IMMUNOCHEMICAL STUDIES
OF AGGREGATING CARTILAGE PROTEOGLYCANS
IN PSEUDOACHONDROPLASIA

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ABSTRACT

The aim of the present investigation was to study the aggregating proteoglycans in rib cartilages from patients with pseudoachondroplasia in comparison with those from age-matched normals. Biochemical and immunochemical analyses using antibodies against different substructures of these molecules permitted an investigation of their contents, structures, sizes, and aggregatability with hyaluronic acid. The structural changes in proteoglycans were characterized by longer chondroitin sulfate chains, with reduced 4-sulfation, unchanged 6-sulfation and increased non-sulfation, which resulted in larger molecules. These chains were attached to proteoglycan core protein by fewer chondroitin 4-sulfate linkage regions and more unsulfated chondroitin linkage regions. There were three populations of proteoglycans of different sizes in normals at all ages (4,11 and 30 years old) and an adult patient (31 years old). These three populations were characterized by an abundance of chondroitin 6-sulfate epitope and fetal-specific epitope 846, of chondroitin 4-sulfate epitope, and of keratan sulfate in order of decreasing sizes. There was an absence of the population of proteoglycans of intermediate size recognized in young patients (4 and 12 years old). There were also decreased contents of the hyaluronic acid-binding region and keratan sulfate in the adult patient. Hyaluronic acid content was considerably increased in these patients. The latter changes may reflect differences in turnover of proteoglycans in the cartilage matrix in pseudoachondroplasia.

RÉSUMÉ

Nous avons, dans cette étude, analysé la composition, la structure, la dimension et la capacité de former des agrégats avec l'acide hyaluronique des protéoglycans du cartilage costal de patients pseudo-achondroplasiques âgés de 4, 12 et 31 ans en utilisant des anticorps reconnaissant différents épitopes de ces macromolécules. Nous en avons comparé les caractéristiques avec celles observées pour les protéoglycans d'échantillons humains normaux d'âge comparable. La présence de chaînes de chondroitine sulfate plus longues et moins sulfatées, particulièrement en position 4, sont les modifications marquantes observées. De plus ces chaînes sont liées à la protéine centrale par moins de régions chondroitine 4-sulfate et plus de régions non sulfatées. Les tissus normaux aux trois âges étudiés (4,11,30 ans) et celui d'un patient adulte (31 ans), contiennent 3 types de protéoglycanes de masses moléculaires différentes. Ces troix populations se distinguent par la présence, en grande quantité de l'épitope chondroitine 6-sulfate et de l'épitope 846 spécifique au foetus, de l'épitope chondroitine 4-sulfate, et de l'épitope kératane sulfate en ordre de masse décroissante. Par contre le protéoglycane chondroitine 4-sulfate, de masse intermédiaire, est absent des tissus de jeunes patients (4,12 ans). Finalement, nous avons noté une diminution de la concentration de la région polypeptidique charnière avec l'acide hyaluronique et de celle du kératane sulfate dans le cartilage du patient adulte. Ces modifications structurales suggèrent un métabolisme différent des protéoglycanes de la matrice du cartilage dans ces cas de pseudo-achondroplasie.

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ABBREVIATIONS

BFPG	Bovine fetal proteoglycan
BSA	Bovine serum albumin
CHASE	Chondroitinase
CS	Chondroitin sulfate
C0S	Chondroitin
C4S	Chondroitin 4-sulfate
C6S	Chondroitin 6-sulfate
di 4S	Unsaturated disaccharide of C4S
di 6S	Unsaturated disaccharide of C6S
EDTA	Ethylenediamine tetraacetic acid
$F(ab')_2$	Divalent antigen binding subunit of IgG
HA	Hyaluronic acid
HABR	Hyaluronic acid-binding region
HAPG	Human adult proteoglycan
HFPG	Human fetal proteoglycan
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K_{av}	Partition coefficient for gel chromatography
KS	Keratan sulfate
Mab	Monoclonal antibody
N	Normal
NRS	Normal rabbit serum
PBS	Phosphate buffered saline
P	Pseudoachondroplasia
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate
UA	Uronic acid

