Proteoglycan Synthesis in Bovine Intervertebral Disc Cells Cultured in Alginate Beads: Variation with Age and Modulation by TGF-β1.

Terrence François Heathfield, BSc.
Biochemistry Unit
Orthopaedic Research Lab (Royal Victoria Hospital)
Department of Surgery
Division of Experimental Surgery
McGill University, Montreal
November, 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science.

ABSTRACT

The major aim of this work was to develop a cell culture system in which changes in the rate of synthesis of intervertebral disc (IVD) matrix molecules could be evaluated in cells of animals of different ages. In particular, the synthesis of the large aggregating proteoglycans (PGs) and of decorin (DCN) and biglycan (BGN) were investigated. In addition, the potential use of growth factors to modulate the anabolism of these molecules was also studied. The results of these studies have demonstrated that the age related decline in PG biosynthesis observed in human disc tissue can be reflected in a bovine disc cell culture model. The three dimensional alginate bead cell culture model was effective in maintaining the phenotype of disc cells from both annulus fibrosus (AF) and nucleus pulposus (NP) regions in the presence of serum. Differences in phenotype of these respective cells were illustrated *in vivo* by the patterns of resident PGs obtained from tissue extractions. In the AF of young animals, the predominant form observed was a small PG form while in the NP of all age groups, aggrecan was the major species. In vitro collagen analyses confirmed the different phenotype of cells from the AF and NP regions. Also, TGF-β1 was effectively used to stimulate over all PG synthesis in NP cells from and young adult bovine. After 10 days in culture, TGF-β1 treated cells showed similar relative amounts of the small PGs compared to aggrecan as those determined from tissue extractions.

RÉSUMÉ

L'objectif principal de ce travail était de développer un modèle de culture cellulaire permettant d'évaluer le taux de synthèse des molécules de la matrice du disque intervertébrale (DIV) pour les cellules d'animaux d'âge différents. En particulier, la synthèse des protéoglycanes (PGs) de haut poids moléculaire à capacité d'agrégation, ainsi que celle de la décorine (DCN) et du biglycane (BGN), a été étudiée. De plus, la capacité des facteurs de croissance à moduler l'anabolisme de ces molécules a été testée. Les résultats de ces travaux ont montré que la diminution de la biosynthèse des PGs qui est observée avec l'âge dans le DIV humain peut être reproduite dans les cultures du disque bovin. Le modèle de culture tridimensionnel en billes d'alginate a permis de maintenir le phénotype des cellules issues de l'annulus fibrosus (AF) et du nucléus pulposus (NP) du disque, ceci en présence de sérum. Des différences de phénotype entre les cellules de l'AF et du NP ont pu être mises en évidence après l'analyse des profils des PGs résidents obtenus à partir d'extraits tissulaires. Dans l'AF des animaux jeunes, la forme dominante est un PG de faible poids moléculaire alors que dans le NP, quel que soit l'âge des animaux, l'aggrécane est la forme la plus représentée. Les analyses du collagène réalisées in vitro ont permis de confirmer la différence de phénotype entre les cellules du NP et de l'AF du DIV. L'addition de TGF-β1 a permis de stimuler la synthèse de l'ensemble des PGs par les cellules du NP du disque de bovins jeunes ou adultes. Après 10 jours de culture, les cellules traitées avec le TGF-β1 présentent un niveau du PG de faible poids moléculaire rapporté à l'aggrécane comparable à celui mesurer à partir des extraits tissulaires.

ACKNOWLEDGMENTS:

I express my gratitude to all those who encouraged me during the years I spent completing this work. To my supervisor, Mauro Alini, who patiently guided me during both the experimental and written phases, many thanks. To those friends who helped me with all other errands required for the submission; Mauro, Nora, Kayvan and others too numerous to mention. My thanks to all my colleagues of the Orthopaedic Research Lab for making my stay a pleasant one.

I also wish to thank those who provided invaluable support: To Dr. Max Aebi without who's support in the founding of the Orthopaedic Research Lab, this would not have been possible. To Dr. A. Robin Poole for his support throughout my experience in scientific research. Also, to Dr. Peter Roughley for his suggestions on how to improve the original version of this work. Many thanks to Dr. Sheila Laverty (Faculté de Médecine Véterinaire, Université de Montréal) for kind help in obtaining tissue for these studies.

I am also indebted to the many newfound colleagues and friends here in Sweden, for their willing help with the various computer software used to generate this thesis: To Pilar Lorenzo, Karen King, Heiko Herwald, Mark Hickery and Hans Tapper. Many thanks to Leif Dahlberg for his patience and for the time allowed to finish the writing of this work.

My gratitude to Sylvie Mordier for all of the encouragement throughout the years and to my mother, Aline who has given me lifelong love and support.

TABLE OF CONTENTS: **Page**

TABLE OF CONTENTS: **Page**

LIST OF ABBREVIATIONS:

AF	Annulus Fibrosus
ANOVA	Analysis of Variance
BGN	Biglycan
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CAPAGE	Composite Agarose-polyacrylamide Gel Electrophoresis
CHAPS	(3-{(3-Cholamidopropyl) Dimethylammonio}-1-Propanesulphonate
Chase ABC	Chondroitinase ABC
CS	Chondroitin Sulphate
DCN	Decorin
DE	Direct Extraction
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene Blue
DNase	Deoxyribonuclease
DS	Dermatan Sulphate
EDTA	Ethylenediaminetetraacetic Acid
FCS	Foetal Calf Serum
GAG	Glycosaminoglycan
GuHCl	Guanidinium Chloride
HA	Hyaluronic Acid
HEPES	N-2-Hydroxyethylpiperazine-N'-ethanesulphonic Acid
IVD	Intervertebral Disc
IGF-I	Insulin-like Growth Factor I
ITS	Insulin, Transferrin and Selenium
IZ	Intermediate Zone
KS	Keratan Sulphate
LSC	Liquid Scintillation Counting
LTBP	Latent TGF-β Binding Protein
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid

LIST OF ABBREVIATIONS (continued):

NP	Nucleus Pulposus
PG	Proteoglycan
PEG	Polyethylene Glycol
PMSF	Phenylmethylsulphonylfluoride
RFA	Rapid Filtration Assay
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
TBST	Tris Buffered Saline with Tween-20
TIMP	Tissue Inhibitor of Metalloproteinases
TGF-β	Transforming Growth Factor Beta

LIST OF FIGURES: **Page**

LIST OF FIGURES (continued): **Page**

LIST OF TABLES: **Page**

1) INTRODUCTION:

1.1) The anatomical structure of the intervertebral disc:

The human spine comprises 23 intervertebral discs (IVDs) which provide an articulation between spinal segments (Hukins D.W., 1988), six of which make up the lumbar region. Discs are commonly divided into two distinct zones each with characteristic properties. Figure 1 shows a top view of an IVD indicating the structures relevant to this work. The annulus fibrosus (AF), rich in collagen, encloses the more gelatinous nucleus pulposus (NP). Between these two zones is a highly active tissue, the intermediate zone (transition zone), which has been described as having a parallel role with respect to the NP as the epiphyseal growth plate to articular cartilage (Humzah M.D et. al., 1988). On each vertebral surface, there are cartilaginous end plates which are comprised of a layer of matrix similar to that found in hyaline cartilage. The end plates contain the NP and are known to be important in the nutrition of the disc. The AF is bound to the vertebral surface via structures known as Sharpey's fibres (Oegema T.R. Jr., 1993).

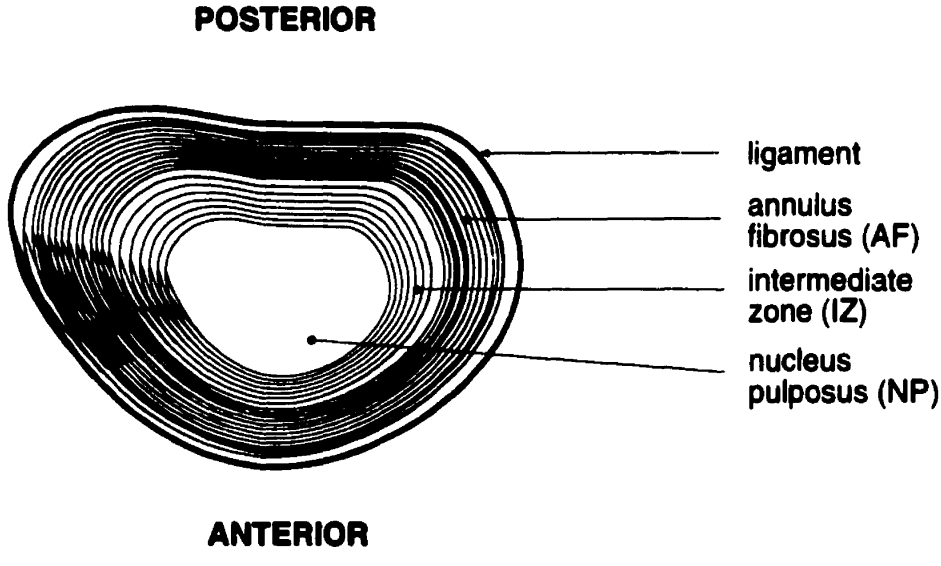

Figure 1. Top view of an intervertebral disc showing the structures relevant to this study.

1.2) Composition of the intervertebral disc matrix:

Proteoglycans: The IVD matrix contains 10-20% per dry weight and approximately 50% proteoglycan (PG) per dry weight of tissue in the AF and NP respectively. Both aggregating and non-aggregating PGs are found in the disc. The most prominent form of PG in the IVD is aggrecan (McDevitt C.A., 1988). This form of aggrecan is slightly smaller than that found in articular cartilage, probably due to different postranslational processing (Buckwalter J.A et. al., 1989). Aggrecan consists of a core protein to which is added chondroitin sulphate (CS) and keratan sulphate (KS) glycosaminoglycan (GAG) chains (Heinegard D., 1977). The core protein is divided into various domains. A short KS binding domain with two CS binding regions further toward the carboxy terminus. Flanking these regions to the N-terminal are two globular domains G1 and G2. G1 is known to be important for aggregation with hyaluronic acid (HA) in conjunction with link protein. The G2 domain does not interact with HA and its function is as of yet unknown (Fosang A.J et. al., 1989). On the carboxy-terminal side of the GAG binding domains is the third globular domain G3, which contains a growth factor binding domain and a lectin-like sequence. The G3 domain is cleaved off soon after secretion of the aggrecan molecule and its function is as of yet unknown (Hardingham T.E et. al., 1992). Figure 2a depicts a representation of the aggrecan monomer and 2b shows the manner in which monomers aggregate with HA in the extracellular matrix via the globular domain G1 (black triangles) and stabilised by link protein (white circles). GAG chains constitute repeating disaccharide units of N-acetyl-glucose or galactosamine linked to glucuronic or iduronic acid or to galactose. Chain lengths vary in the range 20 to 80 repeats depending on the GAG species. Figure 3 shows the detailed structures of the common disaccharide units which make up the GAG chains commonly found in the disc PGs. The substitution with numerous negative charges on these sugars in the form of carboxyl and sulphate groups imparts a high osmotic potential to PG molecules.

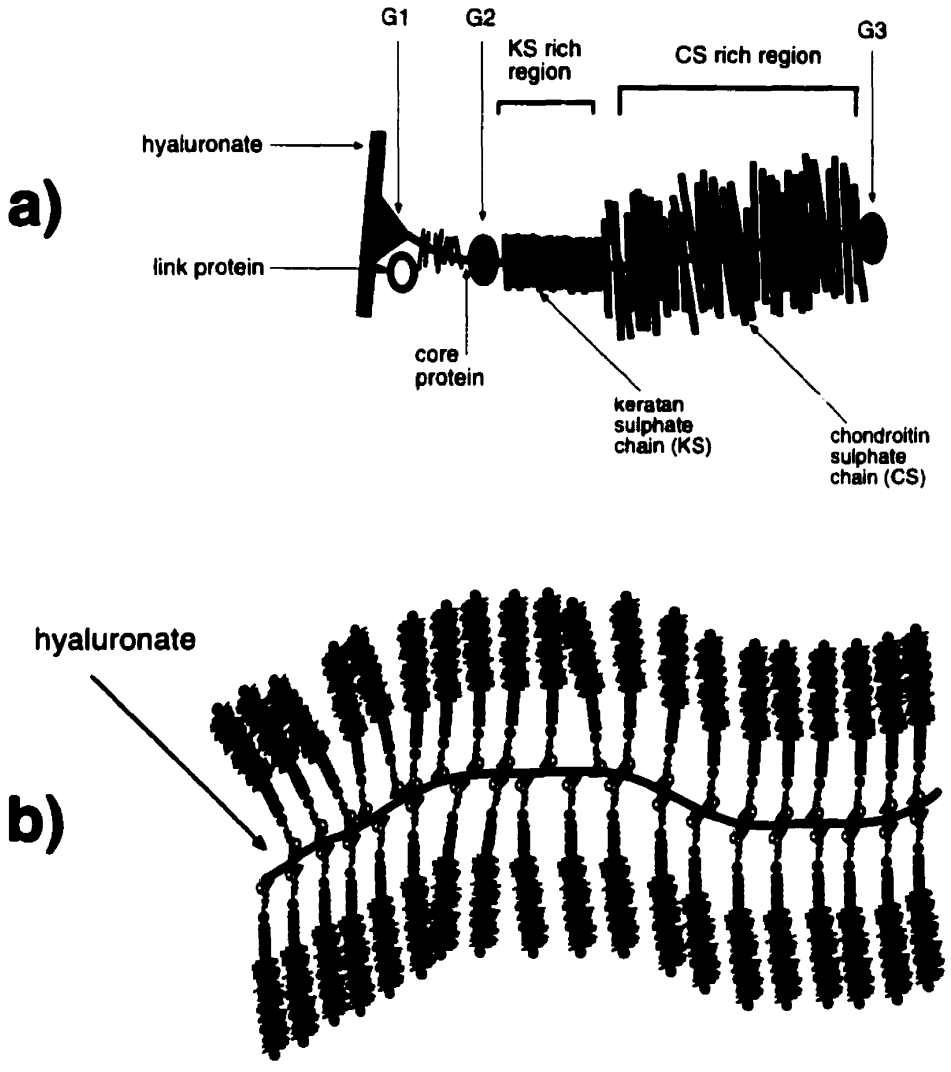

Figure 2. Structure of aggrecan **a)** An aggrecan monomer indicating the major domains of the core protein. **b)** Aggregation of several aggrecan monomers by linkage to hyaluronic acid via the G1 domain (black triangles) and stabilised by link protein (white circles).

Small PGs are comprised of short core proteins, belonging to the ribonuclease inhibitor family, containing leucine rich repeats and one or two GAG chains (Scott J.E., 1996). The core proteins have a molecular mass in the range of 45 kDa to which are attached one or two GAG chains. Decorin (DCN) is substituted with a single dermatan sulphate (DS) chain while biglycan (BGN) has two chains composed of CS or DS. Both DCN (Schonherr E., Hausser H et. al., 1995; Svensson L et. al., 1995) and BGN (Schonherr E., Witsch-Prehm P et. al., 1995) are known to bind to type I collagen. DCN has also been shown to interact with type II collagen, inhibiting its fibrillogenesis (Vogel K.G et al., 1984). One of

the functions of these small PGs has been speculated to be the regulation of collagen fibril diameter (Weber I.T et. al., 1996; Pins G.D et. al., 1997; Danielson K. G et. al., 1997). BGN contains a Transforming Growth Factor β (TGF-β) binding domain which may function as a natural inhibitor or reservoir of this growth factor within the matrix (Hildebrand A et. al., 1994).

Both DCN and BGN have already been identified within the disc (Roughley P.J et. al., 1993). The concentration of the small PGs is highest in the AF, reflecting the association of DCN and BGN in collagen rich areas of the disc (Cole T.C et. al., 1985; Johnstone B et. al., 1993).

PG synthesis can be divided into three major events. The first is the translation of mRNA into the core protein followed by transport from the lumen of the ER to the golgi. Within the golgi, GAG chains are added to the core proteins beginning in the cis-region (Freeze H.H et. al., 1993), and thirdly GAG chain elongation and sulphation in the trans compartment (Wong-Palms S et. al., 1995).

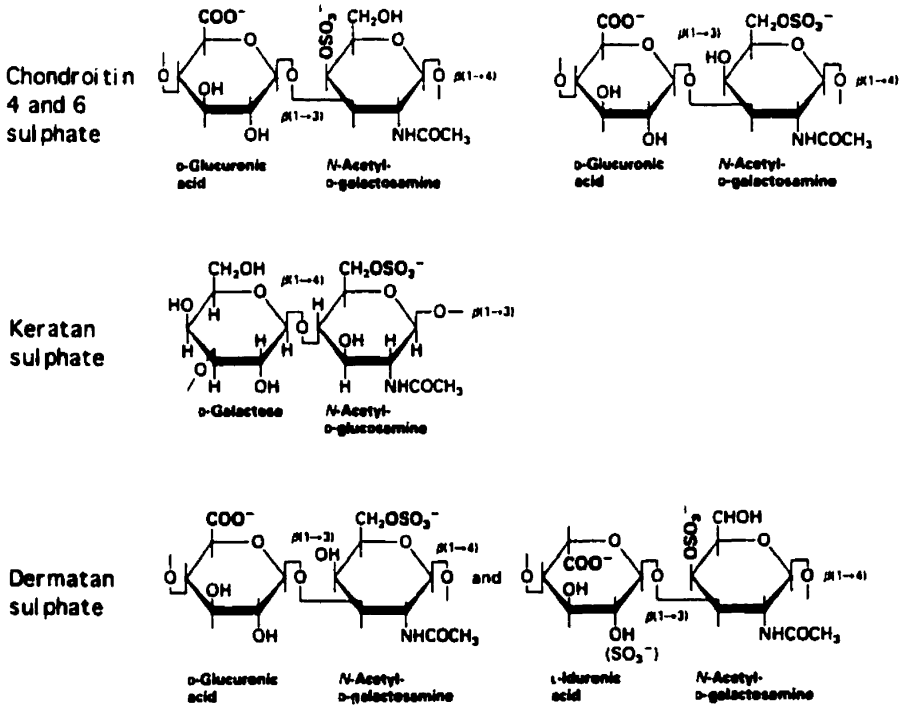

Figure 3. Structures of the disaccharide units which constitute the glycosaminoglycan chains of the various proteoglycans found in the intervertebral disc. (Adapted from Darnell J et. al., 1990. Molecular Cell Biology, 2nd Ed. Scientific American Books. Page 917).

Collagen: Types I and II collagen are the two most prominent collagens found in the IVD matrix. Both are fibrillar molecules comprised of three alpha chains intertwined in a triple helix. Type I collagen is heterogeneous, containing two identical α-1 chains and one α-2 chain. In contrast, type II collagen is made up of three identical alpha chains. Other minor collagens found in the IVD matrix include types III,VI, IX, X and XI.

On a dry weight basis, AF tissue contains 65-70% collagen. The outer AF is enriched in type I collagen and this proportion decreases in the inner lamellae, with an increased type II collagen content (Hukins D.W., 1988; Oegema T.R. Jr., 1993). In contrast the NP contains about 25% collagen by dry weight. Type II collagen concentration is highest in the centre of the NP and decreases radially toward the outer AF. Type I collagen, synthesised by fibroblastic cells, is found in tissues where elasticity is required for their function such as in tendon, skin and bone. Type II collagen, produced by chondrocytic cells, is abundant in cartilage and NP tissues (Miller E.J., 1976; Oegema T.R., 1993).

Cells of the IVD: More than one cell type appears to be present in the different regions of the IVD (Chelberg M. K et. al., 1995). Histological studies have shown that the cells of the AF are more elongated characteristic of fibroblastic cells. In contrast, the cells of the NP tended to be more rounded as are those found in hyaline cartilage. Immunohistochemical studies have also suggested that the molecules secreted by these respective cells are consistent with their morphologies. Type I collagen was localised within the AF while type II collagen was predominant in the matrix of the rounded NP cells.

The NP is thought to be populated by both notochordal and mesenchymal cells. In development, the notochord gives rise to the tissue which becomes the NP (Taylor J.R., 1975). Notochordal cells are gradually eliminated from the cell population in humans before 30 years of age (Humzah M.D et al., 1988). The chondrocytes which replace these cells in the adult NP are derived from the IZ of the young animal (Salisbury J.R et. al., 1988). In young discs, PGs are CS rich and with increasing age, aggrecan molecules tend to have a greater KS to CS ratios (Taylor J.R et. al., 1992; Scott J.E et. al., 1994). In contrast, AF cells are of mesenchymal origin, developing from the perichordal mesenchyme. These cells

eventually secrete the matrix molecules which form the lamellae of the AF (Humzah M.D et. al., 1988).

1.3) Intervertebral disc function:

The ligamentous nature of the AF is important in bending and twisting motions (Ebara S et al., 1996) while compressive forces on the spine are distributed by the NP (Hukins D.W., 1988).

Collagen fibres are known to stretch in other elastic tissues such as tendon and ligament, while they can also resist compression. The importance of these properties in the function of the AF is best illustrated in side bending motions. Type I collagen fibres arranged in parallel to the axis of the spine will be stretched on the side opposite to the direction of the bend and compressed on the same side as the direction of the bend.

The richness in aggregating PG is important in the function of the NP. The osmotic potential of aggrecan molecules is such that they attract water due to their highly negative charge imparted by the sulphate and carboxyl groups present on GAG chains. Since they are unable to diffuse out of the surrounding collagen matrix, water is released during periods of high mechanical load and flows back into the matrix with removal of the load (Kraemer J., 1995).

1.4) Low back pain and IVD degeneration:

Low back pain is the cause for reduced quality of life and major economic loss. Some studies have estimated that up to 80% of the aging population will be affected by this condition (Nachemson A.L., 1985) with an increase in incidence by the fourth decade of life (Buckwalter J.A et. al., 1993). While there is no absolute link between back pain and disc degeneration, a significant proportion of painful discs are found to be degenerated (Vanharanta H., 1994). In the past, discs were seen as being non-innervated and therefore, pain was often attributed to compression on the spinal cord. In fact, the AF was found to be innervated and a link between disc herniation and pain could be established (Vanharanta H., 1994). Magnetic resonance imaging has also shown degeneration measured by reduced image intensity in patients correlated with disc degeneration viewed by

discography of discs in individuals complaining of back pain (Schneiderman G.B et. al., 1987).

Disc degeneration may be a consequence of different pathological changes which occur with age (Buckwalter J.A et. al., 1993). An age and morphological grade related decrease in abundance of total PG per wet (Pearce R.H et. al., 1987) and dry (Antoniou J et. al., 1996) weight of disc tissue has been observed in human lumbar IVDs.

Increased degradation by matrix metalloproteinases (MMPs) has been suggested to compromise the integrity of the disc matrix (Buckwalter J.A et. al., 1993; Buckwalter J.A., 1995). Degenerative changes include apparent breakdown of the molecules important in the function of the disc. The functionality of aggrecan molecules appears reduced in older animals in that there is a loss of capacity of these molecules to aggregate with hyaluronic acid (HA) due to the loss of the G1 domain and increased degradation of link protein with age (Pearce R.H et. al., 1989). Also, an accumulation of non-glycanated core proteins of BGN has been noted in the disc with age. These are thought to represent degraded molecules which have been enzymatically cleaved in the n-terminal region of the core protein, removing the GAG chains (Roughley P.J et al., 1993).

