalN, ManN, and GlcN 3-sulfate were performed in low- (5.5 mM) and high-Glc (25 mM) DMEM simultaneously. The experiments were repeated four times from the cells of four animals. The increase of sulfate concentration in the media caused by the addition of GS was taken into account when the results were calculated on a molar basis.

4.4.7. Reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry (IV)

After treatment, the plates were placed on ice, and the mixtures of the cells and medium were collected into 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 380xg (2000 rpm) for 5 min at 4°C, and the pellets were washed once with ice-cold 1xPBS. The cell pellet was extracted with 300 µl of cold acetonitrile with a subsequent addition of 200 µl of cold H₂O within 2 min. The samples were mixed with a vortex, and then centrifuged at 16060 xg (13000 rpm) for 1 min at 4°C. The supernatant was transferred to a new tube, and stored frozen at -20°C for later analysis. The acetonitrile was vaporized using a vacuum centrifuge and the sample dissolved by vortex mixing in 150 µl of Milli-Q water containing 5 µM *N,N*-methyladenosine 5'-triphosphate (AppCp) as an internal standard prior to analysis. The levels of UDP-Hex, UDP-HexN and UDP-GlcA were analyzed by using reversed-phase column and a Finnigan LTQ quadrupole ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) equipped with an electrospray ionization source (IV).

4.5. Statistical methods

Kruskal-Wallis H- and post-hoc tests were used for testing the significance of differences in biochemical parameters between the untreated and treated groups (**I**, **II**). A non-parametric K-independent test was used to perform statistical analysis for mRNA and [³⁵S]sulfate incorporation data (**III**). Non-parametric Wilcoxon Signed Ranks Test was used to test the significance of differences between the control and treated groups in UDP-sugar analyses (**IV**). A p-value less than 0.05 was considered as statistically significant. SPSS software (SPSS Inc., Chicago, IL) was used for statistical analysis.

5. RESULTS

5.1. Sulfation of chondroitin sulfate disaccharide isoform in bovine and human samples (I, II)

Chondroitin sulfate disaccharide isoforms were analyzed to test the hypothesis that sulfate deficiency was involved in the progression of bovine and human OA. The Mankin scoring method was used to categorize bovine and human samples into three groups according to their stage of OA degeneration.

Bovine samples were prepared from the lateral facet of patellae (n=32). In group I (n=11), the cartilage looked healthy, with a smooth and shiny surface and no evidence of superficial degeneration (Mankin score 0); in Group II (n=11), the cartilage had minor changes in the integrity of superficial cartilage and matrix stainability (Mankin score 1 - 3); and in Group III (n=10), the cartilage displayed clear OA changes (Mankin score 3 - 10).

Human samples (n=74) were prepared from femoral lateral and medial condyles (FLC, n=13; FMC, n=12), tibial lateral and medial plateaux (TLP, n=12; TMP, n=13), femoral groove (FG, n=13), and patellae (PAT, n=11). In Group I, there were 19 samples with a healthy, smooth and shiny surface, no evidence of superficial degeneration (Mankin score 0 - 1); in Group II, there were 33 samples with minor changes in the integrity of superficial cartilage and matrix stainability (Mankin score 2 - 3); and in Group III, there were 22 samples with clear OA changes (Mankin score 3 - 10).

5.1.1. Water content and proteoglycan content

Water content increased and PG content decreased following the development of OA in bovine and human samples (Table 1). The total UA content represents the PG content.

Table 1. Water (% , mean \pm SD) and uronic acid (UA) content ($\mu\text{g}/\text{mg}$ wet weight, mean \pm SD) of bovine and human cartilage samples according to the severity of OA

Group	Bovine			Human		
	Mankin Score	Water content (%)	UA content ($\mu\text{g}/\text{mg}$)	Mankin Score	Water content (%)	UA content ($\mu\text{g}/\text{mg}$)
I	0 - 1	79.9 \pm 2.4	10.2 \pm 3.5	0	70.7 \pm 3.8	7.0 \pm 1.0
II	2 - 3	81.6 \pm 1.2	6.7 \pm 1.5	1 - 3	72.0 \pm 3.5	6.8 \pm 2.0
III	3 - 10	84.2 \pm 2.6 ^{a)}	4.1 \pm 1.2 ^{a,b)}	3 - 10	74.7 \pm 5.2 ^{a)}	4.7 \pm 1.7 ^{a,b)}

Kruskall-Wallis post-hoc test: $p < 0.05$, ^{a)}: Group I vs Group III; ^{b)}: Group II vs Group III.