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CHAPTER I

INTRODUCTION

1.1. OVERVIEW OF GROWTH PLATE

1.1.1. General Properties

The longitudinal growth of developing mammalian long bones is critically dependent on their primary growth plates which undergo endochondral calcification and function as primary ossification centers throughout the period of skeletal growth. On the other hand, the secondary ossification center within the epiphyses of each long bone contributes to the particular shape of the epiphyses which form diarthroidial joints. The growth plates are disc-like cartilagenous tissues interposed between the epiphysis and metaphysis at both ends of developing long bones. The function of the growth plate is characterized by the directional growth of the cartilage away from metaphysis resulting from sequential proliferation, maturation and hypertrophy of the chondrocytes, their synthesis of cartilage matrix and the subsequent provisional calcification of this matrix, which is remodelled and finally incorporated into newly formed woven bone. These overall outcomes determine the lengthening of long bones. These functions are well regulated by chondrocytes within the growth plate and are reflected in the structural organization demonstrated by the histological appearance of this tissue in which chondrocytes exhibit sequential developmental changes. According to these changes, the entire growth plate is divided into four consecutive and distinctive zones, namely the reserve, proliferative, maturing and hypertrophic zones, the latter being adjacent to the bony metaphysis. During their development, growth plate chondrocytes secrete different matrix molecules at different times and modify these molecules, which presumably leads to the consequent provisional calcification of the matrix. In this section, a

brief general overview of the growth plate is provided particularly regarding the structural integrity and organization of this tissue, its morphology, cell biology and biochemistry, which are critically responsible for the development and maintenance of its normal function. Detailed recent overviews of the growth plate should also be consulted (76,134).

1.1.2. Morphology and Cell Physiology of the Growth Plate

Figure 1 is a diagramatic representation of the growth plate. In the reserve zone, chondrocytes are relatively large and arranged randomly. The chondrocytes in the central portion of this zone are thought to originate from epiphyseal vascular tufts, whereas those in the peripheral portion may be derived from the surrounding perichondrium. The chondrocytes are quiescent in their cell cycle and, as the name of this zone indicates, play a role as a reservoir for proliferative chondrocytes.

The chondrocytes in the proliferative zone are flattened or polyspheroid and well aligned in columns parallel to the longitudinal axes of bones. In this zone, where oxygen tension at its highest in all the zones (22) they start to divide rapidly (85) and secrete extracellular matrix vigorously: the latter was demonstrated by sulfate incorporation (72). Longitudinal septa are formed. The well developed intracellular organelles, such as the Golgi apparatus and rough endoplasmic reticulum, also reflect the active synthesis of the extracellular matrix by these chondrocytes. Type II collagen, a major constituent of hyaline cartilage, forms fibrils in the interterritorial matrix in longitudinal septa which are well aligned parallel to the long axis in the lower part of this zone. In contrast, those fibrils in the territorial matrix surrounding the chondrocytes form a fine network surrounding individual cells within each column (26,42).