Studies of the cellularity of the IVD have shown that there is a loss of disc cell viability with age and different growth patterns of these cells. Trout et al showed that while disc cells may appear viable by light microscopy as is used for histology, it is evident that there is a loss in viable cell population only discernible with electron microscopy (Trout J.J et. al., 1982). Cells of foetal humans had almost full viability with many isolated cells, while in adults almost half of the cells were non-viable and grew in clusters termed nests.

Disc degeneration may also be induced by a loss of capacity of the disc to be properly sustained. Work has shown that the cartilaginous end plates, through which nutrition is known to occur undergoe changes with age. The IVD being avascular, derives the required nutrition through the end plate which allows diffusion of metabolites (Maroudas A et. al., 1975; Katz M.M et. al., 1986). With age, the end plates lose their porosity in part due to gradual calcification (Roberts S et. al., 1996). Further evidence supporting the hypothesis of impaired diffusion of metabolites into the disc, is the increased abundance of small KS rich forms of

aggrecan with age which are similar to those found in oxygen deprived areas such as deep zones of cartilage and thickened corneas (Melrose J et. al., 1991).

1.5) Models used to study IVD metabolism:

Human IVD tissue, animal models and in vitro culture methods have been used to study disc metabolism. The use of post-mortem human tissue for biochemical analyses has the advantage of enabling the mapping of events which have occurred over the individuals lifetime. However, human tissue obtained at autopsy for the purpose of cell and tissue culture often has low metabolic activity due to the lag time post-mortem to harvesting. Also, due to the different lifestyles of individuals, a large variation in metabolism can be observed between them.

Animal models provide a readily available source of tissue which allows the study of large groups of individuals with similar lifestyles. Care must however be taken in interpreting results and especially with direct extrapolation to the human situation. Furthermore, the expense of animal care along with ethical considerations are often prohibitive factors in performing large animal experiments.

Most live animal studies of IVD PG metabolism have involved the measurement of synthesis using radiolabelled sulphate. Venn et al. showed that PG synthesis was reduced in 80 day old mice compared to that observed in discs of 30 day old animals (Venn G et. al., 1986). This study did not however examine the synthesis rates in the different regions of the disc. Synthesis of PG in different regions of the IVD were compared in a canine study comparing 2 and 10 year old dogs (Cole T.C et. al., 1986). Significantly lower PG contents were observed in lumbar AF and NP regions of older beagle dogs. Furthermore, decreased ability for aggregation with HA and larger hydrodynamic size of the forms extracted from the AF were reported to occur with age.

IVD explant cultures are complicated by swelling of the tissue which causes rupture of cells or exposure to extreme pressure. Earlier, a model was developed to culture slices of IVD tissue within dialysis bags, thus preventing PG loss and direct contact of polyethylene glycol (PEG) with tissue (Bayliss M.T et. al., 1986; Bayliss M.T et. al., 1988; Johnstone B et. al., 1995). In the first of these studies it was shown that concentrations above 10% PEG brought about a decrease

in PG synthesis and water content within the tissue. This model provided controlled culture conditions for the study of baseline synthesis levels of discs of different ages. It is however limited by the fact that it is difficult to calculate the PEG concentration required to maintain the exact level of hydration found *in vivo*.

Using this system, PG synthesis was studied in dogs subjected to strenuous running exercise (Puustjarvi K et. al., 1994). In this study, following one year of exercise, discs were removed and cultured whole. With the use of PEG, swelling due to release of constraint was minimised. A short fall of this type of model remains that different concentrations of PEG were used for AF and NP tissues respectively. Also, the high concentration of PEG used to control swelling of NP tissue likely had an effect on cell metabolism as it has been shown that $^{35}SO_4^{2-}$ incorporation varies directly with PEG concentration, thus inversely with hydration (Bayliss M.T et. al., 1986).

The alginate bead culture system remains a method which permits the growth of cultured cells in a tree-dimensional environment, more realistic than in cell monolayers. Alginic acid, the major component used to form alginate beads for cell culture, is a glycopolymer composed of guluronic and/or mannuronic acid carbohydrate residues (figure 4) (Haug A et. al., 1966; Atkins E.D.T et. al., 1970).

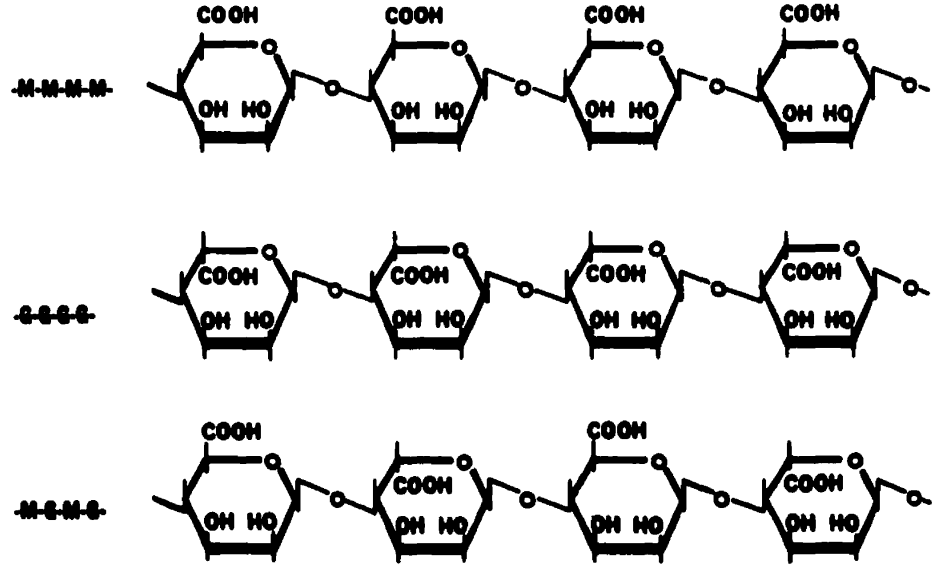

Figure 4. Chemical structure of segments of alginic acid. M=mannuronic acid, G=guluronic acid. (adapted from Alginate Products for Scientific Water Control, Third Edition, page 5).

Upon contact with divalent cations, the carbohydrate chains precipitate by co-operative binding of oxygen on the carboxyl groups with the divalent cations (Grant G.T et. al., 1973).

Alginate microsphere culture has been used to maintain the phenotype of cultured cells by maintaining them in a three-dimensional environment (Benya P.D et. al., 1982; Kolettas E et. al., 1995; Guo J.F et. al., 1989; Grandolfo M. et. al., 1993; Mok S. S et. al., 1994; van Susante J.L.C et. al., 1995). Encapsulated chondrocytes have been shown to synthesise both aggrecan (Mok S.S et al., 1994) and type II collagen (Benya P.D et al., 1982) and to maintain a rounded shape, characteristic of chondrocytes (Guo J.F et al., 1989).

Disc cells were first cultured in alginate microspheres by Maldonado (Maldonado B.A et. al., 1992). In this study, canine IVD cells were cultured by disc region and PG synthesis rates and hydrodynamic sizes were determined. AF cells were found to have higher rates of sulphate incorporation after 48 hours of culture and smaller GAG chains than those of the NP. Also, cells from the AF were reported to be viable for up to 14 days of culture while those of the NP for about 10 days due to loss of notochordal cells. No attempt was made to examine age related differences in this study.

The presence of alginate hinders the study of the majority of the PG synthesised and retained within the matrix during the entire culture period. Our and other studies have shown that most of the PG synthesised by cells detectable by labelling with $^{35}SO_4$ for 24 hours is retained within the alginate bead, often referred to as the further removed matrix (Mok S.S et. al., 1994; Aguiar D.J et. al., 1998). Removal of alginate is often complicated by the chemical properties of the polymer, being insoluble at 40% v/v concentration of most alcohols (Solvent tolerance claimed by the manufacturer) used to precipitate proteins and insoluble at low pH (Mok S.S et. al., 1989; Haug A et. al., 1967).

Methods used to recover cultured cells from alginate beads have been previously described (Guo J.F et. al., 1989; Mok S. S et. al., 1994; Petit B et. al., 1996). Briefly, alginate microspheres are depolymerised using a solution containing sodium citrate and cells are recovered by centrifugation. While this method is practical when studying cell viability and morphology, difficulties arise when attempting to investigate the proteins secreted into the alginate matrix.

Electrophoresis, Western blotting and some column chromatographic analyses are often only possible for proteins found in close proximity to recovered cells or those that are lost into medium.

With the difficulty in obtaining of fresh post mortem human discs, an alternate source of tissue is necessary in order to develop culture conditions which could be used to establish a culture system to study human tissue upon rare availability. The bovine coccygeal disc has been characterised and used in culture to compare its biochemical and biomechanical properties to those of other animals (Ohshima H et. al., 1989; Oshima H et. al., 1993). In the following study we chose to use the bovine tail disc, being a readily available tissue source from a large animal (Ghosh P et. al., 1977). As previously mentioned, direct extrapolation to the human situation of data from these studies is an acknowledged limitation when using tissue from a quadrapedal animal. Furthermore, the forces exerted on the coccygeal segments of the spine are likely quite different from those experienced by human discs.

1.6) Use of growth factors to modulate disc cell metabolism:

Few studies have explored the effects of growth factors on IVD cells (Thompson J.P et. al., 1991; Osada R et. al., 1996; Gruber H.E et. al., 1997). Thus far, most studies focusing on growth factor effects on IVD cells have been applied using tissue explant cultures (Thompson J.P et. al., 1991) or monolayer cultures (Yoo J.U et. al., 1992; Osada R. et. al., 1996).

Most of the knowledge of growth factor actions on chondrocytes is derived from an extensive number of studies using articular cartilage chondrocytes. Recent focus has been placed on the following growth factors: Basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-I) and transforming growth factor beta (TGF-β) (Trippel S.B et. al., 1995). These factors have been shown to have effects on synthesis, degradation, and mitogenesis of articular cartilage chondrocytes.

IGF-I has been shown to effectively increase PG synthesis in cultured chondrocytes (Barone-Varelas J et al., 1991) and NP cells (Osada R et al., 1996), though an age related decline in efficacy was observed in both of these studies. Similar, effects have been observed with bFGF, however this growth factor is also

known to simulate interleukin-1 effects in these cells (Chandrasekhar S et al., 1989).

TGF-β was chosen for its previously described efficacy in increasing PG synthesis in chondrocytes (Morales T.I et al., 1988) the cell type thought to populate the adult NP. Also, there is well documented evidence that this growth factor upregulates the expression of tissue inhibitor of metalloproteinases (TIMPs), this being important for future studies using this system focusing on matrix degradation (Gunther M et al., 1994; Su S et al., 1996).

Of the five isoforms of TGF-β, three have been identified in mammals (Sporn M.B et. al., 1990; Lawrence D.W., 1996). TGF-β 1-3 are comprised of 12.5 kDa homodimers which are present in the matrix as large, inactive complexes when bound to the 200 kDa Latent TGF-β Binding Protein (LTBP) (O'Kane S et. al., 1997). TGF-β has been shown to be present in articular cartilage (Morales T.I et. al., 1991) and is known to be stored in latent form, bound to the LTBP (Dallas S.L et. al., 1995) and to thrombospondin (Murphy-Ullrich J.E et. al., 1992).

TGF-β1 is known to have different effects on articular cartilage chondrocytes, these depending on the concentrations of the growth factor and the state of cell differentiation (Galera P et. al., 1992; van der Kraan P et. al., 1992; van den Berg W., 1995). Chondrocytes are known to synthesise (Morales T.I et. al., 1991) and to have receptors for TGF-β (Glansbeek H.L et. al., 1993). The chondrocytic phenotype with production of type II collagen and aggrecan, was shown to be induced in foetal rat mesenchymal cells by this factor (Seyedin S.M et. al., 1985; Seyedin S.M et. al., 1986). PG synthesis was shown to be increased in a dose dependant manner with TGF-β in organ cultures of bovine articular cartilage (Morales T.I et. al., 1988). Also, increased mitogenicity with chondrocyte maturity has been described (Guerne P-A et. al., 1995).

TGF-β has also been shown to regulate the expression of the small PGs DCN and BGN in fibroblasts (Bassols A et. al., 1988). These PGs are known to bind TGF-β and to act as potential natural inhibitors (Hildebrand A et. al., 1994; Hardingham T.E et. al., 1992; Imai K et. al., 1997).

Some studies have shown that TGF-β can also be pathogenic when injected into the joints of mice, producing inflammation and inducing osteophyte formation (van Beuningen H.M et. al., 1994). In the IVD, inflammation is of less

concern than in an articulation where there is a synovial lining. While this study did not relate the incidence of osteophyte formation to different doses of the growth factor, it does demonstrate the importance of careful choice of the dose to be used. In cell culture it has been shown that concentrations greater than 40 pM (1 ng/ml) induced a significant increase in PG synthesis in cultured articular chondrocytes (van der Kraan P et. al., 1992).

Recently, human AF cells were cultured in alginate beads and were tested for response to the growth factor TGF-β1 (Gruber H.E et. al., 1997). In this study, disc cells were grown in primary monolayer cultures and then encapsulated into alginate beads. This was certainly necessary in order to obtain a sufficient number of cells due to the low cell yields obtained with human disc tissue. Increased mRNA levels were observed for BGN though not for DCN in TGF-β treated cultures. Again, this work did not attempt to elucidate whether there is an age related decrease in synthesis in matrix molecules, which would necessitate modulation with growth factors.

1.7) Rationale:

The study described in this work was proposed in order to examine one of the many potential factors involved in IVD degeneration. Synthesis of the predominant matrix molecules has been shown to decrease with age in human discs (Antoniou J et al., 1996). In this study, markers for newly synthesized aggrecan molecules [846 epitope] (Rizkalla G et. al., 1992), the propeptide markers of newly synthesized type I collagen [commercially available], and type II collagen [CPII epitope] (Hinek A et. al., 1987; Poole A.R et. al., 1995) were used to demonstrate the reduction in abundance of these markers in human discs with age (Antoniou J et al., 1996). These results were expressed on a per dry weight of tissue basis. It was thus important to verify whether this reduction in the amount of newly synthesized molecules could be wholely explained by a loss of viable cells populating the disc with age (Trout J.J et. al., 1982) or could also be demonstrated on a per cell basis.

PGs make up an important proportion of the disc matrix and are a likely target for the disruption of normal disc function. While other aspects involved in disc degeneration such as extracellular matrix degradation and disc cell nutrition

are recognized to be equally imporant, focus of study was placed on the synthesis of PGs by disc cells at different ages. Another principal aim of this study was to establish a serum-free cell culture model in which growth factors, or other pharmacological agents, used to modulate disc cell synthesis, could be tested. This serum-free system can potentially be used to study degradative processes of the same molecules and also to study the effects of enzyme inhibitors on such events.

In order to best select potential modulating growth-factors, the phenotype of disc cells of the AF and NP regions needs to be determined. Little is known about the cell types which populate the different regions of the IVD. Histological studies have suggested the presence of two cell types (chondrocytes and fibroblasts) (Chelberg M.K et al., 1995). In this study, the morphology and synthetic products (collagen and PG) will be evaluated in both alginate and monolayer culture.

2.1) HYPOTHESES:

2.1.1) The compromised integrity of the extracellular matrix of the IVD observed with ageing, results from an impairment in the synthetic activity of the disc cells, which may act as one predisposing factor in subsequent disc degeneration.

2.1.2) The impaired synthetic activity of aged disc cells can be modulated by TGF-β1.

2.1.3) At least two different cell populations are present in the IVD tissue.

2.2) SPECIFIC AIMS:

2.2.1) To measure the synthesis of PG in disc cells, and its variation with age, a serum-rich system will be applied in order to culture IVD cells in vitro. Cells from AF and NP regions will be isolated from foetal (7-9 months of gestation), calf (less than 4 months old) and adult (greater than 6 years of age) bovine and encsapsulated into alginate beads. Cultured cells will be labelled with $^{35}SO_4$ for 24 hours and incorporated radioactivity quantified by scintillation counting. All measurements will be equalized to DNA content determined on parallel cultures.

2.2.2) To determine if TGF-β1 can be used to modulate IVD cell biosynthesis, a serum-free culture system will be applied to the same cell types as described in 2.2.1. TGF-β1 treated cells will be compared to controls in serum-free medium with respect to sulphate incorporation and cell proliferation. Effects on small PG synthesis will be semi-quantitatively evaluated by electrophoresis and Western blotting.

2.2.3) Differences in phenotype of cells from the respective regions of the IVD will be evaluated on both fresh tissue and cultured cells. The PGs synthesised in culture will be compared to those found *in vivo* by composite gel electrophoresis. The types of collagen synthesised will be determined by autoradiography and fluorography of ^{3}H-proline labelled collagens in both monolayer and alginate bead culture. Cell morphology will be observed in high density monolayer culture since chondrocyte-like cells are known to maintain a polygonal shape under these conditions, while fibroblasts will flatten out to form a sheet.

3) MATERIAL and METHODS:

3.1) Tissue and cell isolation:

Bovine tail (coccygeal) segments from foetal (7 to 9 months gestation), calf (120 days old) and adult (older than 7 years) were obtained from a local slaughter house and processed within 1 to 2 hours. Discs were dissected out, stripped of ligament and separated into AF and NP regions. The intermediate zone was discarded along with a small strip of both AF and NP on each side to ensure the identity of each respective region. Tissue was minced and placed into Dulbeccos Modified Eagle's Medium (DMEM) containing 44 mM $NaHCO_3$, 20 mM HEPES buffer, 100 units/ml of penicillin G and 100 µg/ml of streptomycin sulphate (all from Gibco BRL), 150 mg/L of gentamycin sulphate (Sigma), with 2.5 µg/ml amphotericin B (Fungizone, Gibco BRL). Tissue was washed once with the above medium (DMEM/fungizone) and twice with DMEM/fungizone containing ten times concentration penicillin/streptomycin, followed by a last wash in DMEM/fungizone.

Cells were isolated from AF and NP tissues respectively by sequential enzyme digestion as follows. Minced tissues were agitated at 37°C for 90 minutes in a mixture of 0.2% pronase from streptomyces griseus (Calbiochem-Novabiochem Corp., La Jolla, CA.) and 0.004% deoxyribonuclease I, type II from bovine pancreas (DNase), (Sigma) in DMEM at 2.5 ml enzyme solution per gram of tissue. AF tissue was then digested with 0.04% collagenase II from clostridium histolyticum, 0.01% hyaluronidase, type V from sheep testes and 0.004% DNase (all from Sigma) in DMEM containing 10% foetal calf serum (FCS) (FetalClone III from Hyclone Laboratories, Inc.). NP tissue was treated with 0.01% collagenase IA from C. histolyticum, 0.01% hyaluronidase and 0.004% DNase in DMEM containing 10% FCS. Tissue was agitated at 37°C for 9 hours with 5 ml of enzyme solution per gram of tissue. See Table 1 for quick reference of enzyme concentrations.

	collagenase IA	collagenase II	hyaluronidase	DNase
AF	----	0.04%	0.01%	0.004%
NP	0.01%	----	0.01%	0.004%

Table 1: Quick reference for concentrations used in sequential enzyme digestion of the tissues from different IVD regions. Tissue was pre-digested with 0.2% w/v pronase and 0.004% w/v deoxyribonuclease (DNase). See text for details.

3.2) Cell culture:

Digests were filtered through nylon gauze and cells recovered by centrifugation at 1500 rpm on a Beckman CS6 table top centrifuge. Cell number and viability was determined by the trypan blue exclusion method. Recovered cells were resuspended in 1.2% w/v alginate at a concentration of 2x 10^6 cells /ml and dripped through a 20 gauge needle into 102 mM $CaCl_2$ solution. Beads were washed twice in sterile 150 mM NaCl solution and twice in DMEM. Cells were cultured at 5 alginate beads per 0.5 ml of DMEM with 10% FCS and 25 µg/ml ascorbic acid from day 2 to 4 and 50 µg /ml from day 4 onward. Cultures were labelled with 25 µCi/ml $H_2{}^{35}SO_4$ (ICN Biomedicals) for 24 hours at days 4 and 9 of culture.

Serum-free cultures were also established in order to test the effects of growth factors. The medium used in these cultures comprised of DMEM supplemented with 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (ITS) (Boeringer Manheim) and ascorbic acid at the same concentration as for cultures in FCS. Cultures treated with TGF-ß1 included serum free medium to which was added 1 ng/ml (40 pM) of recombinant human TGF-ß1 (R&D Systems) from day 2 onward.

Monolayer cultures were also studied in media with and without serum. After isolation, cells were plated at 2 x 10^5 cells per well (high density) in 96 well plates (Costar).

3.3) Cell viability and proliferation:

Cells were dissociated from alginate beads by solubilisation with 55 mM sodium citrate as previously described (Mok S.S et. al., 1994). A quarter volume of four times concentrated proteinase K digestion solution (2 mg/ml proteinase K {Type

XI from tritirachium album, Sigma), 40 mM EDTA, and 400 mM NaH_2PO_4, pH 6.5) was added and samples were incubated at 56°C for 16 hours. Enzyme activity was inhibited by heating at 100°C for 10 minutes and samples were stored frozen at -20°C. DNA content was evaluated using a variation of the fluorescent dye binding assay using bisbenzimide (Hoechst 33258) as previously described (Labarca C et. al., 1980).

Standards were diluted in buffer containing 1 volume of 1.2% w/v alginate, 1 volume 4 x proteinase K buffer and 3 volumes of 55 mM sodium citrate in 150 mM NaCl, pH 7. This was found to be important since alginate-containing samples quantified using a standard curve diluted in water gave exactly twice the expected percentage fluorescence value for a known quantity of DNA.

The method described above was used in all experiments, however, it was important to ensure that these values were a direct reflection of the number of live cells found in the alginate beads. Since DNA from lysed cells would be soluble in the sodium citrate buffer used to solubilise alginate beads, intact cells were recovered with 55 mM sodium citrate as previously described (Mok S.S et. al., 1994). Cells were pelleted at 175 g_{av} and the supernatant containing alginate removed. The pellet was then washed with the same citrate buffer and then digested with 200 µl of 1 times proteinase K digestion solution. Samples treated in this manner were compared to parallel cultures in which alginate was not removed. These samples were assayed with DNA standards diluted in H_2O. With the knowledge that measures of fluorescence in presence of alginate yield exactly twice the expected fluorescence, values obtained for samples with alginate were divided by 2. Ten pairs of samples were compared and a correlation coefficient (r^2) determined as shown in figure 6.

3.4) Quantification of newly synthesised proteoglycans:

Alginate beads were harvested at days 5 and 10 of culture and solubilised at 4°C for 24 hours with 10 volumes of 4 M guanidinium chloride (GuHCl) containing 0.05% w/v (3-{(3-cholamidopropyl) dimethylammonio}-1-propanesulfonate) (CHAPS), (Sigma) with the following protease inhibitors: 5mM ethylenediaminetetraacetic acid (tetrasodium salt) (EDTA), (BDH), 1mM iodoacetamide , 10 µg/ml pepstatin A and 1mM phenylmethylsulfonyl fluoride (PMSF) (from Sigma). Medium was made up to a final concentration of 4 M GuHCl by addition of an equal volume of 8M GuHCl in 100mM NaOAc pH 5.8 immediately

upon thawing from storage at –20°C and agitated at 4°C for 30 min prior to assay. Initially, GuHCl solubilised PGs were applied to PD-10 columns (Bio-Rad Laboratories) and equilibrated with 4M GuHCl and 1 ml fractions collected. Fractions from the void volume were pooled and radioactivity measured by liquid scintillation counting (LSC). In later experiments, radiolabelled PGs in both medium and alginate bead extracts were quantified using a rapid filtration assay based on the precipitation of PG by alcian blue and quantified by LSC as previously described (Masuda K et. al., 1994).