5.1.2. Proteoglycan structure and chondroitin sulfate disaccharide isoform analysis in bovine and human sample (I, II)

Agarose gel electrophoresis was used to analyze the PG structure in bovine samples. Two major aggrecan bands were observed from the samples in Group I, while the heterogeneity of the extracted cartilage PG was increased following the progression of OA, evaluated by the Mankin score of cartilage (I, Fig.1).

Monosulfated Di-6S and Di-4S disaccharides were the most abundant isoforms in bovine (I, Table 2) and human (II, Table 1) samples. The non-sulfated Di-0S isoform accounted for about 2-8% of the total disaccharide content in bovine samples (Table 2), and about 1% in human samples (Table 2). The relative amount of Di-0S was significantly higher in group I than in groups II and III in bovine samples (Table 2). No changes were found in the relative amount of Di-0S in human samples (Table 2).

The biosynthesis rate of PGs is known to vary in a site-dependent manner even within the same joint (Parkkinen et al. 1990) and, theoretically, this could lead to a limited availability of the substrates needed for GAG synthesis in the most active sites of articular cartilage. Therefore, a site-specific correlation between Di-0S and Mankin scores or UA content was analyzed with human samples. The correlation between the relative amount of Di-0S and Mankin scores was significant only in the medial femoral condyle area. However, Kruskal-Wallis H- and post-hoc test revealed that showed there

were no statistically significant differences in the percentage of Di-OS between the different sample sites. Furthermore, no significant correlations were observed in the comparison between Di-OS and total UA content per wet weight.

Table 2. The proportion of non-sulfated chondroitin sulfate (Di-OS) of bovine and human cartilage samples according to the severity of OA (% , mean \pm SD).

Group	Bovine		Human	
	Mankin score	Di-OS (%)	Mankin score	Di-OS (%)
I	0 - 1	8.1 \pm 3.4	0	1.2 \pm 1.5
II	2 - 3	2.9 \pm 2.9 ^{a)}	1 - 3	1.0 \pm 1.0
III	3 - 10	1.5 \pm 0.8 ^{b)}	3 - 10	1.1 \pm 1.2

Kruskall-Wallis post-hoc test: $p < 0.05$, ^{a)}: Group I vs Group II; ^{b)}: Group I vs Group III

5.2. Effect of glucosamine and glucosamine sulfate on aggrecan and hyaluronan synthase mRNA expression and [³⁵S]sulfate incorporation (III, IV)

The effect of GlcN and GS on chondrocyte GAG synthesis was studied at concentrations from 10 μ M to 1 mM to compare the physiologically available level (10 μ M) to a non-physiologically high level (1 mM) often used in the previous studies.

5.2.1 Effect of different forms of sulfated and non-sulfated hexosamines on aggrecan mRNA expression

The phenotype of cultured cells at the end of the experiment was typical for the chondrocytes since they expressed abundant levels of procollagen (α_1) II and aggrecan mRNAs, i.e. typical of chondrocytes. Northern blot analysis of the steady-state levels of aggrecan mRNA expression detected no remarkable changes in its expression levels after the chondrocytes were treated with 1 mM GlcN, GalN, ManN, GlcN-3S, GlcN-6S, GalN-6S in high-Glc DMEM for 24 h (III, Fig.1). The average of aggrecan band intensities of 13 parallel experiments after normalization against 28S ribosomal RNA in Northern blot analysis revealed that GS salt in high-Glc DMEM did not increase aggrecan mRNA levels (Table 3). Furthermore, parallel experiments with the cells from

12 individual animals showed that 10 μ M and 1 mM GS in low-Glc DMEM did not increase aggrecan mRNA expression after normalization against GAPDH by QRT-PCR (Table 3).