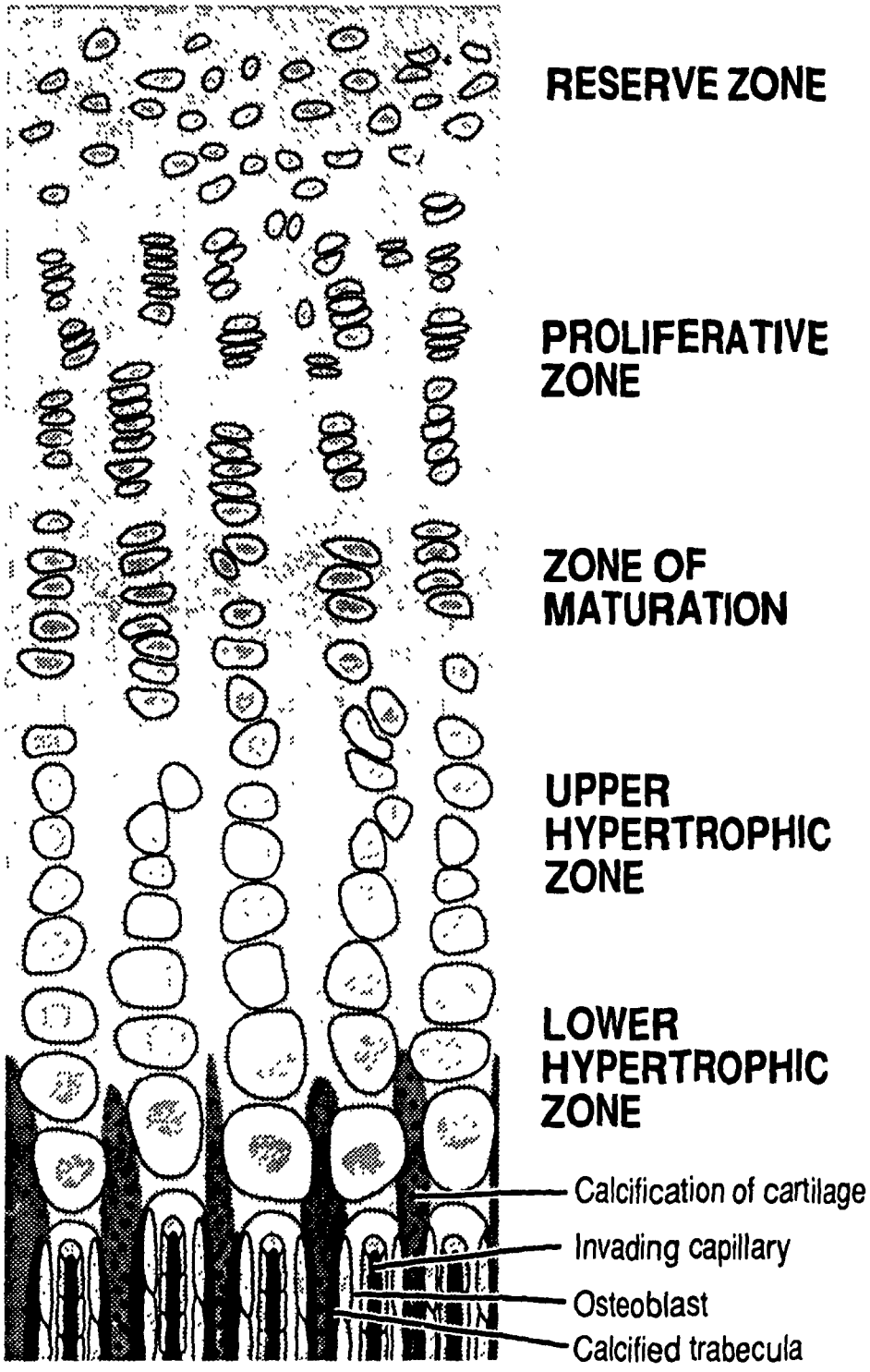


Figure 1 : Diagramatic Representation of Growth Plate

In the lowermost part of this zone, the chondrocytes suddenly start to expand multidirectionally, forming the maturing zone. This zone is followed by the hypertrophic zone where the aligning cells are at their largest, particularly in their height, thereby contributing to the axial growth of this tissue. This zone has drawn much attention not only because the cells exhibit their unique property of hypertrophy but also it is in the lower part of this zone that the matrix calcifies. During the process of hypertrophy, the cells enlarge 5-10 fold (80). This is accomplished by an increase of the volume of cytoplasm as well as nuclei and cell organelles (26,80). Because of these changes in morphology similar to vacuolar degeneration, this zone used to be named the zone of degeneration. However, recent morphological (78), fluorescein uptake (46), immunohistochemical (91,126) and in situ hybridization (95,120,149) studies have indicated that the cells are metabolically active and synthesize various matrix molecules (see section 1.1.3.).