3.5) Composite agarose-polyacrylamide gel electrophoresis of proteoglycans:

In order to establish profiles of PGs found *in vivo*, tissues of the AF and NP regions of the tail and lumbar segments were extracted, immediately after dissection with 4M GuHCl for 48 hours. Extracts were loaded onto PD-10 columns (BioRad) and the void volume recovered in column buffer which comprised of 50 mM ammonium acetate, pH 7. Samples were concentrated and stored frozen until analysed. In later experiments, PGs were directly precipitated from the extraction solution with 75% v/v 2-propanol, final concentration. Recovered pellets were resuspended in distilled water and analysed for chondroitin sulphate (CS) content using a variation of the dimethylmethylene blue (DMMB) assay (Farndale R.W et. al., 1986). Samples were equalised to 5 μg CS per lane in 0.5% sodium dodecyl sulphate (SDS) and applied to composite agarose polyacrylamide gels (CAPAGE) as previously described (Heinegard D et. al., 1985).

For visualisation of PGs from cultured cells, alginate bead extracts freed of alginate were resuspended in 0.5% w/v SDS and electrophoresed as above. Medium samples were precipitated and analysed in the same manner. Loads were equalised as cpm per lane, generally 15000 cpm which gave an adequate signal after 24 hours. Gels were dried and exposed on Kodak XAR film (Kodak).

To further analyse the precipitated PG from culture, samples were digested with chondroitinase ABC (Chase ABC) and papain. Dried extracts were rehydrated in 50 mM sodium acetate, pH 6 containing 0.1 units/ml Chase ABC. from Seikagaku Corp., Tokyo, Japan and incubated for 4 hours at 37°C. For papain digestion, samples were resuspended in 50 mM sodium acetate, 5 mM EDTA, 5 mM cysteine-HCl, pH 6 and incubated overnight at 65°C. Some iodoacetamide inhibited samples were

digested with Chase ABC after papain digestion as described above. All were electrophoresed on CAPAGE in order to verify banding patterns after digestion.

3.6) Removal of alginate for gel electrophoresis:

Difficulties due to the presence of alginate in samples prompted the search for a method to selectively remove alginate, while leaving PGs in solution. Initial experiments focused on alcian blue precipitation as described by Bjornsson,1993 (Bjornsson S., 1993). It became apparent that alginate was not soluble in the dissociation buffer used to dissociate PGs from precipitated alcian blue-PG complexes. In order to evaluate the recovery of known quantities of labelled PGs, alginate was added to radiolabelled PGs from monolayer cultures and removed by precipitation at a final concentration of 33% v/v 2-propanol. PGs were then precipitated from the supernatant at 75% v/v 2-propanol final concentration (data not shown).

To further test whether there was a loss of total PG when precipitating out alginate, the following quantitative experiments were performed. Twelve independent samples of AF and NP cells cultured for 10 days in alginate beads were labelled with $^{35}SO_4$ and extracted with 4M GuHCl as previously described in section 3.4. A small portion of extract (50 µl) was used to quantify by RFA (Masuda K et. al., 1994). The remainder of the sample was freed of alginate and PG precipitated at 75% v/v 2-propanol final concentration as described above. Recovered PGs were resuspended in 50 mM NaOAc, pH 5.8 and a small aliquot used for quantification by LSC. Another portion was used to quantify the total GAG recovered after 10 days of culture by DMMB assay (Farndale R.W et al., 1986).

In order to ensure that the intermediate precipitation step at 33% v/v 2-propanol was adequate in order to precipitate all of the alginate present in samples, the following experiment was undertaken. Four replicates per experimental point of 5 alginate beads per tube were agitated at 4°C for 16-24 hours in 1 ml of 4M GuHCl in 50 mM NaOAc, 5 mM EDTA, 0.05% CHAPS, pH 5.8. A range of concentrations of 2-propanol was established between 9 to 42 % v/v (final concentration). Tubes were agitated for 15 minutes at 4°C and precipitated alginate was recovered by centrifugation at 9000 g_{av}. Pellets were washed twice with 70% cold ethanol and then dried. Alginate was resuspended in dH_2O with heating at 60°C for 5 minutes to ensure

complete solubility, and quantified by DMMB assay (Farndale R.W et al., 1986). To ensure that the standard curve of the assay prepared with alginate instead of CS was linear, known doubling concentrations of alginate were measured by DMMB, these yielding values between 0 and 0.3 OD at A530.

The validity of this precipitation method was demonstrated for PGs from alginate cultured samples electrophoresed on both composite and SDS-PAGE gels. Alginate beads containing NP cells were labelled and extracted as described in section 3.4. Each sample was divided into 400 µl aliquots; one precipitated with 75% v/v 2-propanol and the other with an intermediate 33% v/v 2-propanol precipitation with subsequent addition of 2-propanol to 75% v/v. Pellets were washed twice with 80% v/v EtOH and dried. Dried pellets were resuspended in 50 mM NaOAc, pH 6 and heated to 40°C for 10 minutes to ensure complete solubilisation.

For CAPAGE, a portion of each sample was taken to which was added an equal volume of 2 x sucrose buffer as described by Heinegard (Heinegard D et al., 1985). Following composite gel electrophoresis and fixing in 40% MeOH, 7% HOAc, gels were stained with 0.1% w/v toluidine blue in 3% v/v HOAc. Destaining was achieved with 3% v/v HOAc. Dried gels were exposed to Kodak XAR film to resolve radiolabelled bands.

Aliquot of the same samples were also electrophoresed on SDS-PAGE. A thirty microliter aliquot of each treatment (with or without intermediate 33% 2-propanol precipitation) was treated with 0.1 units/ml Chase ABC and 0.01 units/ml keratanase II, final concentrations, and incubated for 4 hours at 37°C. Enzyme activity was stopped by addition of a quarter volume of 4 x Laemmli sample buffer containing β-mercaptoethanol. Samples were electrophoresed on 10% polyacrylamide gels and then transferred onto nitrocellulose as previously described (Laemmli U.K., 1970; Towbin H.T et al., 1979).

Membranes were blocked with BSA overnight at 4°C. Antibody 3B3, used at 1:300 dilution, was chosen as the first antibody as it is specific for chondroitin sulphate (CS) and dermatan sulphate (DS) stubs which remain after cleavage of GAG chains by Chase ABC (Couchman J.R et. al., 1984). The second antibody was a goat anti-mouse IgG+IgA+IgM (H+L) alkaline phosphatase conjugate (Zymed Laboratories, San Francisco, CA) used at 1:1000 dilution. Reactive bands were resolved using NBT/BCIP alkaline phosphatase substrate kit (Bio-Rad Laboratories).

Five micrograms of bovine foetal aggrecan from epiphyseal cartilage (a kind gift from Dr. A.R. Poole, Joint Diseases Lab, Shriners Hospital, Montreal) was used as a positive control.

In all experiments shown in this work, the following procedure was routinely used to precipitate PGs: Five alginate beads were extracted overnight with 1 ml of 4M GuHCl in 50 mM NaOAc, 5 mM EDTA, 0.05% CHAPS, pH 5.8. Alginate was then removed by addition of 2-propanol, to a final concentration of 33% v/v. Samples were agitated for 15 minutes and then centrifuged at 9000 g_{av} for 10 min. at 4°C. The supernatant was retained and made up to a final concentration of 75% 2-propanol and agitated at 4°C for 1 hour. PGs were recovered by centrifugation at 9000 g_{av} for 20 min. at 4°C. Pellets were washed twice with 1 ml of 80% cold ethanol and stored at –20°C until analysed. A schematic outline of this procedure is shown in figure 5 for quick reference.

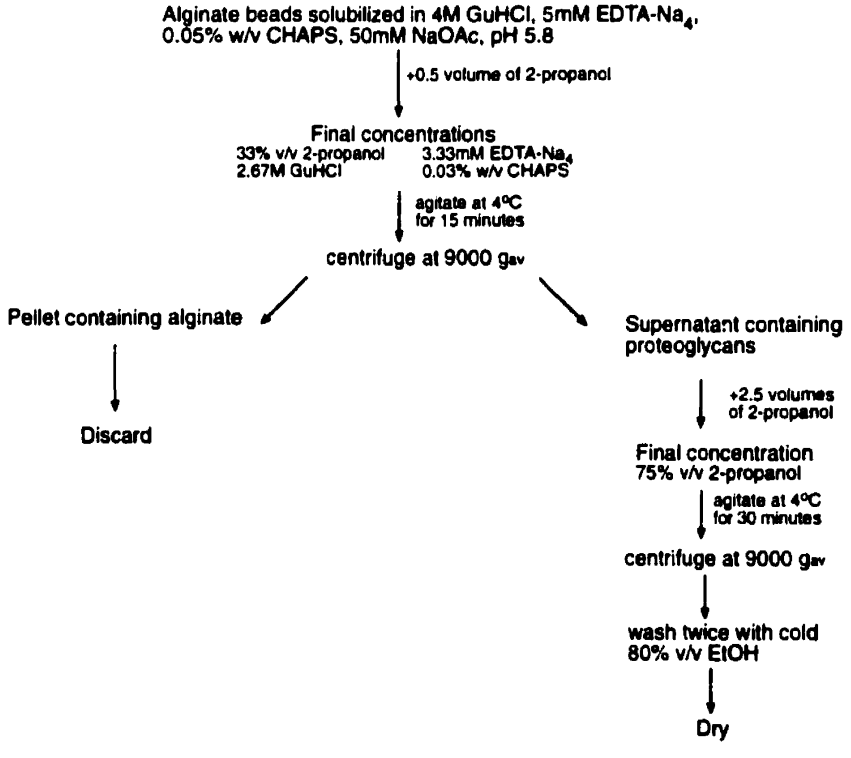

Figure 5. Schematic representation for separation of alginate from proteoglycans. See text for details.

3.7) Analysis of glycosaminoglycan chains by column chromatography:

Dried PGs were resuspended in 200 µl of 50 mM sodium acetate, 5 mM EDTA, 5 mM cysteine-HCl, pH 6 containing 250 µg/ml papain. Samples were incubated at 65°C for 16 hours and were then inhibited with iodoacetamide at a final concentration of 10 mM. Immediately prior to loading on the column, samples were made up to 1 ml with column elution buffer which consisted of 50 mM tris-HCl, 0.5 M NaCl, pH 7.5. PGs were eluted on a 99 x 1 cm column packed with Sepharose CL-6B (Pharmacia, Uppsala, Sweden). One millilitre fractions were collected at 6 ml/hour. V_o and V_t were determined by the elution positions of dextran blue and potassium dichromate respectively.

Elution profiles of ^{35}S-labelled GAG chains were obtained by LSC of fractions at a 1:10 ratio of eluate to scintillation fluid. Non radioactive GAG chains from direct tissue extractions were measured by DMMB assay.

3.8) Core protein analysis of small proteoglycans:

Analysis of the small PGs DCN and BGN, was carried out by Western blotting using specific antibodies to their core proteins (a kind gift from Dr. P.J. Roughley, Genetics Unit, Shriners Hospital, Montreal). Samples comprised of 4M GuHCl extracts of disc AF and NP tissues or of alginate beads containing NP cells after 10 days of culture in FCS, or ITS alone or ITS+TGF-β1. PGs precipitated with 75% v/v 2-PrOH final volume, were resuspended in 50 mM NaOAc, pH 6 and quantified for CS content by DMMB assay. The equivalent of 2.5 µg CS was made up to 30 µl with the same buffer to which was added final concentrations of 0.1 units/ml Chase ABC and 0.01 units/ml keratanase II (Sigma). Samples were incubated for 4 hours at 37°C and the reaction stopped by addition of 10 µl of 4 times concentrated SDS-PAGE sample buffer containing β-mercaptoethanol. Aliquots were heated at 95°C for 5 minutes and stored at -20°C until analysed. The positive control for antibody reactivity was a Chase ABC treated bovine nasal PG extract, (A generous gift from Dr. P.J. Roughley, Genetics Unit, Shriners Hospital, Montreal).

Aliquots containing the equivalent of 2.5 µg CS per lane were run on 10% SDS-PAGE as previously described (Laemmli U.K., 1970) and were electro-transferred onto nitrocellulose at 100 V for 2 hours at 4°C (Towbin H et. al., 1979). Membranes were blocked with 0.2% w/v non-fat dry milk overnight at 4°C prior to

blotting with anti-DCN or anti-BGN antibodies. Both first antibodies were exposed to membranes for 1 hour after which they were washed four times with tris-buffered saline with 0.1 % v/v Tween-20 (TBST). The second antibody, a goat anti-rabbit alkaline phosphatase conjugate, was exposed to the membranes for 1 hour at room temperature. After four washes with TBST, membranes were treated with a chemiluminescent substrate for 5 min. (Bio-Rad Immun-Star™ chemiluminescent protein detection system). Reactive bands were resolved by brief exposure (1 to 2.5 minutes) to high performance chemiluminescence film; Hyperfilm™ ECL™ (Amersham).

3.9) Cell morphology and cell phenotype:

Isolated cells from tail AF and NP were plated as monolayers at high density ($2x10^5$ cells/well) in DMEM+FCS. Cultures were incubated in a humidified incubator at 37°C with 5 % CO_2. Photographs were taken of the cell monolayers at days 5, 10 and 15 of culture.

In order to further identify the phenotype of cultured cells from the AF and NP regions of the disc, profiles of newly synthesised collagens were obtained. Cells in monolayer (high density) or alginate (5 alginate beads per well) were labelled with 25 μCi/ml ^{3}H-proline (Amersham) for 24 hours at days 5 and 10 of culture. Medium was collected and proteins digested with pepsin by addition of an equal volume of 1 mg/ml pepsin A, from porcine stomach (Worthington Biochemical Corp., Freehold, NJ), in 1 N acetic acid. Digestion was carried out at 4°C for 16-24 hours. Radiolabelled collagens were precipitated with $(NH_4)_2SO_4$ at a final concentration of 33% and agitated at 4°C for 16 hours. Collagens were recovered by centrifugation at 10000 rpm and washed twice with 70% v/v cold ethanol. Pellets were dried and then solubilised in Laemmli sample buffer containing β-mercaptoethanol. Samples were heated at 95°C for 5 minutes and applied to 7.5% polyacrylamide gels. Gels were prepared for fluorography using Entensify™ (NEN Dupont, Boston, MA) and then dried under vacuum. Labelled bands were resolved by exposure of gels to Kodak XAR film at -70°C.

3.10) Histological preparation of alginate beads:

To prevent distortion of cells when cutting sections, alginate beads were hardened by exchange of calcium for barium by incubation of beads in 100 mM $BaCl_2$ for 15-20 minutes at room temperature (Mok S.S et. al., 1994). Beads were fixed in 2.5% v/v glutaraldehyde, 10% w/v cetylpyridinium chloride and 2% w/v sucrose for 16 to 24 hours at room temperature. They were then dehydrated with a 50% to 100% v/v ethanol series with a final change in xylol and embedded in paraffin. Five micron sections were cut and mounted onto positively charged slides (Fisher Scientific). Adherence was ensured by heating slides at 55°C. For staining, sections were dewaxed in xylol and rehydrated in a 100 to 50% EtOH series and quickly rinsed in dH_2O. Sections were then stained with 0.5% w/v toluidine blue in 5% v/v ethanol for approximately 1 minute, quickly rinsed in dH_2O and blotted dry. Slides were then dipped in xylol and coverslipped. Slides of alginate beads were photographed at a magnification of 200x.

3.11) Statistical analyses:

Lines of best fit were evaluated by regression analysis and r^2 values obtained. Quantitative data was evaluated statistically by comparison of treatment groups using the non-parametric Mann-Whittney U rank sum test when tests for normality failed. For comparisons of more than one mean of which the variance was significantly similar, a one way ANOVA test was used. Sample means under comparison whose variances differed significantly (in the case where the ANOVA test could not be used), were compared individually using the rank sum test. A p value of less than 0.05 % was considered significant. Error bars represent standard deviations and have been shown in each figure unless the scale used renders them invisible.

4) RESULTS:

4.1) Intervertebral disc cell isolation:

Table 2 shows the yields of cells per gram wet weight of tissue obtained using the method described in section 3.1. From all tissues, an age related reduction in cell yields was observed. The greatest number of viable cells was obtained from foetal AF tissues however these cells rarely survived in alginate beads in culture with FCS. The large difference observed between cell yields of AF and NP cells seen in the foetus AF ($21x10^6$ cells/g) compared to NP ($5.2x10^6$ cells/g) was followed by a rather abrupt equalisation of cell yields of these respective tissues from calf and adult bovine. Differences in the cell yields from calf tissues AF ($5.7x10^6$ cells/g) and NP ($3.94x10^6$ cells/g) were not significant. Similar cell yields from adult tissues AF ($1.23x10^6$ cells/g) and NP ($1.26x10^6$ cells/g) were essentially identical.

	AF	NP
Foetus	21.2 (sd=6.2, n=3)	5.2 (sd=2.21, n=3)
Calf	5.78 (sd=2.32, n=6)	3.94 (sd=1.52, n=6)
Adult	1.23 (sd=0.37, n=5)	1.26 (sd=0.46, n=7)

Table 2: Cell yields from annulus fibrosus (AF) and nucleus pulposus (NP) tissues following sequential enzymatic digestion ($x10^6$ cells/g tissue). sd= standard deviation. n= sample size.

4.2) Cell viability and proliferation in FCS:

DNA content of cells grown in alginate beads was determined using a fluorescent dye binding assay. In order to determine whether it was necessary to remove alginate from solubilized alginate beads prior to assay, the experiment described in section 3.3 was performed. Figure 6 shows the correlation obtained

between samples containing alginate and paired samples assayed following removal of alginate. A highly significant correlation (r^2=0.952) was obtained, indicating that essentially all of the cells remaining within the alginate beads were viable. Values obtained for DNA content were therefore not artificially increased by dead cells remaining within the alginate matrix.

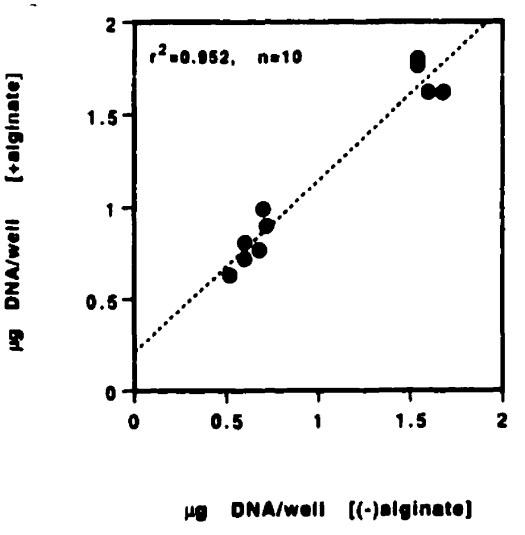

Figure 6. Correlation between sample wells of alginate bead cultures of bovine disc cells which were separated from the alginate matrix prior to assay for DNA content (x-axis), or assayed without separation of cells from sodium citrate solubilized alginate matrix (y-axis).

In cultures used to quantify PG synthesis, no significant difference was observed in DNA content in cultures of calf AF cells, between the two time points of culture (figure 7, left panel). However, a significant 1.5 fold increase ($p<.05$, Rank sum test) in cell number was observed in adult AF cells from day 5 to 10 of culture. No significant difference was observed between age groups. In NP cells, there was again no significant difference in DNA content between age groups. However, significant 1.4 fold and 1.8 fold increases (Both $p<.01$, Rank sum test) in DNA content were observed in calf and adult NP cells respectively from day 5 to 10 of culture. A similar trend toward and increase in cell proliferation was seen in NP cells of the foetus, though with the available sample size, its significance could not be obtained.

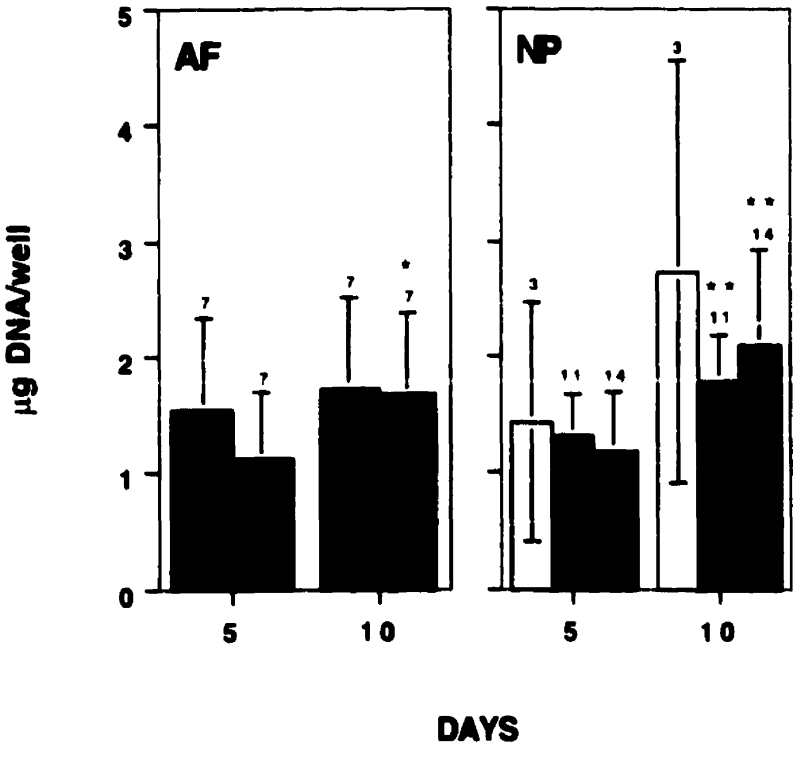

Figure 7. Quantification of DNA in serum-containing alginate bead cultures of bovine annulus fibrosus (AF) and nucleus pulposus (NP). White: foetus, Grey: calf, Black: adult. Error bars indicate standard deviations. Numbers above each bar indicate the sample size. Significant increases were obtained by Mann-Whittney Rank Sum test (* = $p<.05$, ** = $p<.01$) for adult AF (from day 5 to 10 of culture) and similarly for both calf and adult NP respectively.

Although significance of cell proliferation could not be obtained due to a small sample size of foetal NP cells, toluidine blue staining of alginate beads showed that by day 12 of culture, foetal NP cells (Figure 8b) had greater cell numbers than cultures of calf (figure 8d) or adult (figure 8f). Both calf (figure 8c) and adult (figure 8e) AF cells grew as much smaller clusters than those of the NP. Figure 8a shows the appearance of an alginate bead containing cells at day 0 to compare the intensity of staining at day 12 in which there has been deposition of PGs into the alginate matrix.