Table 3. Effect of GS on aggrecan mRNA expression evaluated by Northern blot and QRT-PCR (% , mean \pm SD).

GS-treatment	Relative aggrecan mRNA expression in high-Glc DMEM, Northern blot, n = 13	Relative aggrecan mRNA expression in low-Glc DMEM, QRT-PCR, n =12
Control	100 \pm 0.0	100 \pm 11.0
10 μ M	not determined	104.1 \pm 13.3
100 μ M	108.3 \pm 39.2	not determined
1 mM	114.1 \pm 46.2	114.6 \pm 14.1

Bovine primary chondrocytes were treated with 0, 10, 100 μ M and 1 mM GS in high- or low-glucose DMEM for 24 h. The difference between the control (untreated group) and treated group were evaluated with nonparametric Wilcoxon Signed Ranks Test. No significant changes were observed.

Chondrocytes from 13 individual animals were also treated for 24 h with different concentrations (0, 100 μ M and 1 mM) of Glc added to high Glc DMEM, and the isolated RNAs were analyzed with the probes for aggrecan (**III**, Fig. 2A). Statistical analysis of densitometric data after normalization against 28S ribosomal RNA revealed that Glc did not increase the steady-state level of aggrecan mRNA (**III**, Fig. 2B).

5.2.2 Effect of glucosamine sulfate and glucose on hyaluronan synthase mRNA expression

In Northern blot analysis, the expressions of both HAS-1 and 3 were below the detection level, only the expression of HAS-2 was detectable in three isolations out of thirteen. In one of these, an increase in HAS-2 level was detected when the cells were treated with 100 μ M GS. However, the average of HAS-2 band intensities of three parallel experiments after normalization against 28S ribosomal RNA showed that GS salt (0, 10, 50, 100, 500 μ M and 1 mM) did not increase HAS-2 mRNA levels (**III**, Fig. 3). Furthermore, Glc did not increase the steady-state level of HAS-2 mRNA in these three individual animals.

5.2.3. Effect of different forms of sulfated and non-sulfated hexosamines on glycosaminoglycan synthesis

[³⁵S]sulfate incorporation analysis showed that GAG synthesis in the presence of 1 mM concentration of GlcN, GalN, ManN, and GlcN-3S remained approximately at the control level. High Glc concentration in DMEM might prevent the stimulatory effect of hexosamines. We therefore performed [³⁵S]sulfate incorporation assays for samples cultivated in the presence of hexosamines in low- Glc DMEM. It was observed that in high- Glc medium the presence of 1 mM hexosamine concentration did not change GAG synthesis, while in low- Glc medium, ManN significantly inhibited [³⁵S]sulfate incorporation (**III**, Table 1). The experiments were repeated with the cells from four individual animals.

[³⁵S]sulfate incorporation analysis with the cells from 13 individual animals indicated that none of the GS salt or Glc concentrations increased chondrocyte GAG synthesis after the chondrocytes were cultured with GS or Glc in high- Glc DMEM for 24 h (**III**, Table 2). Furthermore, no increase was visible in GAG synthesis even after the chondrocytes from 10 cell isolates were treated with 100 μM and 1 mM GS or Glc for 72 h (**III**, Table 2). Serum may influence the uptake of the sugars, and could have had a profound effect on transcription. Therefore, the cells were also treated with 100 μM and 1 mM GS in serum-free DMEM for 24 h. Three parallel experiments were conducted but the results clearly showed that GS did not increase GAG synthesis in serum-free conditions (**III**, Table 2). No change was found in the GAG synthesis from [³⁵S]sulfate incorporation analysis with the cells from 12 individual animals after the cells were treated with 10 μM and 1 mM GS in low-Glc DMEM for 24 h (**IV**, Table 1). Furthermore, Glc did not increase GAG synthesis after the cells were treated with 100 μM and 1 mM GS in high- Glc DMEM for 24 h or even 72 h (**III**, Table 2).