The calcification is initiated in the territorial matrix close to the cells in the lower hypertrophic zone and is mainly concentrated in the matrix of the longitudinal septa. The transverse septa usually remain uncalcified. The pericellular zone also never calcifies even in the longitudinal septa (134). There are small vesicular structures enclosed by a trilaminar membrane called matrix vesicles which are concentrated within the matrix of the longitudinal septa from the proliferative zone downward. Their highest concentration is at the level where calcification is initiated. These have been described as primary calcification sites (2,4,5). But there is a negative correlation between the concentration of these vesicles and the degree of mineralization in the lower hypertrophic zone (139). These vesicles are thought to be produced by a budding mechanism at the plasma membrane of adjacent cells (34). They contain

considerable amounts of calcium and phosphate (5,172) and highly concentrated alkaline phosphatase (105,106) which play a crucial role in supplying inorganic phosphate required for the growth of mineral crystals, named crystalline hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (180). Under the electron microscope, primary nulceation sites from which the mineral crystals radiate are commonly observed as being distinct from matrix vesicles (126,133). These primary calcification centers are discussed in relation to proteoglycans in the growth plate (section 1.3.).

Associated with the more extensive calcification of the septa, the hypertrophic cells finally die and all transverse and some longitudinal septa are invaded by the capillaries growing between the longitudinal septa from the bony metaphysis. The terminal hypertrophic cells exhibit a unique morphology of cell death with condensation or apoptosis characteristic of programmed cells death seen in other cell types such as thymocytes (146). The capillary sprouts that invade cartilage were thought to form loop-like structures until recently (155,166). Scanning electron microscopy of the cartilage has revealed that the microvasculatures in the hypertrophic zone have saccular or bulbous structures (9). These capillaries may invade the cartilage directly and supply chondroclasts which digest and absorb the remnant calcified and uncalcified cartilage. The remaining calcified longitudinal septa serve as scaffolds where osteoblasts settle and deposit osteoid which calcifies to form the initial woven bone. The rate of osteoid vascular invasion is so stringently regulated that it balances the overall growth of this tissue to maintain its length during almost the entire period of skeletal growth (80,81).

1.1.3. Biochemistry of the Growth Plate

Together with changes in the morphology of the chondrocytes as well as the matrix of the different zones as discussed above, the corresponding alterations in the structural configuration of matrix molecules have been identified. The growth plate contains various collagenous and non-collagenous macromolecules as constituents of its extracellular matrix. This section provides a general description of the synthesis and degradation of these structural molecules (except large aggregating proteoglycans, discussed in section 1.3.).

COLLAGENS

Type II collagen

As in other hyaline cartialges, type II collagen is the major collagen in the growth plate. It is a fibrillar collagen that, in its mature form, is a homotrimer of α1(II) chains which form a triple helical structure. This molecule functions to preserve the structural integrity of the matrix by resisting tensile force applied internally (swelling pressure generated by proteoglycan, next section) and externally (mechanical stresses and strains) to the tissue (124,127). The level of mRNA for this collagen is high from the reserve down to the lower proliferative zone, reaching to a maximum level in the upper hypertrophic zone. Thereafter it decreases until the lower hypertrophic zone, where a minimum level is expressed (149). Recently, Lee et al. (97) demonstrated the segregation of type II procollagen in dilated rough endoplasmic reticulum of hypertrophic cells using immunogold techniques with antibodies against the carboxy terminal propeptide of this collagen. These observations reflect the shut-down of the synthesis of this protein together with the reduced level of mRNA expression. Since the mechanical stresses and strains applied to the growth plate are probably much less than those to the articular cartilage, which results from their specific