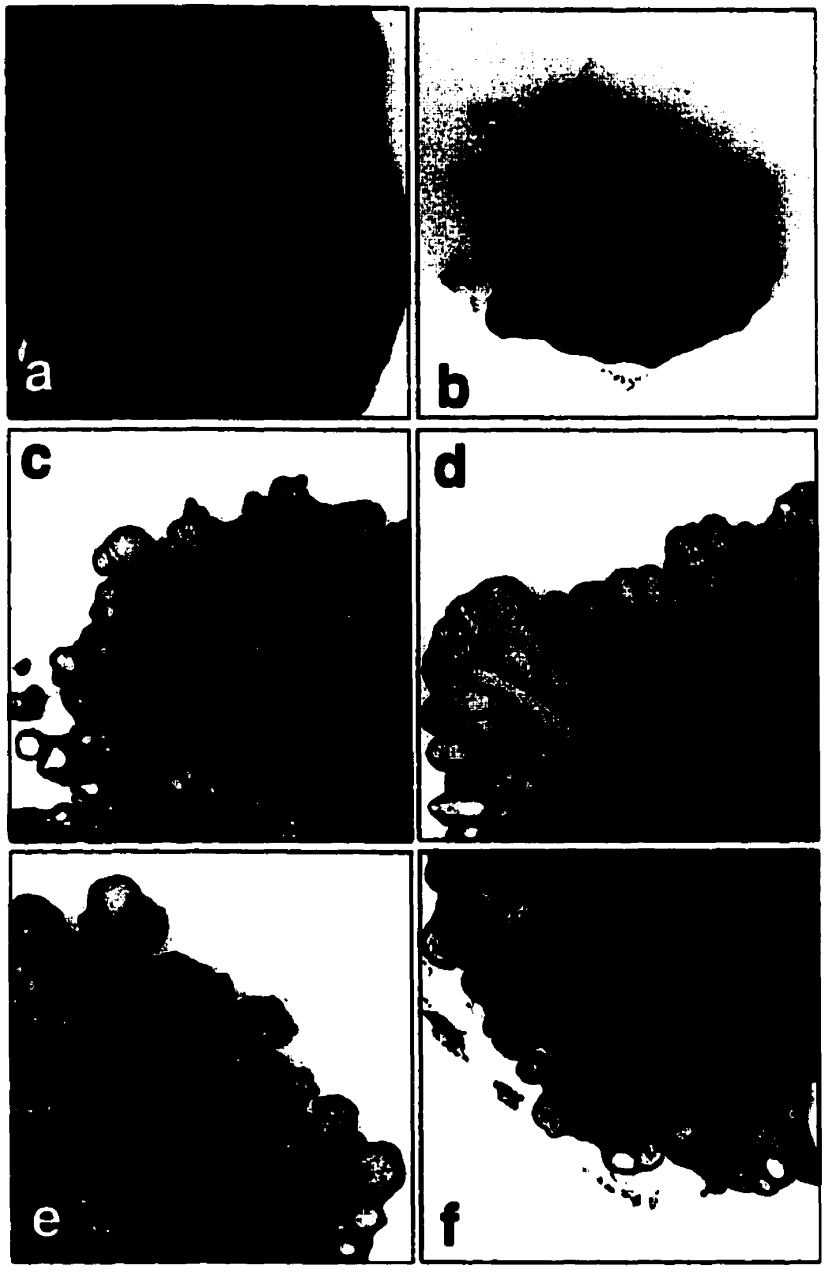

Figure 8. Appearance of disc cells within alginate beads stained with toluidine blue **a)** Calf AF cells immediately after encapsulation (NP had similar appearance). **b)** Foetus NP cells at day 12. Calf AF (**c**), and NP (**d**) at day 12. Adult AF (**e**), and NP (**f**) at day 12. Magnification 200x; sizes of beads appear different due to different levels of cuts throughout the plane of the microsphere.

4.3) Quantification of proteoglycan synthesis of cells cultured in alginate beads:

Quantification of ^{35}S-labelled PGs was performed using the RFA described in section 3.4. Rates of PG synthesis were described as ^{35}S cpm/μg DNA. In AF cells, no difference in sulphate incorporation was observed between calf and adult cells at days 5 and 10 of culture. However, a slight increase in PG synthetic rate (1.5 fold for calf and 1.6 fold for adult; both $p<.01$ by Rank sum test) was observed from days 5 to 10 (figure 9, left panel).

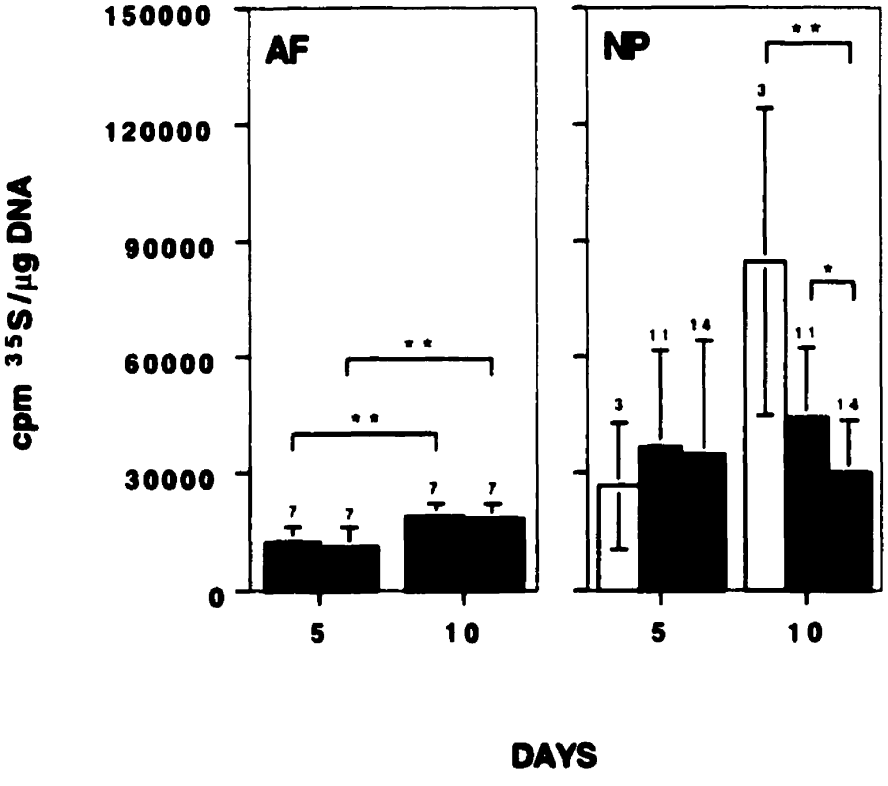

Figure 9. Proteoglycan synthesis in serum-containing alginate bead cultures of bovine annulus fibrosus (AF) and nucleus pulposus (NP). White: foetus, Grey: calf, Black: adult. Error bars indicate standard deviations. Numbers above each bar indicate the sample size. Significant differences obtained by Mann-Whittney Rank Sum test (* = $p<.05$, ** = $p<.01$) are indicated.

Significantly higher sulphate incorporation (1.5 fold) was observed in calf NP cells compared to those of the adult at day 10 of culture, ($p<0.01$, Rank sum test), (figure 9, right panel). Also, 2.8 fold higher synthesis rates seen in the foetal NP compared to those of the adult, were significant ($p<0.05$, Rank sum test),

however due to high variability and low sample size, the 1.9 fold higher PG synthesis rate in foetal cells compared to those of the calf, was not significant. No significant differences were observed between any respective age groups from day 5 to 10 of culture.

In general, synthesis rates were higher in the NP than AF. Calf NP cells incorporated 3 times greater amounts of radioactive sulphate than AF cells at day 5 and 2.4 times more at day 10 (both time points: $p<.01$, Rank sum test). Similarly, adult NP cells had a 2 fold higher synthetic rate compared to the AF at day 5 of culture ($p<.05$) and at day 10, a similar trend was noted though this was not significant.

4.4) Separation of alginate from PGs to enable their visualisation by electrophoresis:

Experiments designed to measure whether any PGs were lost when precipitating away alginate showed no such loss (figure 10). A significant correlation ($r^2=0.993$) was observed between ^{35}S- labelled PG quantified by RFA, used as the "gold standard" and quantities precipitated by 75% v/v 2-propanol precipitation after intermediate 33% v/v 2-propanol precipitation to remove alginate from the solution.

The alginate pellet recovered after 33% precipitation was also counted to further verify that there was no loss of PG. In these experiments, the over all yield of cpm recovered in the alginate pellet accounted for only 1.5% (sd=2%) of the total yield of labelled PGs after 75% 2-propanol precipitation. A correlation between values obtained for the amount of total PG retained in the matrix of alginate beads over the 10 day culture period, measured by DMMB assay, and those obtained for newly synthesised PG by RFA at day 10 is also shown in figure 10 ($r^2=0.766$).

The precipitation of DCN and BGN could also be achieved using this method. Figures 16b and 17 show samples which were precipitated with 2-PrOH. Samples containing small PGs recovered using this method (blotted with specific antibodies for DCN and BGN) from alginate bead cultures are shown in figure 24.

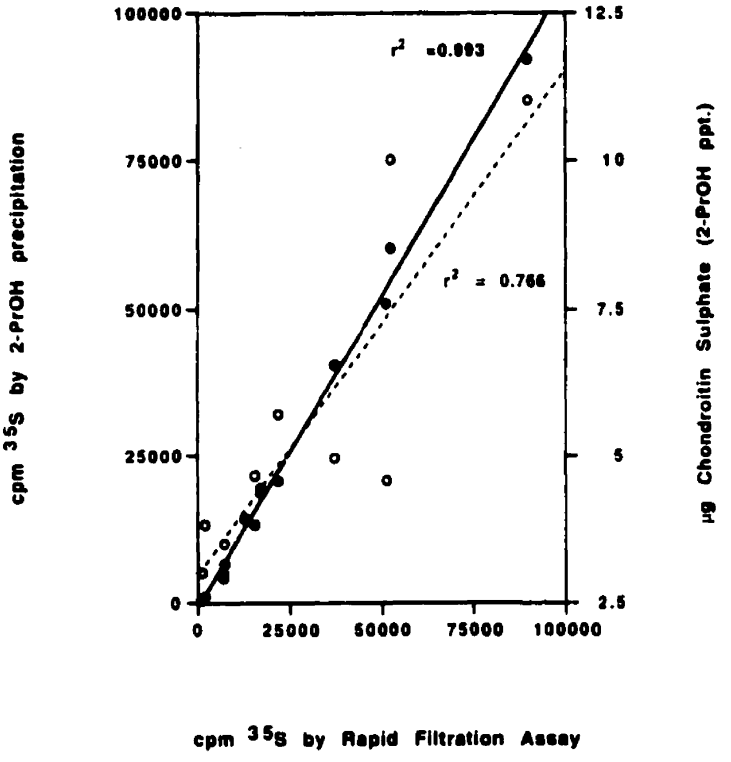

Figure 10. Comparison between cpm ^{35}S recovered from alginate beads by rapid filtration assay (left hand x-axis) and from 75% 2-propanol precipitation following intermediate 33% 2-propanol precipitation to remove alginate and rapid filtration assay (RFA) (y-axis) [solid line, black points; r^2=0.993]. The points shown in white represent the correlation between recovered cpm from RFA and chondroitin sulphate content measured by DMMB assay (right hand x-axis) in the same samples shown on the left hand axis. [broken line, r^2=0.766]. Sample size n=12.

In order to test whether all of the alginate present in the 4M GuHCl extraction solution was precipitated in the intermediate 33% v/v 2-propanol precipitation step, a single quantity of alginate (equivalent to 5 alginate beads) was subjected to increasing concentrations of 2-propanol. At a concentration of 33 % v/v 2-propanol all of the alginate contained in solution was precipitated (figure 11). The DMMB assay was found to have a linear range within the working concentrations of alginate used in these experiments [figure 11 (inset)].

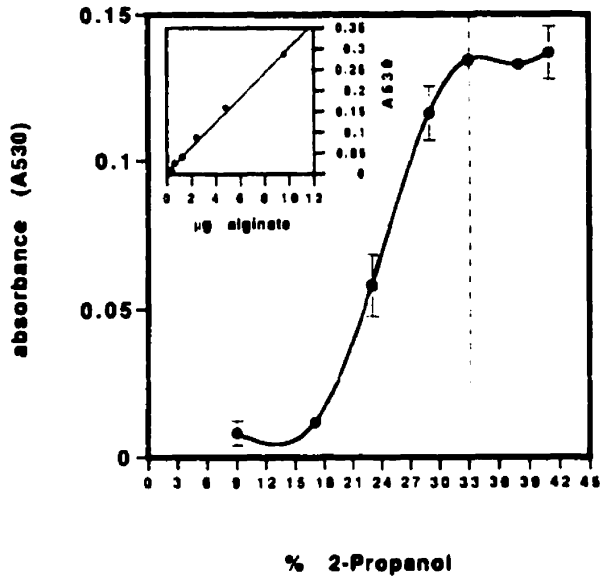

Figure 11. Precipitation of alginate (equivalent to 5 alginate beads) by increasing concentrations of 2-propanol measured by DMMB assay. Points are means of 4 replicates and error bars indicate standard deviations. The broken line marks the concentration used to precipitate alginate in culture experiments. Inset shows the linearity of the DMMB assay when used to detect known concentrations of alginate within the range of optical desities of interest. Error bars are not visible at the scale used.

The quality of visual analysis was greatly improved by this method. Bands of aggrecan down to products smaller than the DCN/BGN standard were recovered (See figure 23b). Toluidine blue stained composite gels showed a smear in lanes in which alginate-containing samples were applied (figure 12a, lane indicated by "-"). Autoradiography yielded clear bands in propanol treated samples (figure 12b "+" lane) and diffuse, further migrating smears in untreated samples (figure 12b "-" lane). Smearing became much worse in the presence of larger amounts of alginate, as was observed in untreated samples of AF cells cultured in FCS or NP cells cultured in serum-free medium (not shown). In these samples, more extract had to be loaded per lane in order to achieve the minimum 7500 cpm per lane.

^{35}S-labelled PGs from monolayer culture to which was added the equivalent amount of alginate contained in 5 alginate beads, yielded identical PG profiles when treated with 2-propanol as those to which no alginate was added (not shown). The same sample from which alginate was not removed yielded a

smear in which no distinct bands could be discerned. A spurious band which stained with toluidine blue was observed in 2-propanol precipitated alginate containing samples if CHAPS was omitted from the 4M GuHCl extraction solution.

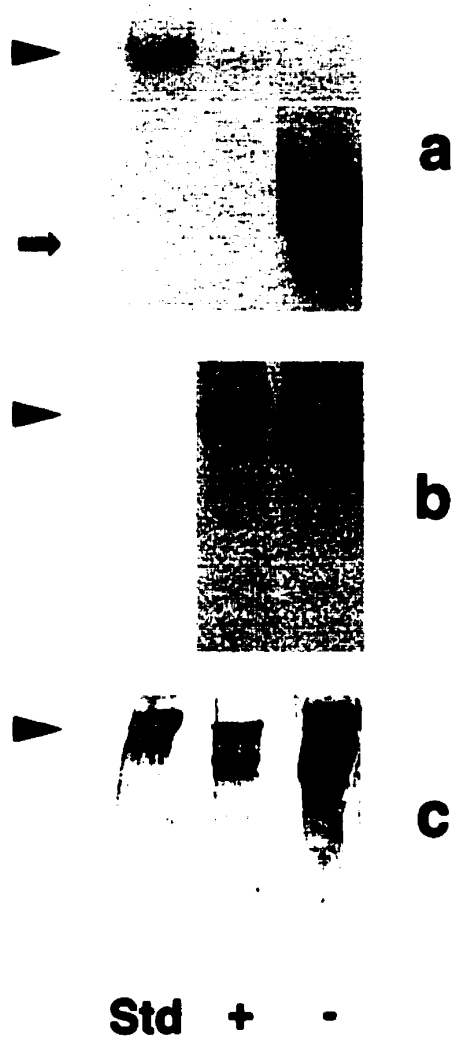

Figure 12. Gel electrophoresis of bovine adult nucleus pulposus samples with or without removal of alginate. First lane (Std) indicates 5μg of standard bovine foetal aggrecan, (+) indicates sample from which alginate was removed and (-) not removed. The arrowhead shows the position of the standard in all panels. **a)** Toluidine blue stained composite gel: Black arrow highlights the smear observed in samples containing alginate. **b)** Autoradiography of the gel shown in "a". **c)** Western blot with 3B3 antibody on the same samples following digestion with chondroitinase ABC and run on 5% SDS-PAGE. Note the distortion of the standard caused by running alginate containing samples on the same gel.

Samples subjected to SDS-PAGE also displayed a great deal of distortion when alginate was not removed. Figure 12c shows the appearance of PGs isolated from within alginate beads with (+) and without (-) removal of alginate. Samples were digested with Ch'ase ABC and electrophoresed on SDS-PAGE followed by Western blotting with the 3-B-3 antibody. It was also observed that in both SDS-PAGE and composite gels, even samples that did not contain alginate were distorted to varying degrees when run on the same gels alongside alginate containing samples. This was especially true of samples run in the outer lanes. The amperage observed in electrophoretic runs of alginate containing samples was sometimes twice that observed for alginate-free samples.

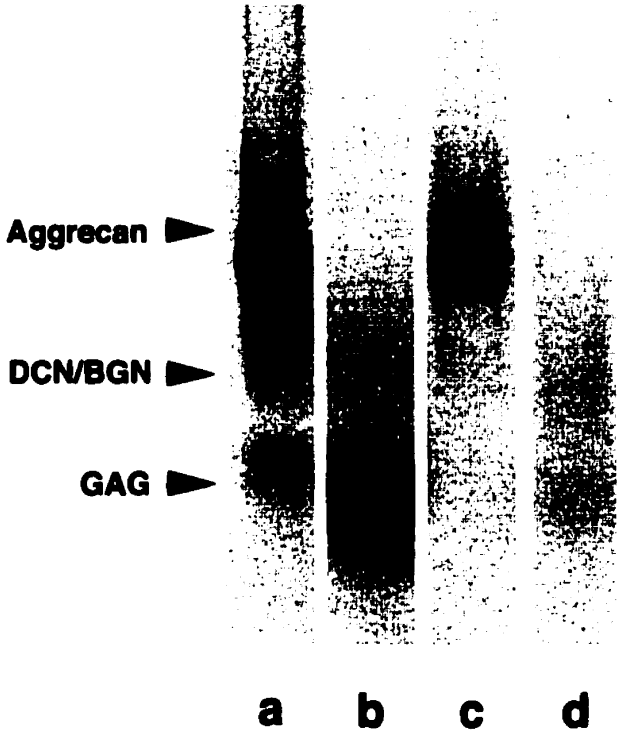

Figure 13. Autoradiography of a composite gel showing the effect of papain and chondroitinase ABC on ^{35}S-labelled proteoglycans recovered by 75% 2-propanol precipitation following intermediate 33% 2-propanol precipitation of bovine nucleus pulposus cells cultured in alginate at day 10 of culture in presence of serum. Lane **a)** no enzyme treatment, **b)** treated with papain, **c)** treated with chondroitinase ABC, **d)** papain followed by subsequent Ch'ase ABC treatment. The position of standards was determined by toluidine blue staining of the following: Aggrecan=bovine foetal epiphyseal, DCN/BGN=bovine nasal decorin and biglycan. GAG highlights the position of glycosaminoglycan chains obtained after papain digestion of proteoglycans.

To further prove that the ^{35}S-labelled compounds resolved on composite gels were actually PGs, alginate-free samples were treated with Ch'ase ABC and papain respectively and subsequently (figure 13). In figure 13b, the effect of papain on the isolated molecules was consistent with that of its effect on large CS PGs, by which all molecules are cleaved into faster migrating products labelled GAG. Ch'ase ABC (figure 13c) which cleaves GAG chains into smaller sugar units, was observed to diminish the presence of ^{35}S-labelled molecules. These two enzymes used sequentially (figure 13d) eliminated the molecules almost entirely.

4.5) Visualisation of proteoglycans produced in culture with FCS:

Initially it was only possible to visualise PGs in monolayer culture. As mentioned in section 3.6, difficulties arising from the presence of alginate in extracts of alginate bead cultures made it unfeasible to visualise PGs synthesised in these cultures. PGs extracted from cell monolayers showed age-related differences. In the NP, the PG profiles of foetal cells consisted of two major bands, the slower migrating one being the most intense (figure 14a). In the calf, the two bands were of equal intensity, while in the adult, these two bands were no longer observed (figure 14a). Also, there appeared to be an age related increase in a fast migrating form which ran below the DCN/BGN standard and close to the position of papain generated GAG chains.

PGs released into the medium were very similar in the foetus and calf, to those observed in the cell layer (figure 14b). In the medium of adult NP cells however, there was evidence of the bands thought to correspond to aggrecan, not observed in the adult cell layer. Also, the fastest migrating band, running close to the position of papain generated GAG chains, was not present in the medium of NP cells cultured in monolayer at any age. It must be noted that while all lanes were loaded with an equivalent number of cpm ^{35}S, for both cell layer and medium, the amount observable in the adult appears to be inferior to that of the foetus and calf possibly due to greater heterogeny of the PGs extracted from adult NP cell monolayers.

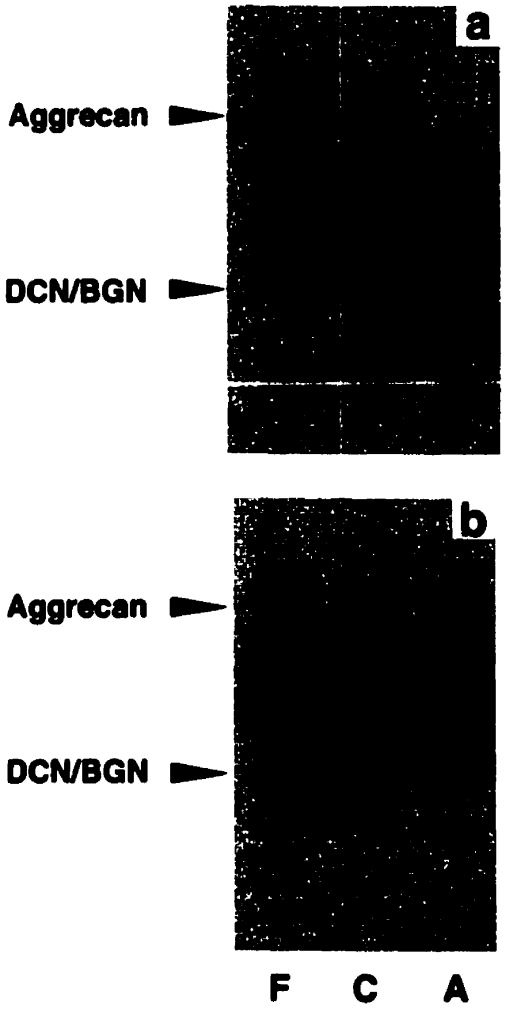

Figure 14. Autoradiography of a composite gel of ^{35}S-labelled proteoglycans synthesized by bovine nucleus pulposus cells after 10 days in serum-containing monolayer cultures. **a)** cell layer and **b)** medium. Loads are equalized to 15000 cpm per lane. F=foetus, C=calf and A=adult. [Aggrecan=the position of bovine foetal epiphyseal aggrecan. DCN/BGN=the position of bovine nasal decorin and biglycan (determined by toluidine blue staining of standards; Not shown).