5.3 Effect of glucosamine, glucosamine sulfate and glucose on intracellular UDP-hexoses, UDP-glucuronic acid and UDP-N-acetylhexosamines

The effect of GlcN and GS on the intracellular UDP-sugar levels at different concentrations was examined, both in low- and high- Glc DMEM. Low Glc medium corresponds to the level of Glc in normal serum and synovial fluid (Tercic and Bozic 2001). The content of UDP-HexN increased after 10-min-treatment at 1 mM GlcN (**IV**, Fig. 2A) and GS (**IV**, Fig. 2B) in low- Glc DMEM, while the content of UDP-GlcA slightly decreased. Similar changes were found in the ratio of UDP-HexN/UDP-Hex and UDP-GlcA/UDP-Hex. However, no changes were observed at 10 min after addition of 10 and 100 μ M GlcN and GS (**IV**, Fig. 2A, B). In high- Glc DMEM, 1 mM GS did not affect the UDP-sugar levels. Glucose at 10, 100 μ M and 1 mM concentrations in low-Glc DMEM had no effect on UDP-sugar levels. Consequently, the following experiments were performed only in low-Glc DMEM.

The levels of UDP-sugars over a time period of 2 h were also studied. The highest level of UDP-HexN was reached at 30 min after addition of 1 mM GS (**IV**, Fig. 3A). Although the absolute level of UDP-HexN began to decrease after 30 min, the UDP-Hex level also declined such that the ratio of UDP-HexN/UDP-Hex showed an increase during the time course with 1 mM GS treatment. However, the level of UDP-GlcA still remained at the control level. Previously, the maximum level of GlcN in serum was found to lie in a range of 1.9-11.5 μ M after oral administration of GS (McAlindon and Biggee 2005). Therefore, we treated the chondrocytes with 10 μ M GS in low-Glc DMEM for 30 and 60 min to investigate whether an exogenous but physiologically relevant concentration of GS could increase the intracellular UDP-HexN and UDP-GlcA levels. The experiment was repeated with chondrocyte cultures from nine animals. However, no changes were noted in the contents of UDP-HexN and UDP-GlcA at the different time points (**IV**, Fig. 3B).

Primary chondrocytes are often cultivated for several days prior to the experimental procedures and this may affect the metabolic balance of the cells. Thus, we investigated whether the duration of the culture time would have any impact on the

response of the chondrocytes to GS. The cells were treated with 10 μ M and 1 mM GS for 30 min after 2 and 8 days culture. After the 2-day-culture period, the content of UDP-HexN increased at 30 min after addition of 1 mM GS, while the contents of UDP-GlcA and UDP-Hex decreased ($p < 0.05$) (**IV**, Fig. 4A). The ratio of UDP-HexN/UDP-Hex was significantly higher in 1 mM GS treatment for 30 min compared with that of the controls, while the ratio of UDP-GlcA/UDP-Hex was significantly decreased ($p < 0.05$). However, treatment with 10 μ M GS for 30 min did not affect the contents of UDP-sugars (**IV**, Fig. 4A).

After culturing the chondrocytes for 8 days, the content of UDP-HexN was significantly increased at 30 min after addition of 1 mM GS (**IV**, Fig. 4B), but no changes could be found in the content of UDP-GlcA (**IV**, Fig. 4B). Similarly to the 2-day-culture period, addition of 10 μ M GS did not have any effect on the UDP-sugar levels of chondrocytes (**IV**, Fig. 4B). The level of UDP-GlcA clearly increased during the longer culture period and, as a consequence, the ratios of the UDP-HexN/UDP-Hex and UDP-GlcA/UDP-Hex were much higher after the 8-day-culture period than those observed after the 2-day-culture period.