anatomical properties, the collagen and its network in the matrix of the growth plate are thought to be less developed. The collagen in this tissue is thus considered to be degraded more easily. This was supported by a recent immunohistochemical study indicating pericellular cleavage of this molecule within the maturing zone identified using antibodies against epitopes exposed on unwound α-chains only when its helix is degraded. This cleavage of type II collagen later spreads out into the entire extracellular matrix of the hypertrophic zone (3). These observations suggest the possibility of reorganization of the matrix molecules. Recent reports have described the presence of net collagenase activity (39) and the pericellular immunolocalization of collagenase (23). These observations indicate the degradation of the matrix together with a decrease of extracellular matrix volume at the time of cellular hypertrophy (26,80).

C-propeptide of type II collagen (chondrocalcin)

This calcium-binding protein, initially called chondrocalcin, has been implicated in the calcification of the matrix since its discovery in the eiphyseal cartilage (36). This molecule is also present in the growth plate and is closely associated with type II collagen fibrils in the proliferative and upper hypertrophic zones. Later, it is selectively concentrated in primary mineralization foci in the lower hypertrophic zone (126,133) suggesting the possible direct involvement of this molecule in the processes of the calcification. N-terminal sequence determination of this protein has revealed that it is identical to the carboxy terminal propeptide (C-propeptide) of type II procollagen (168). It is removed extracellularly, together with an amino terminal propeptide, by specific proteinases to give rise to tropocollagen, which assembles to form collagen fibrils (135).

Type X collagen

The spatial and temporal appearance of type X collagen within the hypertrophic zone suggests its involvement in the processes which lead to matrix mineralization (52,53,95,152). The synthesis of this collagen by hypertrophic cells reaches up to 45% of the total collagen synthesized with a corresponding relative decrease of type II collagen (138). A potential role for this molecule in calcification was further implicated by its association with matrix vesicle fractions isolated from chondrocyte culture medium (57) and a possible transmembrane domain within the molecule (115). However, recent immunoelectron microscopic studies of chick type X collagen have demonstrated a close association of this molecule with type II collagen fibrils rather than primary calcification foci or matrix vesicles, suggesting that it has no direct involvement in mineral growth (133,153).

Type IX collagen

The growth plate contains type IX collagen (112). This collagen is composed of three genetically distinct α-chains forming non-helical (non-collagenous: NC) and helical (collagenous: COL) domains. It is covalently cross-linked to type II collagen (45,168) and is present on type II collagen fibrils (112) lying periodically along their surfaces (170). It appears to have a short and a long arm connected by a flexible hinge (82). The short arm projects out from the surfaces of the fibrils. Sequential analysis of the terminal domain of the short arm has revealed its basic nature (169). This collagen is, therefore, considered to be involved in the supramolecular organization in the matrix of type II collagen fibrils and may interact with other, as yet undetermined, matrix components.

Type XI collagen

Type XI collagen, a minor fibrillar collagen, is also present in the growth plate at a relatively high content of about 20% of the total collagen (179). It consists of three different chains (111) of α1(XI) (14) α2(XI) (90) and α3(XI) or possibly α1(II) (29). Immunohistochemical studies have shown that it is co-localized with types II and IX collagen, forming a collagen network of mixed fibrils in chick embryo sternal cartilage (107). However, very little is known about its function in the matrix.

Non Collagenous Protein

Osteonectin

Osteonectin is a major matrix protein present in bone matrix and is associated with collagen fibrils (164). It has been implicated in the formation of hydroxyapatite in bone tissue (164). Northern blot and in situ hybridization analyses have shown mRNA expression of this calcium-binding protein by chondrocytes throughout the growth plate (95,119). More recently, the selective accumulation of this molecule in the matrix of the hypertrophic zone was reported (120. The possible role of this protein in the calcification of cartilage remains to be established.