With the technique elaborated in section 3.6, it became possible to visualise the profiles of ^{35}S-labelled labelled PGs synthesised by cells cultured in alginate beads. Calf and adult AF cells were able to survive and produce PGs in serum-containing alginate bead cultures. In contrast, few cultures of foetal AF cells survived in three-dimensional culture. Three major bands were observed in PGs extracted from within alginate beads containing AF cells (figure 15a). A striking difference in electrophoretic profile was observed between cultures of AF

cells from young animals (foetus and calf) when compared to those of the adult. Adult AF cells produced a distinct band of a large ^{35}S-labelled PG, while those synthesised by young animals tended to be more heterogeneous (figure 15a). For PGs extracted from NP cells cultured in alginate beads, the same PG profile was observed at all ages, with the presence of a single major band, corresponding to aggrecan (figure 15c). Longer exposures yielded the presence of a fast migrating band migrating close to the position of papain generated GAG chains, as shown for calf and adult in figure 23b.

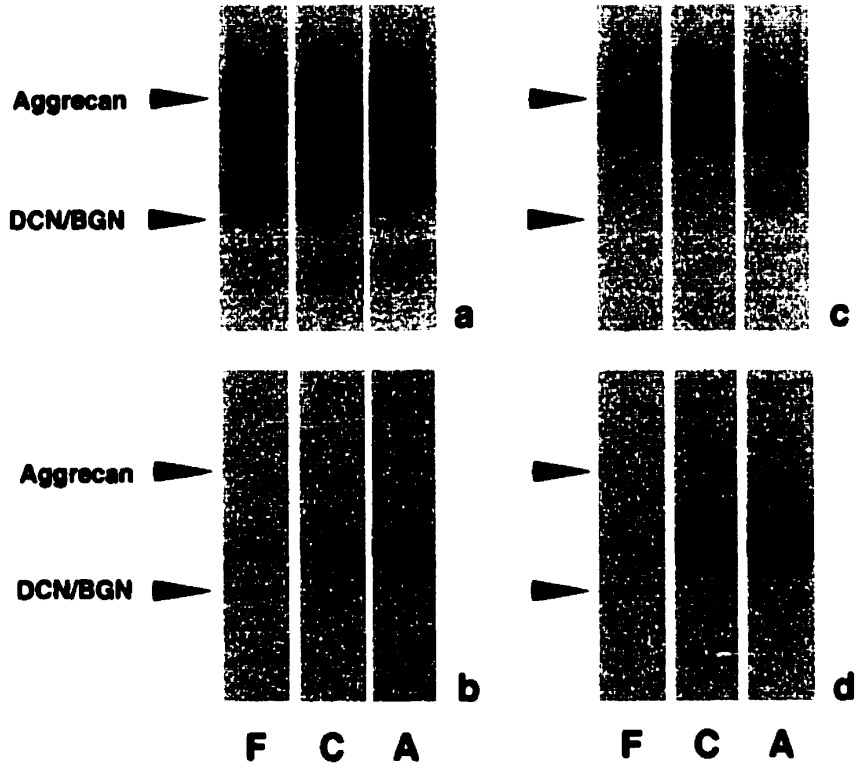

Figure 15. ^{35}S-labelled proteoglycans from bovine disc cells in alginate beads at day 10 in serum-containing culture electrophoresed on composite gels. **a)** annulus fibrosus (AF) alginate beads **b)** AF medium **c)** nucleus pulposus (NP) alginate beads **d)** NP medium F=foetus, C=calf and A=adult. **a** and **c** equalized to 15000 cpm per lane (24 hour exposure). **b** and **d** equalized to 5000 cpm per lane, except foetal annulus medium in **b** which is substantially less, (6 day exposure). [Aggrecan=the position of bovine foetal epiphyseal aggrecan. DCN/BGN=the position of bovine nasal decorin and biglycan (determined by toluidine blue staining of standards; not shown).

PGs were essentially undetectable in the medium of alginate bead AF cell cultures (figure 15b) when directly compared to those of NP cells (figure 15d). In NP cells, there was an age related increase in products observed in the medium. The major form was observed to migrate between the positions of the aggrecan and the DCN/BGN standards (figure 15d). Longer exposures shown in figure 23c revealed the presence of aggrecan in the medium particularly in cultures of adult NP cells. Focusing on NP cells in monolayer, as observed when comparing cell monolayers to medium (figure 14), the presence of the fastest migrating form running close to the position of papain generated GAG chains was only observed within the cell layer (figure 14a). A similar observation was made in alginate bead cultures in that this same band was observed within the alginate matrix (figure 23b) but was not detected in the medium (figure 23c).

4.6) Proteoglycans from direct tissue extraction:

PGs from cultured cells were compared to those found *in vivo*. Extracts from AF and NP tissues immediately after dissection showed both zonal and age-related differences in PG profiles. In general, the major band observed on CAPAGE was of slightly greater mobility than the bovine foetal epiphyseal aggrecan, used as a standard. Also, PG molecules extracted from NP tissues were slightly less mobile than those of the AF (figure 16).

In lumbar tissues, the major differences were observed between different age groups (figure 16a). In the foetus, a single major band was seen, in both AF and NP (NP had to be diluted in order to solubilize all in SDS-PAGE buffer). Banding patterns in the calf were again similar between zones, however a lower band became evident. In the adult, this lower form consistently accounted for most of the PG extractable from NP in both lumbar (figure 16a) and tail (figure 16b) tissues.

Bovine tail NP tissues yielded PG profiles which were similar to those obtained in the lumbar NP (figure 16b). On the other hand, PGs extracted from tail AF tissues showed differences with respect to those of the lumbar AF and NP (both tail and lumbar), especially in younger animals. The major band observed in foetus tail AF tissue, migrated slightly above the DCN\BGN standard. A similar

band was observed in tail AF extracts (figure 16b) from calves, though aggrecan was clearly detectable in these tissues. Adult tail AF extracts resembled those of the NP and in turn, to those obtained from the lumbar AF region (figure 16a).

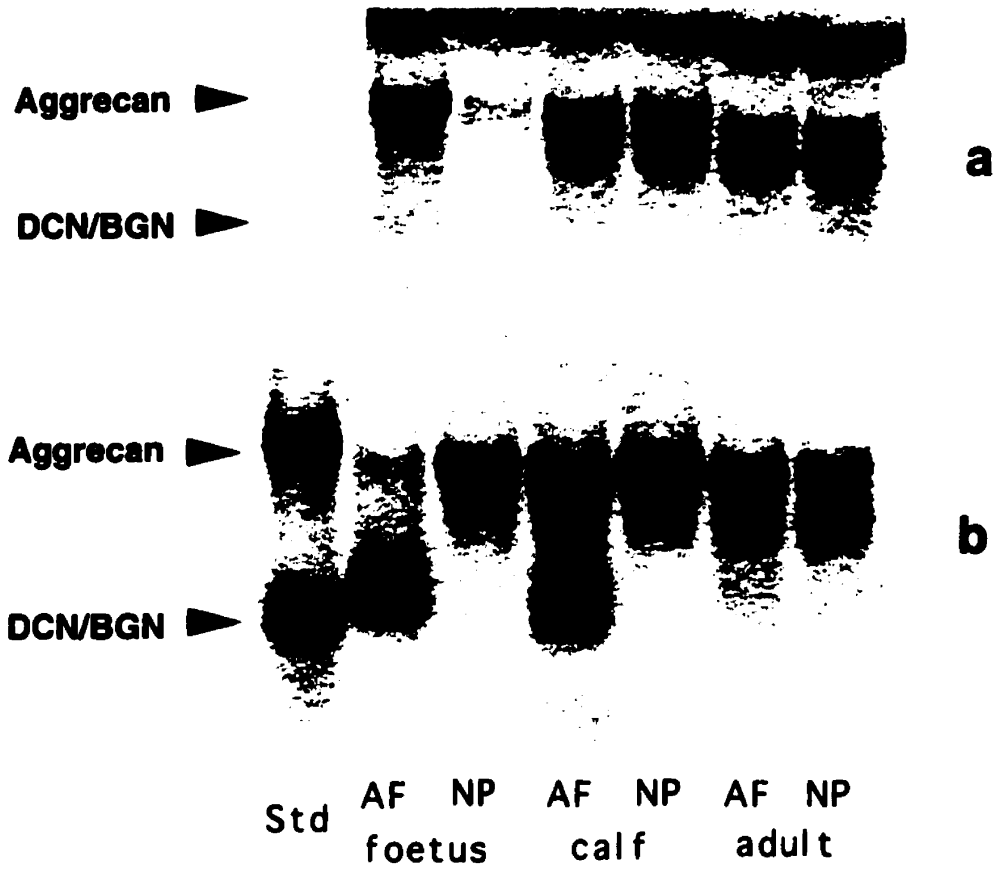

Figure 16. Toluidine blue stained composite gels of GuHCl extracted proteoglycans from annulus fibrosus (AF) and nucleus pulposus (NP) tissues of bovine intervertebral discs. **a)** lumbar and **b)** tail. Samples are equalized to 5μg chondroitin sulphate per lane by DMMB assay with the exception of foetal lumbar NP which had to be diluted 1:20 in order to avoid gelation in the SDS containing sample buffer. Std= standards: Aggrecan=bovine foetal epiphyseal, 5μg; DCN./BGN.= bovine nasal decorin and biglycan, 20μg.

Relative levels of both DCN and BGN were also determined using specific antibodies to their core proteins. Levels of DCN were found to be substantially higher in extracts of AF tissue at all ages than in those of the NP. Levels of DCN were often undetectable with the antibody used against 2.5 μg CS per lane in extracts of NP tissues at all ages (figure 17a). However, in longer exposures of blots to chemiluminescence film, some reaction could be observed (See figure

24a, lanes indicated "DE"). Reactivity with the foetal AF was much higher than that seen in all other tissues since the levels observed were on a substantially lower per CS basis. The standard 2.5 mg CS per lane could not be loaded for these samples since CS levels were undetectable by DMMB assay.

Reactivity of the antibody to BGN was seen in all tissues of the bovine tail IVD (figure 17b). As for DCN, the AF showed much greater reactivity than those of the NP. However with BGN, an age related decrease in relative levels was observed in both tissues. Again, as for DCN, amounts of CS loaded for the foetus AF are much less than those for all other tissues.

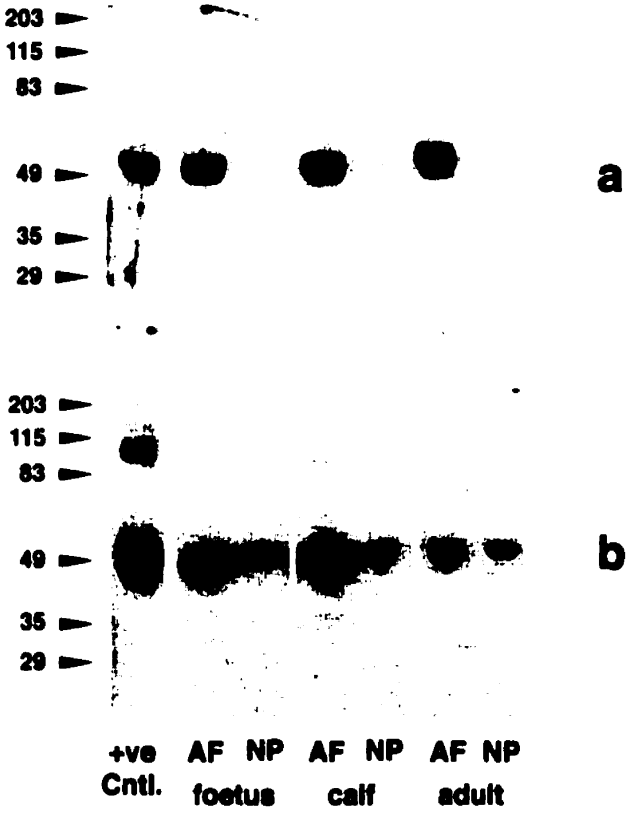

Figure 17. Western blots of small proteoglycans extracted from bovine tail disc tissues with 4M GuHCl and run on 10% SDS-PAGE after treatment of samples with chondroitinase ABC and keratanase II. **a)** blotted with anti-decorin and **b)** anti-biglycan. Reactive bands were resolved using a chemiluminescent substrate for alkaline phosphatase. +ve Cntl.=positive control: bovine nasal PG (reactivity at approximately 100 kDa is thought to be due to dimerization), AF=annulus fibrosus, NP=nucleus pulposus. Loads were equalized to 2.5μg chondroitin sulphate (CS) per lane by DMMB assay except foetal AF in which CS levels were undetectable.

4.7) Determination of GAG chain length in PGs produced by NP cells cultured in FCS (alginate beads):

To determine whether there were differences in length of the GAG chains synthesised *in vitro* vs. *in vivo*, extracts were subjected to chromatography on Sepharose CL-6B resin. PGs extracted from foetal NP tissue had the longest GAG chains (K_{av}=0.57) (figure 18 top), when compared to those from tissue extracts from calf and adult (both K_{av}=0.62), (figure 18 middle and bottom). The same pattern was observed in FCS culture (data not shown). GAG chains of PGs extracted from foetal NP cells cultured in alginate beads were longer (K_{av}=0.50) compared to those of calves and adults (K_{av}=0.62). As shown, no difference in GAG chain length was observed between tissue extractions and PGs synthesised by calf and adult NP cells after 10 days of culture in FCS.

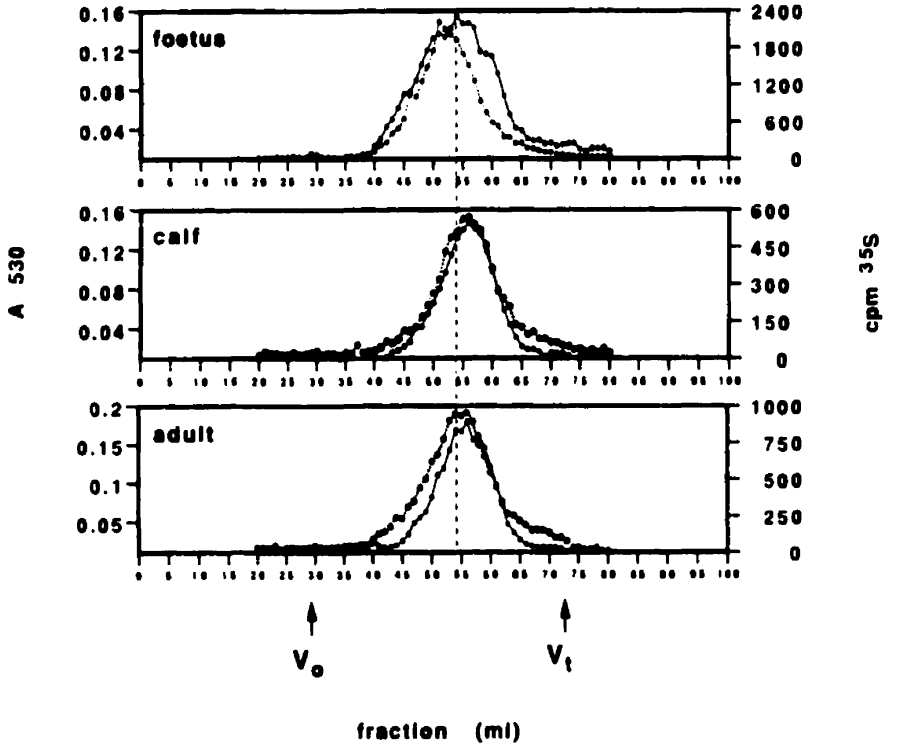

Figure 18. CL-6B column chromatography profiles of glycosaminoglycan chains from PGs extracted from tail nucleus pulposus (NP) tissue [black points with solid line] measured by DMMB assay [left had axis] and ^{35}S-labelled proteoglycans from alginate bead cultures of NP cells at day 10 [white points with dotted line] measured by liquid scintillation counting [right hand axis]. Broken vertical line indicates the position of foetus direct extraction (54th. fraction).

4.8) Cell viability and proliferation in serum-free culture within alginate beads:

AF cells were viable in alginate bead culture with serum however, they could not be maintained in serum-free culture (data not shown). For this reason, the focus was directed to growth factor stimulation of NP cells which were viable in ITS (figure 19). At day 5 of culture, no difference was observed in cell proliferation between cells cultured in ITS (white bars) and ITS+TGF-β1 (black bars). This was true in both calf (left panel) and adult (right panel). No proliferation was observed in ITS cultures of both calf and adult cells at both days 5 and 10. Significant cell proliferation (2 fold, $p<.05$, One way ANOVA) was however observed in TGF-β1 treated cultures of calf cells from day 5 to day 10 of culture. Though a similar increase in cell proliferation was observed in adult NP cells, this was not found to be significant.

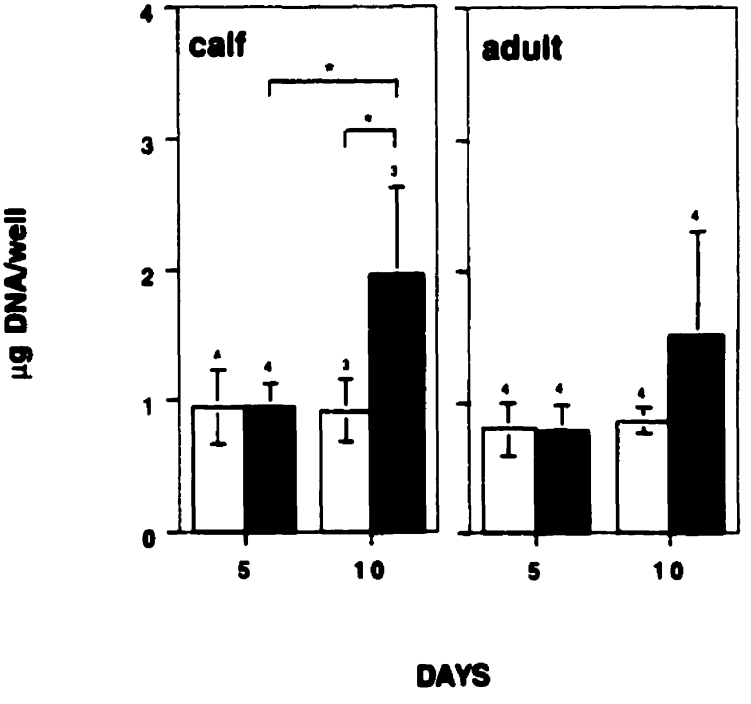

Figure 19. Quantification of DNA in serum-free cultures of bovine nucleus pulposus cells in alginate beads. White bars=serum-free medium (ITS) and black bars= ITS+TGF-β1 at 1 ng/ml. Error bars indicate standard deviations. Numbers above each bar indicate the sample size. Significant differences obtained by One way ANOVA (* = $p<.05$) are indicated.

When comparing treated and untreated groups within each age category, a two fold higher DNA content was observed in ITS+TGF-β1 versus ITS cultured calf NP cells at day 10 ($p<.05$, One way ANOVA). Again, this was not significant for the cells of the adult. Comparison of DNA contents of NP cells from the two respective age groups in serum-free culture to those of NP cells in FCS yielded that there was no difference at day 5. However at day 10, calf NP cells cultured in ITS had 2 times lower DNA content than their counterparts in FCS ($p<.05$, One way ANOVA). For the same comparison, a 2.4 fold less content was observed in adult NP cells at day 10 of culture ($p<.05$, One way ANOVA). These differences between ITS and FCS cultures are a reflection of the increase in proliferation in the latter along with no proliferation seen in ITS cultures. In both calf and adult cells, the difference in DNA content between FCS and TGF treated cultures was not significant.

4.9) Quantification of PG synthesis in serum-free culture with and without TGF-β1:

TGF-β1 was observed to have a positive effect on overall PG synthesis by day 5 of culture as shown in figure 20. ITS cultures (white bars) had significantly lower PG synthesis rates than cultures with TGF-β1 (black bars) and this at both days 5 (3.6 fold in the calf and 3.1 fold in the adult, $p<.05$, One way ANOVA) and 10 (5.9 fold in the calf and 6.2 fold in the adult, $p<.05$, One way ANOVA) of culture.

In comparison to FCS cultures (not shown) at both days 5 and 10, cells of both calf and adult NP cultured in ITS had synthetic rates which were not significantly different. However, TGF-β1 treated cultures differed from cells in FCS in that a 3 fold higher rate was observed in the calf and a 4.3 fold higher rate in the adult ($p<.05$, One way ANOVA).

The proportion of newly synthesised PG produced by NP cells retained within alginate beads and that lost to medium was calculated and depicted in figure 21. The amount of PG released into the medium was generally less than that retained within alginate beads. The highest proportional loss into medium was observed in alginate cultures treated with ITS alone shown as white bars in figure 21. This loss was observed in both calf (left panel) and in adult (right panel), as

early as day 5 of culture and was maintained at day 10. In comparison, serum-free cultures of NP cells treated with TGF-β1, showed a marked decrease in proportion of PG released, this loss ranging from 5-10%, at both time (black bars, figure 21). However this was only significantly different in ITS treated cells of the calf at both days 5 (4.9 fold difference, $p<.05$, Rank sum test) and 10 (7.4 fold difference, $p<.05$, Rank sum test).

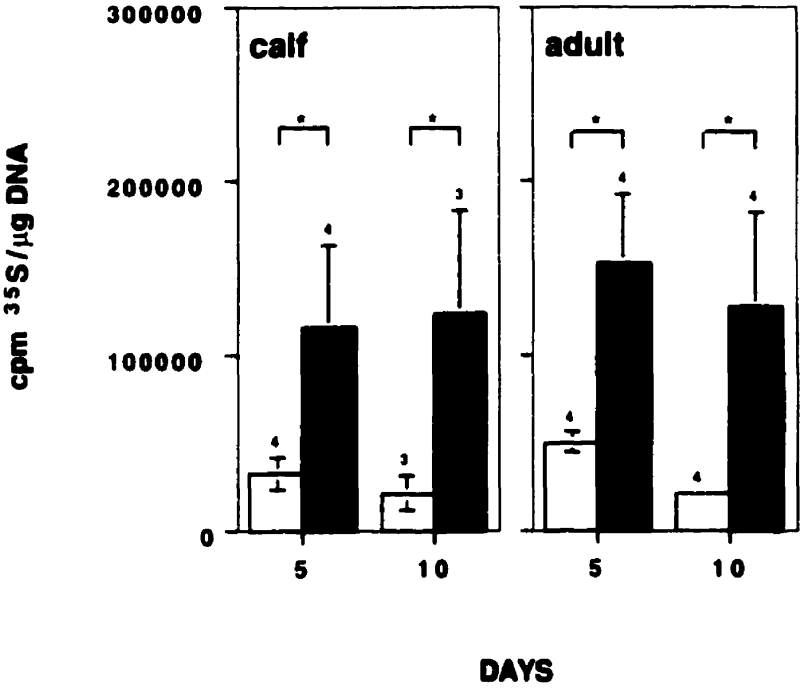

Figure 20. Proteoglycan synthesis in serum-free cultures of bovine nucleus pulposus cells in alginate beads. White bars=serum-free medium (ITS) and black bars= ITS+TGF-β1 at 1 ng/ml. Error bars indicate standard deviations. Numbers above each bar indicate the sample size. Significant differences obtained by one way ANOVA (* = $p<.05$) are indicated.

Figure 21 also includes the proportions calculated for NP cells grown in FCS shown as grey bars. Newly synthesised PG release into medium was significantly higher in FCS cultures than in those in ITS+TGF-β1 in the calf at day 10 (3.9 fold difference, $p<.01$, Rank sum test) and in the adult at both time points (day 5: 3.8 fold difference, day 10: 2.5 fold difference, $p<.01$, Rank sum test). No significant difference in PG release was obtained between FCS and ITS cultures at

either age group or time point in culture. Statistics were not very powerful due to low numbers (n=2-4) however, it was noted that results obtained by CAPAGE of PG released into medium (enumerated in section 4.10) are in accordance with the trends observed. No difference could be reported between respective treatments at both time points of culture and this for both calf and adult cells.