6. DISCUSSION

6.1. Articular cartilage proteoglycans are not undersulfated in bovine and human osteoarthritis (I, II)

Glucosamine, GS and/or CS have been used to treat OA patients as chondroprotective drugs to reduce the pain and to improve the function of the affected joints (Rovetta 1991; Bassleer et al. 1992; Clegg et al. 2006). These substances have been claimed to stimulate the PG production in human articular chondrocytes (Bassleer et al. 1998a; Piperno et al. 2000). However, it has been demonstrated that the plasma level of GS lay in a range of 1.9-11.5 μM after a 1.5g daily dosage (Persiani et al. 2005). *In vitro*, only at a concentration of 50 μM or higher has GS been found to increase PG core protein synthesis and protein kinase C activities, while reducing the phospholipase A2 activity (Bassleer et al. 1998a; Piperno et al. 2000). Glucosamine sulfate has also been reported to increase the expression of mRNA encoding the type II IL-1 β receptor (Gouze et al. 2002).

Recent experiments performed with [^3H]-GlcN for metabolic labeling of CS indicated that exogenous GlcN did not stimulate CS synthesis at the concentration present around the cartilage after oral administration of normal doses of this compound, or even up to 1 mM (Mroz and Silbert 2003 and 2004). It is well known that GlcN is not a uniform substrate for GAG synthesis in the cartilage, and it can't be used directly for GAG synthesis without the UDP-derivatization (Silbert and Sugumaran 2002). It has also been suggested that in treating OA patients that sulfate could mediate the therapeutic effect of GS rather than GlcN alone on account of increased sulfate concentration in blood and synovial fluid after oral administration (Hoffer et al. 2001). Previously, the serum levels of Di-4S and Di-0S were observed to be increased in OA patients compared with the healthy individuals. However, in synovial fluid, Di-0S was not detectable (Uesaka et al. 2001). Nonetheless, Di-0S has been shown to be present in human (Bayliss et al. 1999) and equine (Brown et al. 1998) articular cartilage PGs. With respect to horses, it was concluded that synovial fluid CS was not indicative of cartilage

CS, and it rather represented turnover products of a subpopulation of PGs within the matrix (Brown et al. 1998). The same conclusion appears to be valid for human synovial fluid, although little is known about the degree of sulfation of human synovial fluid CS. Recently, the possibility that blood sulfate levels could contribute to OA by decreasing cartilage chondroitin sulfation was investigated (Blinn et al. 2006). That study indicated that fasting and ingestion of protein-free meals could lead to a decline in the concentration of sulfate in serum (Blinn et al. 2006).

In normal bovine articular cartilage, Di-OS represents a much higher proportion of CS isoforms than in humans, while in early OA cartilage in cows a statistically significant decrease in Di-OS was evident. This indicated that the degree of sulfation was increased in bovine OA cartilage (**I**). In macroscopically normal human articular cartilage CS, the proportion of Di-OS was only $1.2 \pm 1.5\%$ (**II**) compared with $8.1 \pm 3.4\%$ (**I**) present in the normal bovine CS. Even if the proportion of Di-OS would increase considerably in human cartilage, it is very unlikely that it would dramatically affect the water-binding capacity of PGs and, thus, worsen its functional quality (**II**).

A deficiency in the amount of synovial fluid reaching the articular cartilage may explain the possible sulfate deficiency. Under such conditions, also the availability of other nutrients would be decreased, which would slow down the cartilage metabolism. However, there are also conditions when a sulfate deficiency could develop without any change in the availability of other nutrients into the synovial fluid. Several nonsteroidal anti-inflammatory drugs, such as acetaminophen (or paracetamol) and ibuprofen, are used to alleviate the symptoms of OA. Some of these drugs can evoke a decrease in the serum sulfate level (de Vries et al. 1990). Recently, it was shown that the serum sulfate level can decrease in spite of the simultaneous increase in serum Glc after fasting and ingestion of 75 g Glc (Blinn et al. 2006). These are some situations which may in principle predispose the articular cartilage to synthesis of undersulfated GAGs, i.e. a low sulfate concentration in the medium would lead to the synthesis of undersulfated GAGs (Brand et al. 1989; van der Kraan et al. 1989). There are known to be species-specific responses in the sensitivity to sulfate deficiency, for example, murine cartilage is less sensitive to sulfate deficiency than its bovine counterpart (van der Kraan et al.