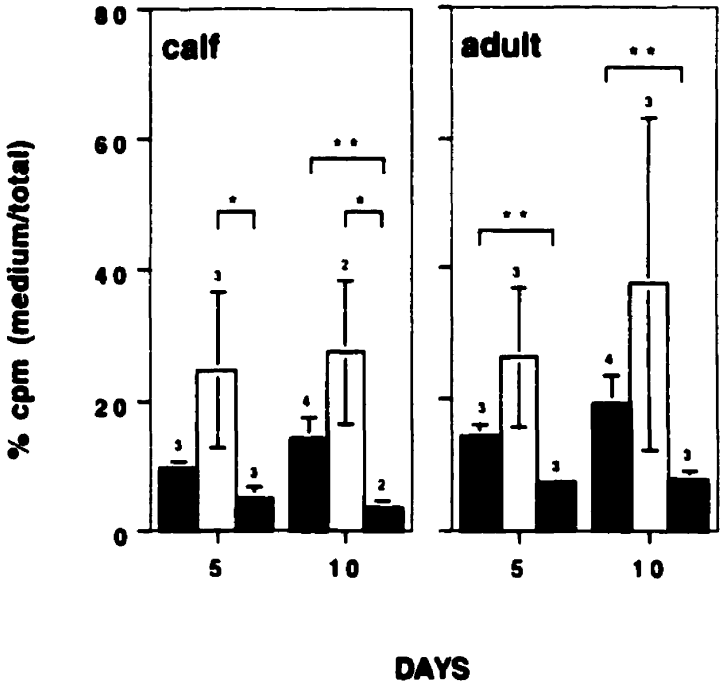

Figure 21. Percentage of labelled proteoglycan released into medium compared to total (within alginate beads + released into medium) from bovine nucleus pulposus cells. Grey bars=FCS cultures, white bars=serum-free medium (ITS) and black bars= ITS+TGF-β1 at 1 ng/ml. Error bars indicate standard deviations. Numbers above each bar indicate the sample size. Significant differences obtained by Mann-Whittney Rank Sum test (* = $p<.05$, ** = $p<.01$) are indicated.

4.10) Visualisation of PGs synthesised in serum-free culture:

PGs produced in serum-free culture could initially only be visualised in monolayer culture due to the difficulties in resolving those produced in alginate bead culture. Little difference was observed between PGs produced by NP cells in ITS and TGF culture of young animals (not shown). These were also not very different from those obtained in cultures with serum. In the adult however, culture

in ITS or ITS+TGF-β1 revealed the presence of bands migrating slightly below the aggrecan standard (figure 22a). In the medium, shown in figure 22b, the electrophoretic profiles were similar in all treatments and age groups (foetus and calf not shown).

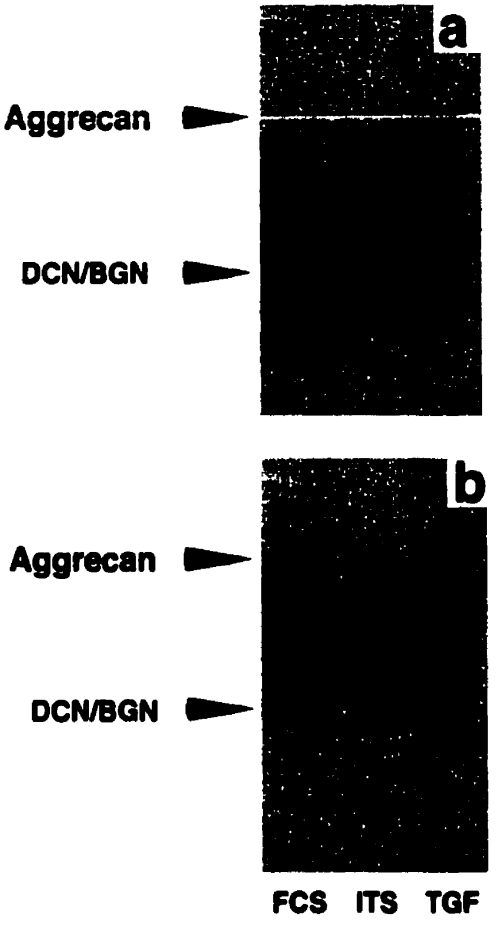

Figure 22. Autoradiography of composite gel electrophoresis of bovine adult tail nucleus pulposus newly synthesized PG at day 10 of monolayer culture in: FCS=cultures with serum; ITS=serum-free medium; TGF=ITS+TGF-β1 at 1 ng/ml. **a)** cell layer and **b)** medium. Loads were equalized to 15000 cpm per lane. Similar results were obtained for foetus and calf NP cells (not shown). [Aggrecan=the position of bovine foetal epiphyseal aggrecan. DCN/BGN=the position of bovine nasal decorin and biglycan (determined by toluidine blue staining of standards; Not shown).

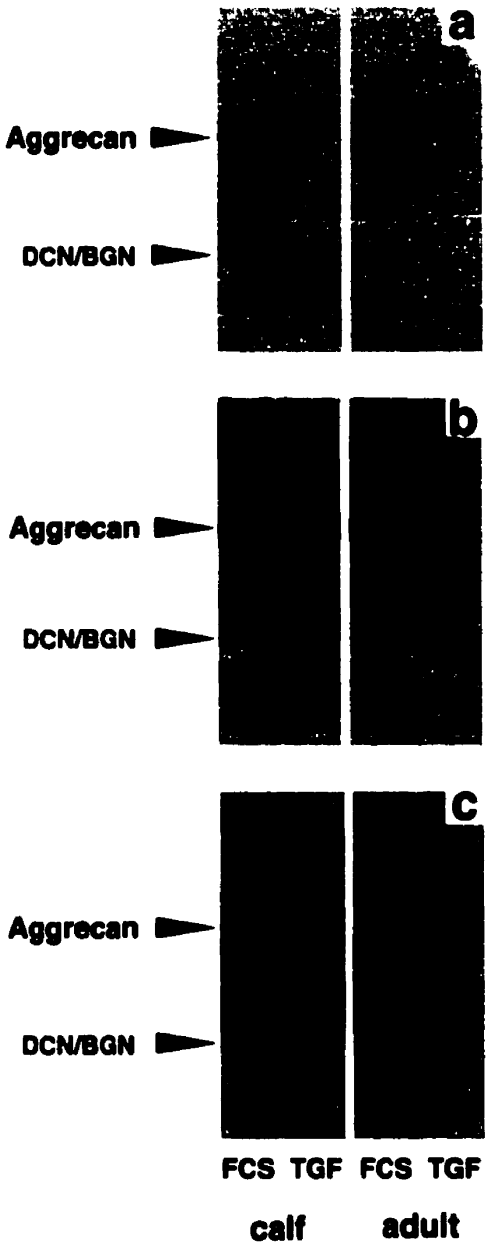

Figure 23. Composite gel analysis of PGs synthesized by nucleus pulposus cells encapsulated in alginate beads (DAY 10). **a)** Toluidine blue stained gel (PGs extracted from beads), **b)** Autoradiography of the same gel shown in **a**. Loads equalized to 15000 cpm per lane, with 48 hour exposure. **c)** Autoradiography of PGs precipitated form medium (lanes loaded on a per culture well basis). [Aggrecan=the position of bovine foetal epiphyseal aggrecan. DCN/BGN=the position of bovine nasal decorin and biglycan (determined by toluidine blue staining of standards; Not shown). FCS= cultures with serum ; TGF=ITS+TGF-β1 at 1 ng/ml. Similar results were obtained for ITS alone (not shown) as those shown for FCS.

Using the technique described in section 3.6, PGs could be separated from alginate and visualised on CAPAGE. The most prominent band observed in alginate beads was aggrecan which was present in large enough quantity to be stained with toluidine blue (figure 23a). Similar electrophoretic profiles were observed in ITS and FCS cultures, for this reason, results for ITS cultures are not shown in figure 23. Autoradiography of CAPAGE yielded a slightly larger form of aggrecan produced by NP cells treated with TGF-β1, also observed with toluidine blue staining, and this in both calf and adult (figure 23b). The fastest migrating band seen in FCS and ITS cultures, migrating below the DCN/BGN was not observed in alginate bead extracts of growth factor treated NP cells. A proportional increase in intensity of a band which migrated alongside the DCN/BGN standard was noted in TGF-β1 treated cultures when compared to those in FCS and ITS (not shown) (figure 23b).

PGs released into the medium of TGF-β1 treated cultures were smaller than those from NP cells cultured in FCS and ITS cultures as shown in figure 23c. The single band observed with TGF-β1 treatment co-migrated with the DCN/BGN standard. Those released from FCS and ITS cultures consisted of a major band which migrated slower than the DCN/BGN standard and another band corresponding to aggrecan. The reduction in amount of newly synthesised PG lost to the medium from the alginate matrix in cultures treated with TGF-β1 compared to those in serum or ITS is appreciable for adult cells (figure 23c).

4.11) Analysis of small PGs in NP cells cultured in alginate beads:

Levels of DCN and BGN were determined by Western blot analysis using specific antibodies to their core proteins. Levels of DCN were undetectable in FCS and ITS cultures of young animals, though in adults, very small amounts were seen (figure 24a, ITS not shown). Treatment with TGF-β1 increased the amount of DCN produced in both calf and adult NP cells at day 10 of culture. Levels found *in vivo* of the respective molecules could be compared from tissue extractions (DE) run alongside as a reference in figure 24.

Similarly, BGN levels were undetectable in culture with serum at all age groups (figure 24b). This was also true for calf and adult NP cells in ITS culture.

TGF-β1 treated cultures showed a marked increase in levels of BGN in both calf and adult NP cells. These levels were similar to those observed in tissue

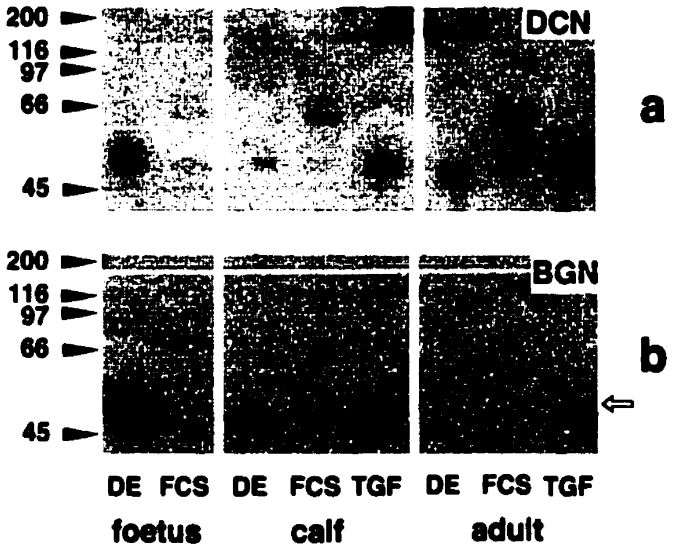

Figure 24. Western blot analysis with **a)** anti-decorin and **b)** anti-biglycan antibodies on chondroitinase ABC and keratanase II treated tissue extracts and extracts of alginate beads at day 10 of culture from bovine tail nucleus pulposus. White arrow indicates the position of the band thought to correspond to pro-biglycan. DE=direct tissue extraction, FCS= cells cultured in presence of serum [Similar results obtained in serum-free culture (ITS), not shown]. TGF=ITS+TGF-β1 at 1 ng/ml. Reactive bands were resolved using a chemiluminescent substrate for alkaline phosphatase.

extracts of calf NP tissue. A slower-migrating band was observed in calf and of even greater intensity in the adult (indicated by a white arrow at the right edge of figure 24b). This top band may be a form of BGN in which the propeptide has not yet been removed.

4.12) GAG chain analysis of serum-free cultures:

GAG chains synthesised in serum-free culture were of the same size in both ITS (white points) and ITS+TGF-β1 (black points), K_{av}=0.50 (figure 25, from adult NP). This is comparatively larger than those of the adult NP cells cultured in FCS and also those isolated from tissue (both K_{av}=0.62). This value compares exactly to that obtained for foetal NP cells cultured in FCS. Extracts from calf NP cells in serum-free medium alone or with TGF-β1 were not applied to column chromatography for comparison.

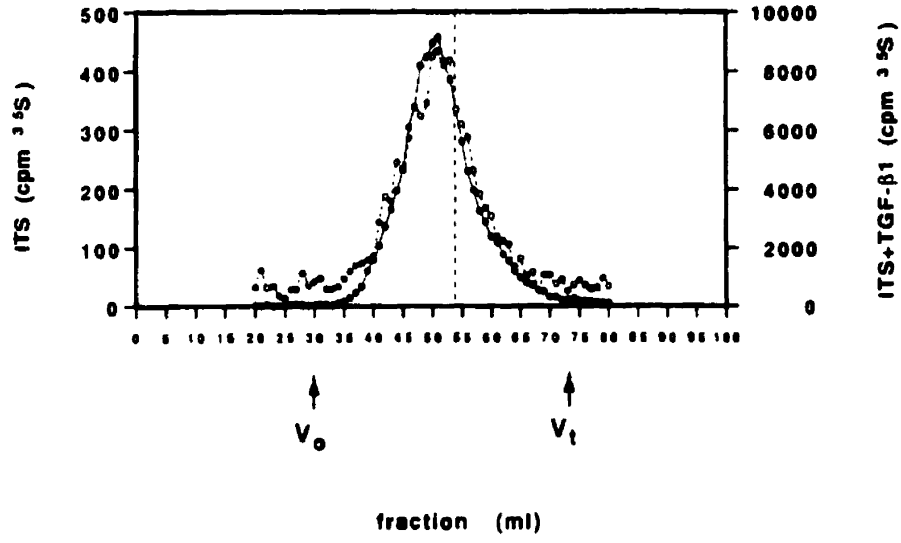

Figure 25. CL-6B column chromatography profiles of proteoglycans extracted from adult tail nucleus pulposus cells in alginate beads cultured in serum-free medium (ITS) [White points with dotted line] and ITS=TGF-β1 [Black points with solid line]. Broken vertical line indicates the position of foetus direct extraction (54 ml.) for reference [also shown on figure 17 top].

4.13) Evaluation of disc cell phenotype from AF and NP regions:

Growth of cells in high density monolayer culture showed differences in gross morphology. Cells from both the AF and NP regions were round upon isolation as shown in figure 26a and 26b. By day 5 of culture, AF cells of the calf had flattened out into a sheet of fibroblasts (figure 26c) while AF cells of the adult still remained essentially polygonal (figure 26d). Cells of the calf and adult NP remained in a polygonal shape characteristic of chondrocytes (figure 26e and f).

Collagen profiles were used at day 10 of culture to determine the phenotype of cells since photography yielded poor resolution due to thickening of monolayers. Figure 27a shows the electrophoretic profiles of ^{3}H-labelled collagens from culture media of AF and NP cells grown in monolayer culture. Type I collagen was present in AF cells of both calf and adult, while no α2 chain was observed in collagens synthesised by NP cells (figure 27a). Collagens from alginate bead cultures, shown in figure 27b, had similar electrophoretic patterns as those from monolayers for AF and NP regions respectively.

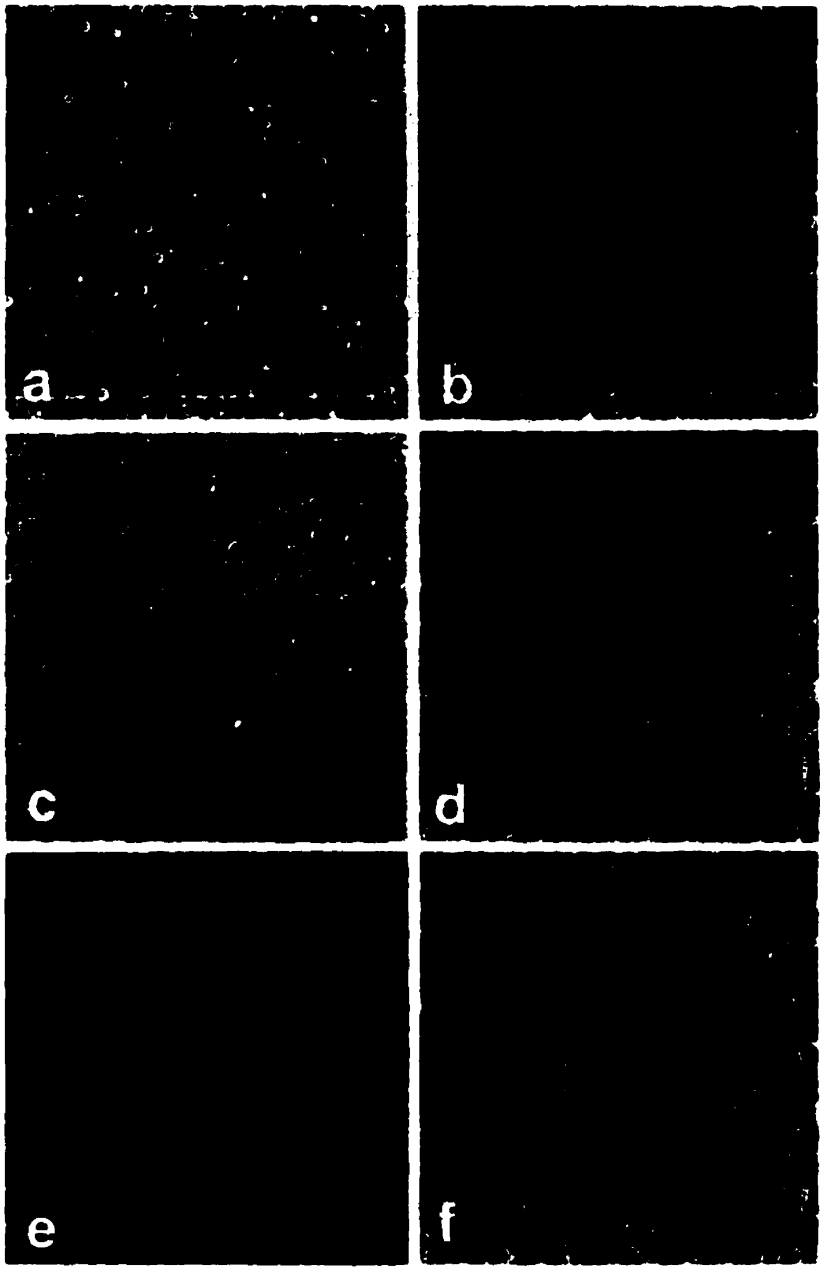

Figure 26. Photographs of bovine tail intervertebral disc cells in high density monolayer culture. Adult **a)** AF day 2; **b)** NP day 2; **e)** AF day 5; **f)** NP day 5. Calf **c)** AF day 5; **d)** NP day 5. Cells of the calf AF and NP at day 2 (Not shown) had similar appearance to those of the adult. Magnification 320 x.

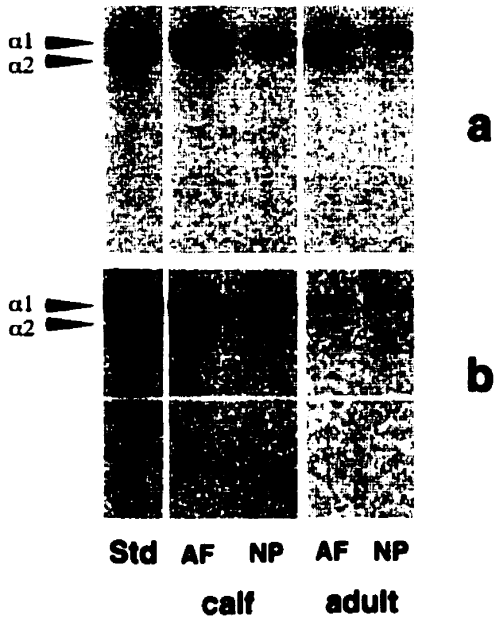

Figure 27. Autoradiography and fluorography of ^{3}H labelled collagens precipitated from pepsin treated medium at day 10 of culture (with serum), produced by bovine disc cells **a)** monolayer culture and **b)** alginate bead culture. Std=standard ^{3}H-labelled Type I collagen, AF=annulus fibrosus, NP=nucleus pulposus. Arrowheads indicate the positions of the collagen α1(I) or α1(II) chains and α2(I) chain.

5) DISCUSSION:

The major aim of this study was to develop a cell culture system in which changes in the rate of synthesis of IVD matrix molecules could be evaluated in cells of animals of different ages. This work focused on the synthesis of small interstitial and large aggregating PGs and the potential use of growth factors to modulate the anabolism of these molecules.

Growth factor stimulation of isolated IVD cells has been shown using TGF-β and IGF-I. Osada et al. effectively used IGF-I on adult bovine NP cells in monolayer culture to stimulate PG synthesis. This study again only involved the stimulation of cells from a single age and was performed on subcultured cells seeded in monolayers (Osada R et al., 1996). Gruber et al. showed that human AF cells grown in alginate bead culture were responsive to TGF-β1 with an elevated expression of BGN and little effect on levels of DCN by ten days of culture (Gruber H.E et al., 1997). This study was based on surgical material from few patients. Furthermore, cells had to be grown in monolayer culture and passaged after isolation, presumably in order to expand cells numbers. Cell phenotype is known to be affected by culture conditions and it is not clear for how long cells were expanded prior to encapsulation in alginate. We have also performed alginate bead cultures of both human AF and NP cells over a fifteen day period (not described in this thesis). In our experience, there is a great deal of variation between human samples therefore, many would have to be processed to obtain conclusive results.

Our findings demonstrate the successful culture of IVD cells from animals of different ages with a reflection of the decreased aggrecan synthesis in cells from aged animals. This system also has the potential to be used to study the synthesis of other matrix molecules and their degradation. Preliminary work on growth factor stimulation showed effective modulation of PG synthesis in adult NP cells by TGF-β1. Small PG forms were also beneficially affected in that the amount of both DCN and BGN was increased on a per aggrecan basis in growth factor treated calf and adult NP cells. Methodology is described for a simple two stage precipitation method to remove alginate from PG containing GuHCl extracts and the recovery of both small and large PG forms.

With regards to methodology, two major issues had to be resolved in order to successfully perform the experiments outlined in this work. First, the cell isolation procedure had to be designed to yield the optimal conditions for cell viability and number. Secondly, a method to separate both small and large PGs from alginate had to be developed to enable the qualitative evaluation of the molecules synthesised by encapsulated cells. These two issues will be discussed in detail in the paragraphs which follow.

It was observed that cell yields could be optimised by using different types of collagenase during the sequential enzyme digestion procedure. While the numbers of recovered AF cells were not very different using type IA or II collagenases, cell viability was often less than 50% when using type IA. For nucleus cells, the yield was low using type II collagenase.

Differences in the Class I and II collagenases from C. histolyticum have been described in characterisation studies (Lwebuga-Mukasa J.S et. al.,1976). The primary difference between the two classes is that the type II collagenase has a much broader specificity than the type I enzyme (Van Wart H.E et. al., 1985). The finding that Class II collagenase was effective in producing cell yields with greater viability than Class I in isolations of AF cells, may suggest that the packing of collagen fibrils hinders the binding of the latter to potential cleavage sites. In contrast, class II collagenase may disrupt the collagen matrix at many more sites thus allowing further cleavage of previously shielded sequences. While explants from both AF and NP tissues were observed to swell during their initial immersion into medium, AF tissue remained swollen following the initial digestion with pronase. The observation that type I collagenase yielded lower cell viabilities in AF tissue may be due to its lower efficiency in breaking up the AF matrix, rich in type I collagen.