1989), while human articular cartilage appears to be rather susceptible to deviations from physiological sulfate concentration (van der Kraan et al. 1990). The medication history of our patients was not known. However, the very low level of Di-OS in human articular cartilage observed in this study suggests that under normal and OA conditions, the sulfate levels of the synovial fluid were sufficiently high to ensure the synthesis of normal GAGs.

6.2. Glucosamine, glucosamine sulfate and glucose do not increase the steady-state level of aggrecan and hyaluronan synthase mRNA expression and glycosaminoglycan synthesis (III)

Aggrecan is a large CS-PG in the cartilage providing osmotic resistance for the cartilage to absorb compressive loads. Chondroitin sulfate is a major constituent of GAGs, while GlcN is a major building block of the CS disaccharide units in cartilage. The suggested positive effect of GS on OA has been associated with the involvement of GlcN in GAG synthesis. However, there are some recent reports that have questioned whether a higher synthesis rate of GAGs can be achieved after oral administration of GlcN or GS (Mroz and Silbert 2003 and 2004). Certain industry-independent clinical trials with GlcN or GS have shown that GlcN or GS is no better than placebo in the treatment of OA (Rindone et al. 2000; Hughes and Carr 2002; McAlindon 2003; Cibere et al. 2004; McAlindon et al. 2004). One trial was discontinued because there were no evident symptomatic benefits of GS (Cibere et al. 2004). Weak research design, the many different GS or GlcN preparations used, and industry bias have confused the evaluation of the results of clinical trials (Distler and Anguelouch 2006; Reginster 2007; Reginster et al. 2007; Vlad et al. 2007), leading to conclusion in some recent studies that GS or GlcN may not be effective in the treatment of OA (Vlad et al. 2007).

We investigated whether exogenous administration of various concentrations of hexosamine and GS salt would increase the steady-state level of aggrecan and HAS mRNA expression, and GAG synthesis, in bovine primary chondrocytes. However, our study could detect no positive responses from these compounds (III).

Our finding that 1 mM hexosamines or 100 μ M and 1 mM GS, GlcN or Glc did not increase the aggrecan mRNA expression or the GAG synthesis in bovine primary chondrocytes is in line with the studies performed with MC615 mouse (Mroz and Silbert 2003) and human chondrocytes (Mroz and Silbert 2004). A concentration of 1 mM GlcN appeared to inhibit GAG synthesis in human chondrocytes (Mroz and Silbert 2004). A donor-dependent variation in the response of primary chondrocytes to GS has been previously reported (Dodge and Jimenez 2003). Therefore, we tested cells from 25 different animals using two different techniques, Northern blot analysis and QRT-PCR, for this part of the study. The results showed that GS salt did not increase the PG expression in bovine primary chondrocytes (**III, IV**)

Glucose is a general precursor for cellular GAG biosynthesis. It is taken up by the Glc transporter family proteins into the chondrocytes from the synovial fluid. Inside the cell, it is converted into Glc 6-phosphate which is the building block for the synthesis of CS, and HA (Kelly 1998; Mobasher et al. 2002). Chondrocytes are highly glycolytic cells and need a steady Glc supply to ensure their viability and extracellular matrix synthesis (Otte 1991; Lee and Urban 1997). It has been estimated that chondrocytes have an excess ability to form GlcN from endogenous Glc (Mroz and Silbert 2003). Thus, exogenous GlcN after oral or intravenous administration does not stimulate CS synthesis at concentrations higher than those normally present in the joint (Mroz and Silbert 2003). Although the level of Glc in the body fluids is strictly regulated within a relatively narrow concentration range, fluctuations in the blood Glc concentrations do occur at times, e.g., after meals. Glucose level in synovial fluid is usually within 0.55-1.1 mM range of the serum Glc level (4.2-6.1 mM), although in inflammatory joint a reduction of more than 2.8 mM concentration can occur (Brannan and Jerrard 2006).