The reduced rates of cell recovery by sequential enzyme digestion observed from young to old animals is concurrent with the reduction in cell number in discs of older animals (Buckwalter J.A et. al., 1989). AF cells were smaller upon isolation than those of the NP and were only viable in alginate beads when cultured in presence of serum. The small size of AF cells upon isolation along with the

inability of these cells to survive in serum-free culture suggests inherent differences between these and NP cells.

The second difficulty to be addressed was the removal of alginate from PG containing samples. The observation that alginate is insoluble in a buffer comprising of 33% v/v 2-propanol and 2.67 M GuHCl made it possible to selectively separate alginate from PG which remains soluble under these conditions (Bjornsson S., 1993; Masuda K et. al., 1994). It was also found that CHAPS was essential in order to fully precipitate the alginate present in samples. Omission of this detergent was observed to yield poor resolution of the small PGs on autoradiography along with the presence of a fast migrating band which stained with toluidine blue but did not contain radioactivity. This suggests that without CHAPS, some alginate molecules remained in solution at 33% v/v 2-propanol.

The value of 40% alcohol, solvent tolerance (the alcohol concentration at which alginate begins to precipitate) for alginate as quoted by the manufacurers is for the compound dissolved in water. Therefore, the presence of divalent cations in the solution does not appear to have been considered in the manufacturers determination of solvent tolerance. The high charge on GuHCl is likely to alter the solvent properties so as to allow complete precipitation of alginate around 30% v/v alcohol. 2-propanol was used in these experiments though at 33% v/v EtOH, complete precipitation was also observed. At concentrations below 33% v/v, EtOH was less effective than 2-propanol in partially precipitating alginate.

The interference of alginate in the DMMB assay described by Farndale et al. has been recognized (Farndale R.K et. al., 1986; Enobakhare B.O et. al., 1996). For our purposes, this interaction was of benefit since it allowed us to quantify alginate remaining in samples under study. In this application of the assay it was however vital to establish that there was a linearity within the range of measurement of alginate for the working concentrations used. Thus the plateau in the curve obtained beyond 33% v/v final concentration 2-propanol truly indicates that the maximum amount of alginate in solution has been precipitated at this concentration of alcohol. Furthermore, no staining of a band corresponding to the migration position of alginate could be detected by toluidine blue staining, again

demonstrating total precipitation of alginate at 33% v/v final 2-propanol concentration.

As expected, the distortion of bands observed on composite gels was increased with greater concentrations of alginate in samples. Also, when attempting to equalize sample lanes (on a per cpm or per μg CS basis), different quantities of alginate were inevitably present in each sample. Samples containing the greatest quantities of alginate were observed to migrate further than those with less. In samples where the total cpm was very low, therefore requiring the equivalent of all five alginate beads to be loaded on the gel, the radioactivity was found to be diffused throughout the gel. Also, the presence of a single sample containing alginate on a gel was sufficient to disrupt the migration of samples in the outer lanes, often loaded with alginate-free standards.

Medium samples were also found to contain alginate, though expectedly in much smaller amounts than in GuHCl extracts. This was however enough to disrupt migration since relatively large quantities of medium were required to be loaded in order to obtain a sufficient signal. It was found that the precipitation method to remove alginate yielded a significant improvement in band resolution.

Similar difficulties were overcome for SDS-PAGE. The quantity of alginate in samples made it initially impossible to resolve any electrophoretic profiles. Both large MW proteins; aggrecan core protein (in the range of 300 kDa) and small MW; DCN and BGN (in the range of 40 kDa) could be resolved following removal of alginate with 2-PrOH.

Another minor methodological issue involving the compatibility of the fluorescent dye binding assay to quantify DNA content of alginate bead cell cultures was also adressed. The results obtained when comparing paired samples either with cells isolated from within alginate beads after ten days in culture or with beads simply solubilized prior to proteinase K digestion, were significantly correlated. The higher values obtained with alginate still present in the sample only accounted for about 5 percent of the total value (the "gold standard" was taken as the values obtained for samples free of alginate). This indicates that the fluorescence measurements obtained are a true reflection of the cell number of live cells present at each time point.

Since samples containing alginate were found to yield exactly double the percent fluorescence emitted by Hoechst 33258 when bound to, it was necessary to include it when diluting standards. This was observed in a manner which was not dependant on the concentration of alginate in solution in the range of concentrations used (1-3 mg/ml, final concentration). Bisbezimide is known to bind to A-T pairs within the minor groove of DNA molecules (Martin R.F et. al., 1983). Following the binding of the positively charged moiety of bisbenzimide, hydrogen bonding between its hydroxyl group and those present on DNA may occur (Jin R et al., 1988), however, some bonding my also occur with the polar groups on alginate sugars.

Patterns of cell growth were determined using the fluorescent dye binding assay as described above. In culture with serum, viability was observed in both AF and NP cells. By the end of the culture period, cells of the foetus were generally (though not statistically significant) in highest number. Calf and adult NP cell numbers were not significantly different at both time points measured.

The age-related decrease in PG synthetic rate of NP cells by day 10 of culture reflects the pattern observed *in vivo* in humans (Antoniou J., et al., 1996). While the sample size of foetal NP is low due to the difficulty to obtain sufficient cells from a single animal, all values obtained were higher than any of those observed for single calf or adult cells. Rates of newly synthesized PG loss into the medium showed a trend toward higher release in the adult NP compared to calf, though this did not significantly alter the pattern observed. The obtention of this pattern at day 10 of culture prompted our choice to focus on this time point for analysis of growth factor treated cells. Due to the low yield of foetal NP cells, growth factor stimulation was only performed on calf and adult cells.

The type of PG synthesized by AF cells in alginate beads from each age group as evaluated by CAPAGE indicated that adult cells produced a greater proportion of aggrecan (on an equal cpm basis) than did young animals. This reflects the PG profiles obtained by direct tissue extraction, showing that with increasing age, the cells of the AF produce aggrecan as the major PG form. Greater amounts of a smaller form of PG migrating above the DCN/BGN standard

observed in FCS cultured AF cells also reflects the higher quantity of these small PGs seen in the AF relative to the NP *in vivo*. At all ages NP cells in culture with serum produced a single form of aggrecan most of which was retained within the alginate bead. Furthermore, the larger form migrating alongside the bovine epiphyseal aggrecan standard in cells of the AF in alginate beads, shows that there is a consistent difference at all ages between AF and NP cells in this culture system.

The similarity of newly synthesized PG from cultured NP cells at all ages separated on CAPAGE indicates that these cells conserve the potential to produce similar molecules at all ages. While we did perform toluidine blue staining of gels in order to visualize the PGs resident over the full culture period, no other bands than intact aggrecan were observed. Differences in GAG chain length were however observed. The alginate bead culture system allowed the GAG chain length of aggrecan synthesized by calf and adult NP cells to remain constant for up to 10 days of culture. A previous study showed that GAG chains from cultured foetal NP cells were longer than those synthesized *in vivo* (Mitchell D et. al., 1981) as we have observed. It is known that aggrecan from foetal animals is substituted with CS chains while that of adults is increasingly substituted with shorter KS chains (Webber C et al., 1987).

AF cells secreted very little newly synthesized PG into the medium. In contrast, NP cells showed an age related increase in labelled PG release into the medium and even a loss of seemingly intact aggrecan in adult cells. Since the cell number was not significantly different between calf and adult cells, this reduced ability to retain newly synthesized molecules in alginate beads may be due to alterations in the process of extracellular matrix assembly. This increase in release of PG fragments into the medium with age in serum-rich alginate bead cultures of NP cells may be a reflection of an age related increase in processing as seen *in vivo*. An increased MMP/aggrecanase activity could be suggested however further studies would be necessary to better explain why large amounts of seemingly intact aggrecan are released (as viewed on CAPAGE). Other investigators have shown that disc cells cultured in alginate beads in presence of serum, release approximately 70% of the collagen produced throughout the culture period (Chiba K et. al., 1998). A reduction in synthesis of small PGs observed in cultures with

serum may in part explain this release since collagen fibril assembly would likely be less efficient without adequate quantities of these regulatory molecules.

As mentioned previously, gel electrophoresis was initially only possible for PGs extracted from monolayer cultures. Monolayer cultures are a less realistic model than alginate beads to study intervertebral disc cell metabolism, being two dimensional and having a high concentration of cells in close proximity unlike the *in vivo* state. However, the electrophoretic profiles showed that some processing of newly synthesised aggrecan could be observed, reflecting the patterns seen *in vivo*. Cell monolayers of adult NP cells contained almost no intact aggrecan indicating intense enzymatic activity soon after synthesis. This observation can be compared to the *in vivo* state in which electrophoretic profiles show that the aggrecan extractable from adult disc tissues can be separated into more than one form suggesting increased processing.

The different PG profiles of AF tissue (tail vs. lumbar) seen in young animals suggest a difference in environment or cell type populating these respective tissues at the different levels of the spine. Contrary to the lumbar AF region, the tail AF region of the foetus was rich in small PGs and almost devoid of aggrecan. Significant loads would not be expected on foetal tail AF tissue, but would be expected to have an increased effect on the response of cells to their mechanical environment in the calf and especially in the adult. It could be speculated that this is part of the developmental process which prepares the structures of the animal's spine for the forces which will be placed upon it immediately after birth. The disc tissues of the lumbar region may therefore mature earlier than those of the the tail. The appearance of large quantities of small PGs relative to that of aggrecan (as equalized per lane by CS) indicates the presence of a tissue which is more ligament-like in the tail region than the more cartilage-like tissue seen in the lumbar spine. The PG content of adult bovine collateral ligament has been described by Hey as being essentially 20% large aggregating PGs and 80% small PG (Hey N.J et. al., 1990). Similarly, Vogel has shown that there was a change in relative proportion of PG types in regions experiencing different loads in bovine adult tendon (Vogel K.G et. al., 1985). Regions subjected to greater compression are enriched with large CS PGs compared to those areas not subjected

to these kinds of forces. In the same way it may be that the reduction in the proportion of small PGs compared to aggrecan observed with age in the AF *in vivo* may reflect the mechanical load experienced by the tissue.

This characteristic appears to change quite drastically in the period of young age (calf) and that of adulthood where PG from AF regions of tail and lumbar discs appear very similar in the adult animal. No differences were observed between PGs from the NP regions of tail and lumber discs.

The aggrecan residing in the AF migrated faster than that of the NP and this most noticeably in young animals. Since differences in GAG chain length were not determined between these two zones, it is not known whether this is due to different processing of the core protein or that of GAG chain length. Very preliminary data, using the 3B3 antibody and an antibody specific for the G1 domain, suggests that the aggrecan core protein isolated from adult tissues was processed to a greater extent than that of younger tissues (data not shown).

As the cell culture system we have described has focused on cells isolated from the tail region of the spine, further study of the small PGs were carried out on this region only. The age and tissue-related differences in the tail region observed by toluidine blue staining were confirmed using antibodies to the core proteins of DCN and BGN. With respect to DCN and BGN, a significantly greater quantity was observed in foetal AF tissue when compared to both calf and adult. In light of the association between DCN and collagen fibrils (Danielson K.G et. al., 1997), these results suggest a much higher collagen content per CS (aggrecan) in young than in older AF tissue. Similarly, the observation that DCN was barely detectable in NP tissues at all ages reflects the difference in collagen content between these two zones (25% dry weight NP, 80-90% dry weight AF) (Antoniou J et al., 1996).

Phenotypic differences between cells of the zones of the disc under study could be clearly identified in monolayer culture. It is known that fibroblast like cells flatten out rapidly when plated on plastic surfaces and produce type I collagen. The cells of the AF rapidly adopted a fibroblast-like morphology in monolayer and this was in contrast to those of the NP, which remained mostly polygonal throughout the 10 days of culture. Cells from calf discs became

fibroblastic in a much shorter delay than those of the adult indicating that there probably is age related differences as well.

In contrast to those of the AF, cells from NP zones of both calf and adult remained chondrocytic in monolayer culture as confirmed by collagen profiles, where an absence of the α2(I) chain was observed in the medium. Similarly, articular cartilage chondrocytes have been shown to maintain their phenotype (production of type II collagen) for up to 16 days in high density culture (Kuettner K.E et. al., 1982). This difference in cell phenotype could also be maintained in alginate bead cultures with survival of both chondrocytes and fibroblasts in culture with serum.

Following the observation that the pattern of reduced aggrecan synthesis with age observed in humans could be demonstrated in a cell culture model, the objective then became to stimulate synthesis in aged cells with growth factors. Other studies have shown that TGF-β is an important regulator of PG synthesis in chondrocytes. TGF-β having been shown to bind to DCN and BGN and to act as a potential storage reservoir within the tissue (Yamaguchi Y et. al., 1990; Hildebrand A et. al., 1994; Imai K et. al., 1997), levels of which may in part explain why there is less synthesis of aggrecan and greater over all catabolism with age (Edwards D.R et. al., 1996; Zeng G et. al., 1996). The reduction in available TGF-β has also been associated wth decreased over all PG synthesis in calf articular cartlage explant cultures (Morales T.I., 1992). Furthermore, the observation that exogenous TGF-β added to these cultures increased the levels of BGN synthesis suggests a relationship between this small PG and the growth factor.

TGF-β has also been described as having a mitogenic effect in fibroblasts and chondrocytes. In our cultures, NP cells survived but did not proliferate in serum-free medium. In both calf and adult however the rapid increase in cell number observed from day 5 to 10 show that these cells were sensitive to the mitogenic effect of TGF-β1 as previously reported in fibroblasts (Jeoung D-I et. al., 1995) and in articular chondrocytes (Skantze K.A et. al., 1985). Ishikawa et. al. described how human fibroblasts were succeptible to the mitogenic effect of TGF-β via a pathway involving the PDGF receptor (Ishikawa O et. al., 1990).

Due to the presence of insulin in our serum-free medium, it can not be ruled out that the observed mitogenic effect is due to a synergy between the two factors. The lack of cell proliferation observed in serum-free cultures discounts the possibility that insulin alone can be mitogenic at the concentration used in this cell culture system.

While no mitogenic effect was seen after 2 days of exposure to TGF-β1 (day 5), an increase in PG synthesis was observed by this time. This increase was maintained at a steady state until the end of the culture period. This rapid increase in PG synthesis has also been reported in cultured articular cartilage (Morales T.I et. al., 1988; Morales T.I., 1994) and tendon fibrocartilage explants (Vogel G.V et. al., 1992) explants and cultured chondrocytes treated with this growth factor (van der Kraan P et. al., 1992).

The potential of TGF-β1 in stimulating synthesis of matrix molecules is illustrated by the effect on DCN and BGN synthesis. The total amount of these small PGs (synthesised throughout the entire culture period and retained within alginate beads) relative to the total amount of aggrecan present was found to be increased for both small PGs in cells treated with the growth factor. This ratio is comparable in TGF-β1 treated cells to that observed in direct extractions of NP tissues from young animals.

Highest levels of a larger form of the BGN core protein were observed in alginate bead extracts of adult NP cells treated with TGF-β1. Neither form could be detected without Ch'ase ABC treatment indicating that both forms appear to be substituted with GAG chains (data not shown). The antibody to BGN is directed to a sequence in the C-terminus and it has been found that there is decreased reactivity to the intact molecule possibly due to hindrance of the epitope by GAG chains (Roughley P.J et. al., 1993). This may represent a less processed form of the core protein which is also observed in calf TGF-β1 treated NP cells, though with less abundance. Presence of pro-forms of BGN have been shown to increase with age in human NP tissue (Roughley P.J et. al., 1996).

The effect of TGF-β1 in reducing the loss of aggrecan from the alginate bead matrix may be due to the upregulation of the synthesis of small PG. While the synthesis of other matrix molecules was not studied in depth in this work, there is evidence that collagen synthesised by chondrocytes encapsulated in alginate beads

is normal with respect to lysyl- and prolyl- hydroxylation (Beekman B et. al., 1997). These studies however did not establish whether collagen fibrils were normal in appearance with respect to fibril diameter. The dramatic reduction in the loss of PG into medium and the observation that only small newly synthesised PG are released in TGF-β1 treated cultures suggests that a large proportion of the aggrecan synthesised is incorporated into the newly formed matrix within the alginate bead.

The greatest relative loss of PG into the medium is seen in serum-free medium alone, and this at both days 5 and 10. At day 5 the difference is probably due to the low synthesis rate of all matrix molecules in cells cultured in ITS compared to those in ITS+TGF-β1. By day 10 not only the total synthesis is increased in TGF-β1 treated cultures, but the synthesis expressed per cell also. This elevated rate may in part account for the high relative retention of newly synthesised PG molecules by increasing the amount of matrix surrounding cells. Also, it is clear that at the end of the culture period, the cell number of adult NP cells cultured in serum is not different than that of those exposed to TGF-β1.This indicates that retention of PG within the matrix is likely due to anabolic effects of the growth factor other than its mitogenicty.

The slower migrating form of aggrecan observed in TGF-β1 treated cultures could not be explained by a difference in GAG chain length. This observation was independent of age and was however not confirmed in monolayer cultures of tail NP cells at any age. Aggrecan migration was also retarded in monolayer cultures of bovine foetal and calf lumbar NP cells (data not shown) and also in cells of human lumbar discs from all regions (AF, IZ and NP) cultured in presence of TGF-β1. No surviving bovine adult lumbar cells could be recovered for study. Toluidine blue staining of recovered PGs retained within alginate beads after 10 days of culture showed that this form persisted within the matrix and was not degraded to a smaller form. Differences in sulphation of GAG chains were not examined. A difference in charge caused by altered sulphation could explain the reduced migration of aggrecan from TGF-β1 treated cultures on CAPAGE.

GAG chains produced by cells treated with ITS or ITS+TGF-β1 were of longer size than any observed in culture with serum. This increase in size was also observed in GAG chains produced by cells of tendon fibrocartilage treated with

TGF-β (Vogel K.G et. al., 1992). Furthermore this study showed that there was an increase in the ratio of small to large PG in growth factor treated cells. Our work has shown that there is an increased relative (to aggrecan) and total (per cell) production of small PGs in NP cells treated with TGF-β1. Schonherr et. al. showed the same pattern in GAG chains of versican (a large aggregating CS PG) produced by smooth muscle cells in which a similar increase in chain length was observed in both TGF-β and PDGF treated cultures (Schonherr E et. al., 1991). This elongation appears to occur irrespectively of the treatment with TGF-β1 and that the presence of insulin is sufficient to produce this elongation, indicating that either growth factor affects the ligand which initiates this effect. While the performance of CL-6B chromatography was hindered by the low radioactivity due to low ^{35}S incorporation present in ITS treated cells (even with pooling of four samples), the profiles which could be obtained suggest that chain length is identical in ITS and ITS+TGF-β1 treated cultures.

Since aggrecan isolated from cells cultured in ITS did not migrate differently than that from cells cultured in FCS, but migrated less than that from TGF-β1 treated cells, this could suggest a difference in the number of chains present on the molecule as previously described to occur in GAG chains of syndecan (a large heparan and chondroitin sulphate cell surface PG) from TGF-β1 treated epithelial cells (Rapraeger A., 1989). This was demonstrated by assessing the number of Ch'ase ABC generated stubs present on the core protein and found an estimated threefold increase in the number of sites recognised by an anti-CS monoclonal antibody. This increase in number of chains was also coupled with an overall increase in size as observed in our study.

While this study of different aged cells focused on stimulation of synthesis with TGF-β, the system can clearly be used to test other growth factors and pharmaceutical agents. Further work with this system could involve the study of collagen synthesis which is also known to be reduced in aged individuals (Antoniou J.A et al., 1996). Furthermore, the degradation of matrix molecules could also be evaluated biochemically and using molecular biology techniques to evaluate gene expression of MMPs.

This work further supports the potential of growth factor stimulation as a potential non-invasive therapy for IVD degeneration. The NP is non-vascularised

and therefore would allow such local therapy without rapid clearance of the agent. Studies are under way to establish the feasibility of using gene therapy in the IVD (Nishida K et. al., 1998) which could lead to therapies not requiring continuous administration of growth factors.

6) CONCLUSION:

NP cells from bovine IVDs of different aged animals showed an age related decrease in PG synthesis in alginate bead culture, a pattern which reflects what has previously been described in human discs *in vivo*. This demonstrated the validity of the culture system which can now be used to study the effect of growth factors on the metabolism of disc cells.

The phenotype of disc cells of the AF and NP regions was also compared, by analysing their collagen profiles and their morphological appearance in monolayer culture. The results demonstrate that the phenotype of these respective cells is different. AF cells are fibroblast-like producing type I collagen and flatten out in high density monolayer culture. On the other hand, NP cells are chondrocyte-like, synthesising type II collagen and maintaining a rounded shape when grown in cell monolayers.

TGF-β1 supplied to cells in serum-free culture was effective in increasing PG synthesis in old animals to a level higher than that of young animals cultured with serum. The growth factor also increased the synthesis of small PG relative to aggrecan in adult NP cells. This demonstrates that even cells from adults are able to regain synthetic capacity similar to that observed in those of young animals, given the required signal.

7) REFERENCES:

Aguiar D.J., Johnson S.L. and Oegema T.R. (1998) Notochordal cells regulate nucleus pulposus matrix integrity. Transactions of the 44th Annual Meeting, Orthopaedic Research Society 23, 680.

Antoniou J., Goudsouzian N.M., Heathfield T.F., Winterbottom N., Steffen T., Poole A.R., Aebi M. and Alini M. (1996) The human lumbar endplate. Evidence of changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, aging and degeneration. Spine 21, 1153-1161.

Antoniou J., Steffen T., Nelson F., Winterbottom N., Hollander A.P., Poole A.R., Aebi M. and Alini M. (1996) The human lumbar intervertebral disc: evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation and degenertion. Journal of Clinical Investigation 98, 996-1003.

Atkins E.D.T., Mackie W. and Smolko E.E. (1970) Crystalline structures of alginic acids. Nature 225, 626-628.

Barone-Varelas J., Schnitzer T.J., Meng Q., Oten L. and Thonar E. J-M. A. (1991) Age-related differences in the metabolism of bovine articular cartilage explants maintained in the presence of insulin-like growth factor 1. Connective Tissue Research 26, 101-120.

Bassols A. and Massague J. (1988) Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. Journal of Biological Chemistry 263, 3039-3045.

Bayliss M.T., Johnstone B. and O'Brien J.P. (1988) 1988 Volvo award in basic science. Proteoglycan synthesis in the human intervertebral disc. Variation with age, region and pathology. Spine 13, 972-981.

Bayliss M.T., Urban J.P., Johnstone B. and Holm S. (1986) In vitro method for measuring synthesis rates in the intervertebral disc. Journal of Orthopaedic Research 4, 10-17.

Beekman B., Verzijl N., Bank R.A., von der Mark K. and TeKoppele J.M. (1997) Synthesis of collagen by bovine chondrocytes cultured in alginate; posttranslational modifications and cell-matrix interaction. Experimental Cell Research 237, 135-141.

Benya P.D. and Shaffer J.D. (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30, 215-224.

Bjornsson S. (1993) Simultaneous preparation and quantification of proteoglycans by precipitation with alcian blue. Analytical Biochemistry 210, 1-10.

Buckwalter J.A. (1995) Aging and degeneration of the human intervertebral disc. Spine 20, 1307-1314.