Hyaluronan is one of the major constituents of the cartilage PGs, and together with lubricin, it is primarily responsible for the lubricating and shock-absorbing properties of synovial fluid. Hyaluronan content has been shown to decrease in OA cartilage (Thonar et al. 1978; Rizkalla et al. 1992) and also in synovial fluid from OA patients (Belcher et al. 1997). In an experimental OA model, HA in particular was decreased in the early stage of OA (Manicourt and Pita 1988), while in joint immobilisation-associated atrophy, HA and aggrecan appeared to be coordinately regulated (Haapala et al. 1996).

Hyaluronan has been used to alleviate joint pain of patients with OA (Altman and Moskowitz 1998; Altman 2000), even though conflicting results on its effectiveness in easing the symptoms have been reported. It is premature to draw any conclusions on the effect of GS on HAS expression, since in Northern blot analysis HAS-2 was detectable only in three donors, and HAS protein levels were not measured. As far as we are aware, no previous reports are available on the effect of GlcN or GS on any of the three known HAS mRNAs. Theoretically, the increase in HAS-2 mRNA expression could be beneficial for cartilage, with the assumption that also HA synthesis would be increased. Nevertheless, our results indicated that GS or Glc did not increase HAS-2 mRNA levels.

6.3. Physiologically relevant level of glucosamine sulfate does not enhance chondroitin sulfate synthesis by increasing the intracellular levels of UDP-N-acetylhexosamine and UDP-glucuronic acid (IV)

Glucosamine, which is intracellularly produced from endogenous Glc, is one of the basic sugar structures used for the synthesis of CS via conversion of fructose-6-phosphate to GlcN-6-P by the enzyme GFAT (Page 29, Fig. 4). GlcN-6-P is then rapidly converted into the GlcNAc-6-P by acetyl-CoA:GNPNAT (Fig. 4). Exogenous GlcN or GS supplemented to the cultured cells can enter this metabolic pathway *via* conversion to GlcN-6-P. N-acetylglucosamine-6-P is further converted *via* N-acetylglucosamine-1-phosphate into UDP-GlcNAc, and by epimerase into UDP-GalNAc. These nucleotide-activated sugars, together with UDP-GlcA, are utilized in the assembly of GAG chains (Fig. 4). The CS polysaccharide chain is composed of GlcA and GalNAc, and KS of galactose and GlcNAc, while HA consists of GlcA and GlcNAc (Fig. 4). It has been shown that supplemental GlcN can affect cellular metabolism in adipocytes (Marshall et al. 2004). However, that report did not show how the levels of UDP-GlcA and UDP-GalNAc behaved, or if GlcN at concentrations available after oral administration of GS had any effect on the cultured cells. Some previous *in vitro* studies have shown that the addition of GlcN or GS to culture medium increases the PG synthesis by chondrocytes (Setnikar et al. 1984 and 1986; Bassleer et

al. 1998a). One recent study demonstrated that exogenous GlcN effectively protected chondrocytes from the arthritogenic effects of IL-1 β (Gouze et al. 2006). However, the concentrations of GS or GlcN used in these studies were too high to be physiologically achievable *in vivo* after oral administration.

Our results in this study show that two concentrations, 10, 100 μ M GS salt did not increase UDP-HexN and UDP-GlcA levels. The UDP-GlcA level was clearly lower than that of UDP-HexN, though both components are required for GAG synthesis (IV). Therefore, it is important to note that even when the level of UDP-HexN increased after 1 mM GlcN or GS, the level of UDP-GlcA remained at the control level or even decreased. This finding raises the question of whether this increase in UDP-HexN can actually accelerate GAG synthesis rate if the intracellular content of one of the two essential components required for the GAG assembly remains unchanged. Furthermore, it must be remembered that 1 mM GS is too high a concentration to be achieved in the extracellular and intracellular fluids after administration of normal GlcN or GS doses (Biggee et al. 2006).