Buckwalter J.A., Savio L.-Y., Goldberg V.M., Hadley C.E., Booth F., Oegema T.R. and Eyre D.R. (1993) Soft-tissue aging and musculoskeletal function. Journal of Bone and Joint Surgery (American) 75-A, 1533-1548.

Buckwalter J.A., Smith K.C., Kazarien L.E., Rosenberg L.C. and Ungar R. (1989) Articular cartilage and intervertebral disc proteoglycans differ in structure: an electron microscopic study. Journal of Orthopaedic Research 7, 146-151.

Chandrasekhar S. and Harvey A.K. (1989) Induction of interleukin-1 receptors on chondrocytes by fibroblast growth factor: A possible mechanism for modulation of interleukin-1 activity. Journal of Cellular Physiology 138, 236-246.

Chelberg M.K., Banks G.M., Geiger D.F. and Oegema J.T.R. (1995) Identification of heterogeneous cell populations in normal human intervertebral disc. Journal of Anatomy 186, 43-53.

Chiba K., Anderson G.B.J., Masuda K., Schmid T.M. and Thonar E.J.-M.A. (1998) Metabolism of collagen synthesized by nucleus pulposus and annulus fibrosus cells cultured in alginate beads. Transactions of the 44th Annual Meeting, Orthopaedic Research Society 23, 682.

Cole T.C., Burkhardt D., Frost L. and Ghosh P. (1985) The proteoglycans of the canine intervertebral disc. Biochimica et Biophysica Acta 839, 127-138.

Cole T.C., Ghosh P. and Taylor T.K. (1986) Variations of the proteoglycans of the canine intervertebral disc with aging. Biochimica et Biophysica Acta 880, 209-219.

Couchman J.R., Caterson B., Christner J.E. and Baker J.R. (1984) Mapping by monoclonal antibody detection of glycosaminoglycan in connective tissues. Nature 307, 650-652.

Dallas S.L., Miyazono K., Skerry T.M., Mundy G.R. and Bonewald L.F. (1995) Dual role for the latent transforming growth factor beta binding protein in strorage of latent TGF-beta in the extracellular matrix and as a structural matrix protein. Journal of Cell Biology 131, 539-549.

Danielson K.G., Baribault H., Holmes D.F., Graham H., Kadler K.E. and Iozzo R.V. (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. Journal of Cell Biology 136, 729-743.

Darnell J., Lodish H. and Baltimore D. (1990) In Molecular Cell Biology, 2nd Edition. pp 917. Scientific American Books, Inc., New York.

Ebara S., Iatridis J.C., Setton L.A., Foster R.J., Mow V.C. and Weidenbaum M. (1996) Tensile properties of nondegenerate human lumbar anulus fibrosus. Spine 21, 452-461.

Edwards D.R., Leco K.J., Beaudry P.P., Atadia P.W., Veillette C. and Riabowol T. (1996) Differential effects of transforming growth factor-beta1 on the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in young and old human fibroblasts. Experimental Gerontology 31, 207-223.

Enobakhare B.O., Bader D.L. and Lee D.A. (1996) Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. Analytical Biochemistry 243, 189-191.

Farndale R.W., Buttle J.D. and Barrett A.J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochimica et Biophysica Acta 883, 173-177.

Fosang A.J. and Hardingham T.E. (1989) Isolation of the N-terminal globular domains of cartilage proteoglycans. Identification of G2 domain and its lack of interaction with hyaluronate and link protein. Biochemical Journal 261, 801-809.

Freeze H.H., Sampath D. and Varki A. (1993) Alpha- and beta-xylosides alter glycolipid synthesis in human melanoma and Chinese hamster ovary cells. Journal of Biological Chemistry 268, 1618-1627.

Galera P., Redini F., Vivien D., Bonaventure J., Penformis H., Loyau G. and Pujol J.-P. (1992) Effect of transforming growth factor-beta1 (TGF-beta1) on matrix synthesis by monolayer cultures of rabbit articular chondrocytes during the dedifferentiation process. Experimental Cell Research 200, 379-392.

Ghosh P., Taylor T.K. and Braund K.G. (1977) The variation of the glycosaminoglycans of the canine intervertebral disc with aging. I. Chondrodystrophoid breed. Gerontology 23, 87-98.

Glansbeek H.L., van der Kraan P.M., Vitters E.L. and van den Berg W.B. (1993) Correlation of the size of type II transforming growth factor (TGF-beta) receptor with TGF-beta responses of isolated bovine articular chondrocytes. Annals of the Rheumatic Diseases 52, 812-816.

Grandolfo M., D'Andrea P.D., Paoletti S., Martina M., Silvestrini G., Bonnuci E. and Vittur F. (1993) Culture and differentiation of chondrocytes entrapped in alginate gels. Calcified Tissue International 52, 42-48.

Grant G.T., Morris E.R., Rees D.A., Smith P.J.C. and Thom D. (1973) Biological interactions between polysacharides and divalent cations: The egg-box model. FEBS Letters 32, 195-198.

Gruber H.E., Fisher J.E.C., Desai B., Stasky A.A., Hoelscher G. and Nanley J.E.N. (1997) Human intervertebral disc cells from the annulus: Three dimensional culture in agarose or alginate and responsiveness to TGF-beta 1. Experimental Cell Research 235, 13-21.

Guerne P.-A., Blanco F., Kaelin A., Desgeorges A. and Lotz M. (1995) Growth factor responsiveness of human articular chondrocytes in aging and development. Arthritis and Rheumatism 38, 960-968.

Gunther M., Haubeck H.D., van de Leur E., Blaser J., Bender S., Gutgemann I., Fischer D.C., Tschesche H., Greiling H., Heinrich P.C. and Graeve L. (1994) Transforming growth factor beta 1 regulates tissue inhibitor of metalloproteinases-1 expression in differentiated human articular chondrocytes. Arthritis and Rheumatism 37, 395-405.

Guo G.F., Jourdanian G.W. and MacCallum D.K. (1989) Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connective Tissue Research 19, 277-297.

Hardingham T.E. and Fosang A.J. (1992) Proteoglycans: many forms and many functions. FASEB Journal 6, 861-870.

Haug A., Larsen B. and Smidsrod O. (1966) A study of the constitution of alginic acid by partial acid hydrolysis. Acta Chemica Scandinavica 20, 183-190.

Haug A., Myklestad S., Larsen B. and Smidsrod O. (1967) Correlation between chemical structure and physical properties of alginates. Acta Chemica Scandinavica 21, 768-778.

Heinegard D. (1977) Polydispersity of cartilage proteoglycans: Structural variations with the size and buoyant density of the molecules. J Biol Chem 252, 1980-1989.

Heinegard D., Sommarin Y., Hedbom E., Weislander J. and Larsson B. (1985) Assay of proteoglycan populations using agarose-polyacrylamide gel electrophoresis. Analytical Biochemistry 151, 41-48.

Hey N.J., Handley C.J., Ng C.K. and Oakes B.W. (1990) Characterization and synthesis of macromolecules by adult collateral ligament. Biochimica et Biophysica Acta 1034, 73-80.

Hildebrand A., Romaris M., Rasmussen L.M., Heinegard D., Twardzik D.R., Border W.A. and Ruoslahti E. (1994) Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochemical Journal 302, 527-534.

Hinek A., Reiner A. and Poole A.R. (1987) The calcification of cartilage matrix in chondrocyte culture: studies of the C-propeptide of type II collagen (chondrocalcin). Journal of Cell Biology 104, 1435-1441.

Hukins D.W. (1988) Disc Structure and Function. In The Biology of the Intervertebral Disc. Edited by Ghosh P. pp 2-33. CRC Press, Boca Raton, Florida.

Humzah M.D. and Soames R.W. (1988) Human intervertebral disc structure and function. Anatomical Record 220, 337-356.

Imai K., Hiramatsu A., Fukushima D., Pierschbacher M.D. and Okada Y. (1997) Degradation of decorin by matrix metalloproteinases: Identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. Biochemical Journal 322, 809-814.

Ishikawa O., LeRoy E.C. and Trojanowska M. (1990) Mitogenic effect of transforming growth factor beta1 on human fibroblasts involves the induction of platelet-derived growth factor alpha receptors. Journal of Cellular Physiology 145, 181-186.

Jeoung D-I., Tang B. and Sonenberg M. (1995) Mitogenic response to TGF-beta in 3T3-F442A cells. Biochemical and Biophysical Research Communications 216, 964-969.

Jin R. and Breslauer K.J. (1988) Characterization of the minor groove environment in a drug-DNA complex: Bisbenzimide bound to the poly[d(AT)]-poly[d(AT)]duplex. Proceedings of the National Academy of Sciences of the United States of America 85, 8939-8942.

Johnstone B. and Bayliss M.T. (1995) The large proteoglycans of the human intervertebral disc. Changes in their biosynthesis and structure with age, topography, and pathology. Spine 20, 674-684.

Johnstone B., Markopoulos M., Neame P. and Caterson B. (1993) Identification and characterization of glycanated and non-glycanated forms of biglycan and decorin in the human intervertebral disc. Biochemical Journal 292, 661-666.

Katz M.M., Hargens A.R. and Garfin S.R. (1986) Intervertebral disc nutrition. Diffusion versus convection. Clinical Orthopaedics and Related Research 210, 243-245.

Kim Y.-J., Sah R.L.Y., Doong J.-Y.H. and Grodzinsky A.J. (1988) Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Analytical Biochemistry 174, 168-176.

Kolettas E., Buluwela L., Bayliss M.T. and Muir H.I. (1995) Expression of cartilage specific molecules is retainedon long-term culture of human articular chondrocytes. Journal of Cell Science 108, 1991-1999.

Kraemer J. (1995) Natural course and prognosis of intervertebral disc diseases. Spine 20, 635-639.

Kuettner K.E., Memoli V.A., Pauli B.U., Wrobel N.C., Thonar E.J.-M.A. and Daniel J.C. (1982) Synthesis of cartilage matrix by mammalian chondrocytes in vitro. II. Maintenance of collagen and proteoglycan phenotype. Journal of Cell Biology 93, 751-757.

Labarca C. and Paigen K. (1980) A simple, rapid, and sensitive DNA assay procedure. Analytical Biochemistry 102, 344-352.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lawrence D.A. (1996) Transforming growth factor-beta: A general review. European Cytokine Network 7, 363-374.

Lwebuga-Mukasa J.S., Harper E. and Taylor P. (1976) Collagenase enzymes from Clostridium: Characterization of individual enzymes. Biochemistry 15, 4736-4741.

Maldonado B.A. and Oegema J.R. (1992) Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. Journal of Orthopaedic Research 10, 677-690.

Maroudas A., Stockwell R.A., Nachemson A. and Urban J. (1975) Factors involved in the nutrition of the human lumbar intervertebral disc: cellularity and diffusion of glucose in vitro. Journal of Anatomy 120, 113-130.

Martin R.F. and Holmes N. (1983) Use of an 125I-labelled DNA ligand to probe DNA structure. Nature 302, 452-454.

Masuda K., Shirota H. and Thonar E.J.-M.A. (1994) Quantification of 35S-labeled proteoglycans complexed to alcian blue by rapid filtration in multiwell plates. Analytical Biochemistry 217, 1-9.

McDevitt C.A. (1988) Proteoglycans of the Intervertebral Disc. In The Biology of the Intervertebral Disc. Edited by Ghosh P. pp 151-170. CRC Press, Boca Raton, Florida.

Melrose J., Gurr K.R., Cole T.C., Darvodelsky A., Ghosh P. and Taylor T.K. (1991) The influence of scoliosis and aging on proteoglycan heterogeneity in the human intervertebral disc. Journal of Orthopaedic Research 9, 68-77.

Mitchell D. and Hardingham T. (1981) The effects of cyclohexamide on the biosynthesis and secretion of proteoglycans by chondrocytes in culture. Biochemical Journal 196, 521-529.

Miller E.J. (1976) Biochemical characteristics and biological significance of the genetically distinc collagens. Molecular & Cellular Biochemistry 13(3), 165-192.

Mok S.S., Masuda K., Hauselmann H.J., Aydelotte M.B. and Thonar J.-M.A. (1994) Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. Identification of two distinct metabolic matrix pools. Journal of Biological Chemistry 269, 33021-33027.

Morales T.I. (1992) Polypeptide regulators of matrix homeostasis in articular cartilage. In Articular Cartilage and Osteoarthritis. Edited by Kuettner K.E., Schleyerbach R., Peyron J.G. and Hascall V.C. pp 265-279. Raven Press, Ltd., New York.

Morales T.I. (1994) transforming growth factor-beta and insulin-like growth factor-1 restore proteoglycan metabolism of bovine articular cartilage after depletion by retinoic acid. Archives of Biochemistry and Biophysics 315, 190-198.

Morales T.I., Joyce M.E., Sobel M.E., Danielpour D. and Roberts A.B. (1991) Transforming growth factor-beta in calf articular cartilage organ cultures: Synthesis and distribution. Archives of Biochemistry and Biophysics 288, 397-405.

Morales T.I. and Roberts A.B. (1988) Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. Journal of Biological Chemistry 263, 12828-12831.

Murphy-Ullrich J.E., Schultz-Cherry S. and Hook M. (1992) Transforming growth factor beta complexes with thrombospondin. Molecular Biology of the Cell 3, 181-188.

Nachemson A.L. (1985) Advances in low-back pain. Clinical Orthopaedics and Related Research 200, 266-278.

Nishida K., Kang J.D., Suh J.K., Robbins P.D., Evans C.H. and Gilbertson L.G. (1998) Adenovirus mediated gene transfer to nucleus pulposus cells: Implications for the treatment of intervertebral disc degeneration. Transactions of the 44th Annual Meeting, Orthopaedic Research Society 23, 298.

O'Kane S. and Ferguson M.W. (1997) Transforming growth-factor betas and wound healing. International Journal of Biochemistry and Cell Biology 29, 63-78.

Oegema T.R. (1993) Biochemistry of the intervertebral disc. Clinics in Sports Medicine 12, 419-439.

Ohshima H., Tsuji H., Hirano N., Ishihara H., Katoh Y. and Yamada H. (1989) Water diffusion pathway, swelling pressure, and biomechanical properties of the intervertebral disc during compression load. Spine 14, 1234-1244.

Osada R., Ohshima H., Ishihara H., Yudoh K., Sakai K., Matsui H. and Tsuji H. (1996) Autocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insulin-like growth factor on proteoglycan synthesis in bovine intervertebral discs. Journal of Orthopaedic Research 14, 690-699.

Oshima H., Ishihara H., Urban J.P. and Tsuji H. (1993) The use of coccygeal discs to study intervertebral disc metabolism. Journal of Orthopaedic Research 11, 332-338.

Pearce R.H., Grimmer B.J. and Adams M.E. (1987) Degeneration and the chemical composition of the human lumbar intervertebral disc. Journal of Orthopaedic Research 5, 198-205.

Pearce R.H., Mathieson J.M., Mort J.S. and Roughley P.J. (1989) Effect of age on the abundance and fragmentation of link protein of the human intervertebral disc. Journal of Orthopaedic Research 7, 861-867.

Petit B., Masuda K., D'Souza A.L., Otten L., Pietryla D., Hartmann D.J., Morris N.P., Uebelhart D., Schmid T.M. and Thonar E.J.-M.A. (1996) Characterization of the crosslinked collagens synthesized by mature articular cartilage chondrocytes cultured in alginate beads: Comparison of two distinct matrix compartments. Experimental Cell Research 225, 151-161.

Pins G.D., Christiansen D.L., Patel R. and Silver F.H. (1997) Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties. Biophysical Journal 73, 2164-2172.

Poole A.R., Nelson F., Hollander A., Reiner A., Pidoux I. and Ionescu M. (1995) Collagen II turnover in joint diseases. Acta Orthopaedica Scandinavica 266, 88-91.

Puustjarvi K., Lammi M., Helminen H., Inkinen R. and Tammi M. (1994) Proteoglycans in the intervertebral disc of young dogs following strenuous running exercise. Connective Tissue Research 30, 225-240.

Rapraeger A. (1989) Transforming growth factor (type beta) promotes the addition of chondroitin sulfate chains to the cell surface proteoglycan (syndecan) of mouse mammary epithelia. Journal of Cell Biology 109, 2509-2518.

Rizkalla G., Reiner A., Bogoch E. and Poole A.R. (1992) Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. Journal of Clinical Investigation 90, 2268-2277.

Roberts S., Urban J.P., Evans H. and Eisenstein S.M. (1996) Transport properties of the human cartilage endplate in relation to its composition and calcification. Spine 21, 415-420.

Roughley P.J., White R.J., Magny M.C., Liu J., Pearce R.H. and Mort J.S. (1993) Non-proteoglycan forms of biglycan increase with age in human articular cartilage. Biochemical Journal 295, 421-426.

Roughley P.J., White R.J. and Mort J.S. (1996) Presence of pro-forms of decorin and biglycan in human articular cartilage. Biochemical Journal 318, 779-784.

Salisbury J.R. and Watt F.M. (1988) Lack of keratan sulphate in the human notochord. Journal of Anatomy 157, 175-179.

Schneiderman G., Flannigan B., Kingston S., Thomas J., Dillin W.H. and Watkins R.G. (1987) Magnetic resonance imaging in the diagnosis of disc degeneration: Correlation with discography. Spine 12, 277-281.

Schonherr E., Hausser H., Beavan L. and Kresse H. (1995) Decorin-type I collagen interaction. Presence of separate core protein-binding domains. Journal of Biological Chemistry 270, 8877-8883.

Schonherr E., Jarvelainen H.T., Sandell L.J. and Wight T.N. (1991) Effects of platelet-derived growth factor and transforming growth factor-beta1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. Journal of Biological Chemistry 266, 17640-17647.

Schonherr E., Witsch-Prehm P., Harrach B., Robenek H., Rauterberg J. and Kresse H. (1995) Interaction of biglycan with type I collagen. Journal of Biological Chemistry 270, 2776-2783.

Scott J.E. (1996) Proteodermatan and proteokeratan sulfate (decorin, lumican/fibromodulin) proteins are horseshoe shaped. Implications for their interactions with collagen. Biochemistry 35, 8795-8799.

Scott J.E., Bosworth T.R., Cribb A.M. and Taylor J.R. (1994) The chemical morphology of age-related changes in the human intervertebral disc glycosaminoglycans from cervical, thoracic and lumbar nucleus pulposus and annulus fibrosus. Journal of Anatomy 184, 73-82.

Seyedin S.M., Thomas T.C., Thompson A.Y., Rosen D.M. and Piez K.A. (1985) Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proceedings of the National Academy of Sciences of the United States of America 82, 2267-2271.

Seyedin S.M., Thompson A.Y., Bentz H., Rosen D.M., McPherson J.M., Conti A., Siegel N.R., Galluppi G.R. and Piez A. (1986) Cartilage inducing factor-A. Apparent identity to transforming growth factor-beta. Journal of Biological Chemistry 261, 5693-5695.

Skantze K.A., Brinkerhoff C.E. and Collier J.P. (1985) Use of agarose culture to measure the effect of transforming growth factor beta and epidermal growth factor on rabbit articular chondrocytes. Cancer Research 45, 4416-4421.

Sporn M.B. and Roberts A.B. (1990) TGF-beta: Problems and prospects. Cell Regulation 1, 875-882.

Su S., Dehnade F. and Zafarullah M. (1996) Regulation of tissue inhibitor of metalloproteinases-3 gene expression by transforming growth factor-beta and dexamethasone in bovine and human articular chondrocytes. DNA and Cell Biology 15, 1039-1048.

Svensson L.D., Heinegard D. and Oldberg A. (1995) Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4-5. Journal of Biological Chemistry 270, 20712-20716.

Taylor J.R. (1975) Growth of human intervertebral discs and vertebral bodies. Journal of Anatomy 120, 49-68.

Taylor J.R., Scott J.E., Cribb A.M. and Bosworth T.R. (1992) Human intervertebral disc acid glycosaminoglycan. Journal of Anatomy 180, 137-141.

Thompson J.P., Oegema J.T.R. and Bradford D.S. (1991) Stimulation of mature canine intervertebral disc by growth factors. Spine 16, 253-260.

Towbin H., Staehelin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America 76, 4350-4354.

Trippel S.B. (1995) Growth factor actions on articular cartilage. Journal of Rheumatology 22, 129-132.

Trout J.J., Buckwalter J.A. and Moore K.C. (1982) Ultrastructure of the human intervertebral disc: II. Cells of the nucleus pulposus. Anatomical Record 204, 307-314.

van Beuningen H.M., van der Kraan P.M., Arntz O.J. and van den Berg W.B. (1994) Transforming growth factor beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine kee joint. Laboratory Investigation 71, 279-289.

van den Berg W. (1995) Growth factors in experimental osteoarthritis; Transforming growth factor beta pathogenic? Journal of Rheumatology 22, 143-145.

van der Kraan P., Vitters E. and van den Berg W. (1992) Differential effect of transforming growth factor beta on freshly isolated andcultured articular chondrocytes. Journal of Rheumatology 19, 140-145.

van Susante J.L.C., Buma P., van Osch G.J.V.M., Versleyen D., van der Kraan P.M., van den Berg W.B. and Homminga G.N. (1995) Culture of chondrocytes in alginate and collagen carrier gels. Acta Orthopaedica Scandinavica 66, 549-556.

van Wart H.E. and Steinbrink D.R. (1985) Complementary substrate specificities of class I and class II collagenases from Clostridium histolyticum. Biochemistry 24, 6520-6526.

Vanharanta H. (1994) The intervertebral disc: a biologically active tissue challenging therapy. Annals of Medicine 26, 395-399.

Venn G. and Mason R.M. (1986) Changes in the mouse intervertebral disc proteoglycan synthesis with age. Hereditary kyphosis is associated with elevated synthesis. Biochemical Journal 234, 475-479.

Vogel K.G., Paulsson M. and Heinegard D. (1984) Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. Biochemical Journal 223, 587-597.

Vogel K.G. and Heinegard D. (1985) Characterization of proteoglycans from adult bovine tendon. Journal of Biological Chemistry 260, 9298-9306.

Vogel K.G. and Hernandez D.J. (1992) The effects of transforming growth factor-beta and serum on proteoglycan synthesis by tendon fibrocartilage. European Journal of Cell Biology 59, 304-313.

Webber C., Glant T.T., Roughley P.J. and Poole A.R. (1987) The identification and characterization of two populations of aggregating proteoglycans of high buoyant density isolated from post-natal human articular cartilages of different ages. Biochemical Journal 248, 735-740.

Weber I.T., Harrison R.W. and Iozzo R.V. (1996) Model structure of decorin and implications for collagen fibrilogenesis. Journal of biological Chemistry 271, 31767-31770.

Wong-Palms S. and Plaas A.H. (1995) Glycosaminoglycan addition to proteoglycans by articular chondrocytes- evidence for core protein-specific pathways. Archives of Biochemistry and Biophysics 319, 383-392.

Yamaguchi Y., Mann D.M. and Ruoslahti E. (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature 346, 281-284.

Yoo J.U., Papay R.S. and Malemud C.J. (1992) Suppression of proteoglycan synthesis in chondrocyte cultures derived from canine intervertebral disc. Spine 17, 221-224.

Zeng G., McCue H.M., Mastrangelo L. and Millis A.J.T. (1996) Endogenous TGF-beta activity is modified during cellular aging: Effects on metalloproteinase and TIMP-1 expression. Experimental Cell Research 228, 271-276.