Our present findings are in line with the previous studies performed with mouse and human chondrocytes (Mroz and Silbert 2003 and 2004). It has been calculated that chondrocytes can produce a sufficient level of GlcN for CS synthesis from endogenous Glc, and that the exogenous GlcN will be diluted by the GlcN formed from endogenous Glc (Silbert et al. 1989; Mroz and Silbert 2003 and 2004). It was also shown in human chondrocytes that only 9% of galactosamine involved in the synthesis of CS is derived from exogenous GlcN, when the concentration of exogenous radioactively labeled GlcN was 102 μ M (at maximum) in mouse and human chondrocyte cultures (Mroz and Silbert 2003 and 2004).

Interestingly, the intracellular levels of UDP-HexN and UDP-GlcA were clearly higher after the 8-day-culture period than those achieved after the 2-day-culture period, and it seems possible that the level of UDP-GlcA can be the rate-limiting factor of GAG synthesis in newly isolated chondrocytes. A low concentration of GS salt (10 μ M) did not affect the UDP-sugar levels in either the 2- or 8-day-old primary chondrocyte cultures.

7. SUMMARY AND CONCLUSIONS

Glucosamine and/or GS have aroused considerable interest as possible chondroprotective and disease-modifying agents for OA. Today these agents are present in health products and are readily available as over-the-counter dietary supplements in North America. They can also be prescribed as drugs in some European countries. The use of GlcN/GS as a drug to treat OA is currently compensated by national social insurance institutions in some European countries, including Finland. However, the mechanisms to explain how GS might act *in vivo* have still remained mystery.

It has been suggested that GlcN could act as a precursor of GAG biosynthesis and this would increase the biosynthesis of GAGs should it be available in increased amounts in the body. In theory, if GS could enter the metabolic pathway inside the chondrocyte this would require less energy than the pathway utilizing Glc. However, there does not seem to be any direct evidence for cellular transport system for GS. Some studies *in vitro* have investigated the possible molecular mechanisms of GS on OA. However, several studies have been conducted with GS concentrations that cannot be achieved physiologically after a commonly prescribed oral dose. Furthermore, some recent industry-independent clinical trials with GlcN or GS have generated negative results in the treatment of OA.

The effect of oral GS on the sulfate concentration in plasma and synovial fluid has been suggested to be a more plausible mechanism for explaining how GS could manifest its actions as a suggested OA-modifying agent rather than GlcN alone. Indeed, it has been shown that the sulfate concentration of serum and synovial fluid can increase after oral administration of GS. A low sulfate concentration in the culture medium could lead to the synthesis of undersulfated GAGs in cartilage explants.

In conclusion, the results of this thesis demonstrate that articular cartilage PGs were not undersulfated in bovine and human OA. Exogenous GS at physiologically achievable concentration did not increase steady state levels of aggrecan mRNA expression, GAG synthesis or intracellular level of UDP-HexN and UDP-GlcA in bovine primary chondrocytes. Therefore, no mechanism of action of GS could be confirmed in this study.

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Appendix: original publications I-IV

I

Undersulfated chondroitin sulfate does not increase in osteoarthritic cartilage.

Lammi MJ, Qu CJ, Laasanen MS, Saarakkala S, Reippo J, Jurvelin JS, Töyräs J: J Rheumatol 31: 2449-2453, 2004

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II

Human articular cartilage proteoglycans are not undersulfated in osteoarthritis.

Qu CJ, Rieppo J, Hyttinen MM, Lammi MJ, Kiviranta I, Kurkijärvi J, Jurvelin JS, Töyräs J: Connect Tissue Res 48: 27-33, 2007

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III

The lack of effect of glucosamine sulphate on aggrecan mRNA expression and ³⁵S-sulphate incorporation in bovine primary chondrocytes.

Qu CJ, Karjalainen HM, Helminen HJ, Lammi MJ: *Biochim Biophys Acta* 1762: 453-459, 2006

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IV

Effects of glucosamine sulfate on intracellular UDP-hexosamine and UDP-glucuronic acid levels in bovine primary chondrocytes.

Qu CJ, Jauhiainen M, Auriola S, Helminen HJ, Lammi MJ: *Osteoarthritis and Cartilage*
15: 773-779, 2007

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