1.2. CARTILAGE PROTEOGLYCANS

Proteoglycans are a heterogeneous group of specialized glycoproteins which are commonly found in the extracellular matrix of all tissues, especially in the connective tissues where the extracellular matrix is well developed. Hyaline cartilage contains a large amount of these molecules (ranging from 5-10% of the wet weight tissue). Proteoglycans have a common structure characterized by a central core protein to which at least one glycosaminoglycan side chain is covalently bound. This structural unit is named the proteoglycan "subunit" or

"monomer". Proteoglycans can be divided into several classes on the basis of the types and sizes of their glycosaminoglycans and the functional properties of their core proteins, although the number of the individual species within this entire family is, as yet, unknown (127).

Glycosaminoglycans, with the exception of keratan sulfate of the cornea which is branched, have an unbranched chain structure consisting of repeating disaccharide units. These units are composed of hexosamine and hexuronic acid or galactose (in case of keratan sulfate), each disaccharide usually carrying a sulfate group (except for hyaluronic acid) or a carboxylate group (except for keratan sulfate), respectively. This provides the high anionic charge to the proteoglycan molecules. Six different types of glycosaminoglycans have been identified, namely chondroitin sulfate, keratan sulfate, heparan sulfate, dermatan sulfate, heparin and hyaluronic acid. Each of these molecules has different disaccharide components (64,127). A chondroitin sulfate chain usually consists of 20-60 repeating disaccharides (MW: $1 \times 10^4 - 5 \times 10^4$) of a glucuronic acid, and an N-acetyl galactosamine that can be O-sulfated at either the 4 or 6 position or at both positions. Although the sulfated product usually contains approximately a single sulfate group per disaccharide, this 1:1 ratio is changeable, ranging from 0.1 up to 1.3, depending on the tissue (98). Even when the ratio is close to 1, the chains invariably contain some non-sulfated and some disulfated disaccharides (64). Thus, there is a diversity in the chemical properties of the chondroitin sulfate chains within a single proteoglycan molecule. The covalent linkage of chondroitin sulfate to core protein is mediated by oligosaccharides composed of galactose-galactose-xylose. Galactose is bound to the reducing end of glucuronic acid that is linked to chondroitin sulfate and xylose is bound to a serine residue in the core protein of

proteoglycan. Keratan sulfate (MW: 4×10^3 - 2×10^4) consists of repeating disaccharides of galactose and N-acetyl glucosamine which can be sulfated at the 6 position. In cartilage, oligosaccharides covalently link keratan sulfate to a serine or a threonine residue in a core protein (Fig. 2). Hyaluronic acid is a non-sulfated polymer of repeating disaccharides consisting of glucuronic acid and N-acetyl glucosamine. The length of this molecule can vary from 10,000 to 4,000 disaccharide unit lengths (156).

The most abundant and characteristic proteoglycans present in hyaline cartilages are the large aggregating proteoglycans, (now often called aggregating), common features of which are well conserved among various hyaline cartilages such as articular, growth plate, nasal and rib cartilages. This species of proteoglycan was studied in this thesis work and is reviewed in this section (69,71). (The dermatan sulfate proteoglycan which is also present in cartilage is not described in this section). Recently, considerable progress has been made to understand the detailed structure of this highly complex macromolecule. The schematic illustration of the structure is shown in Fig. 3 (71). The average molecular weight of the monomer is about $2\text{-}3 \times 10^6$ (66). Electron microscopic studies of the core protein of the proteoglycan employing rotary shadowing techniques have revealed the presence of three distinct globular domains within the protein (G1, G2 and G3) (175) and two extended regions (E1 and E2) (121). G1 is located at the amino-terminus and is separated from G2 by short strand E1 (25 nm). It is followed by strand E2 (280 nm), which is terminated by G3 located at the carboxyl terminus. However, many proteoglycan monomers isolated from adult articular cartilage have shorter proteins and lack G3 domains (121). These observations correlate well with the peptide mapping and sequence analyses of the core protein (40,114,116,148).

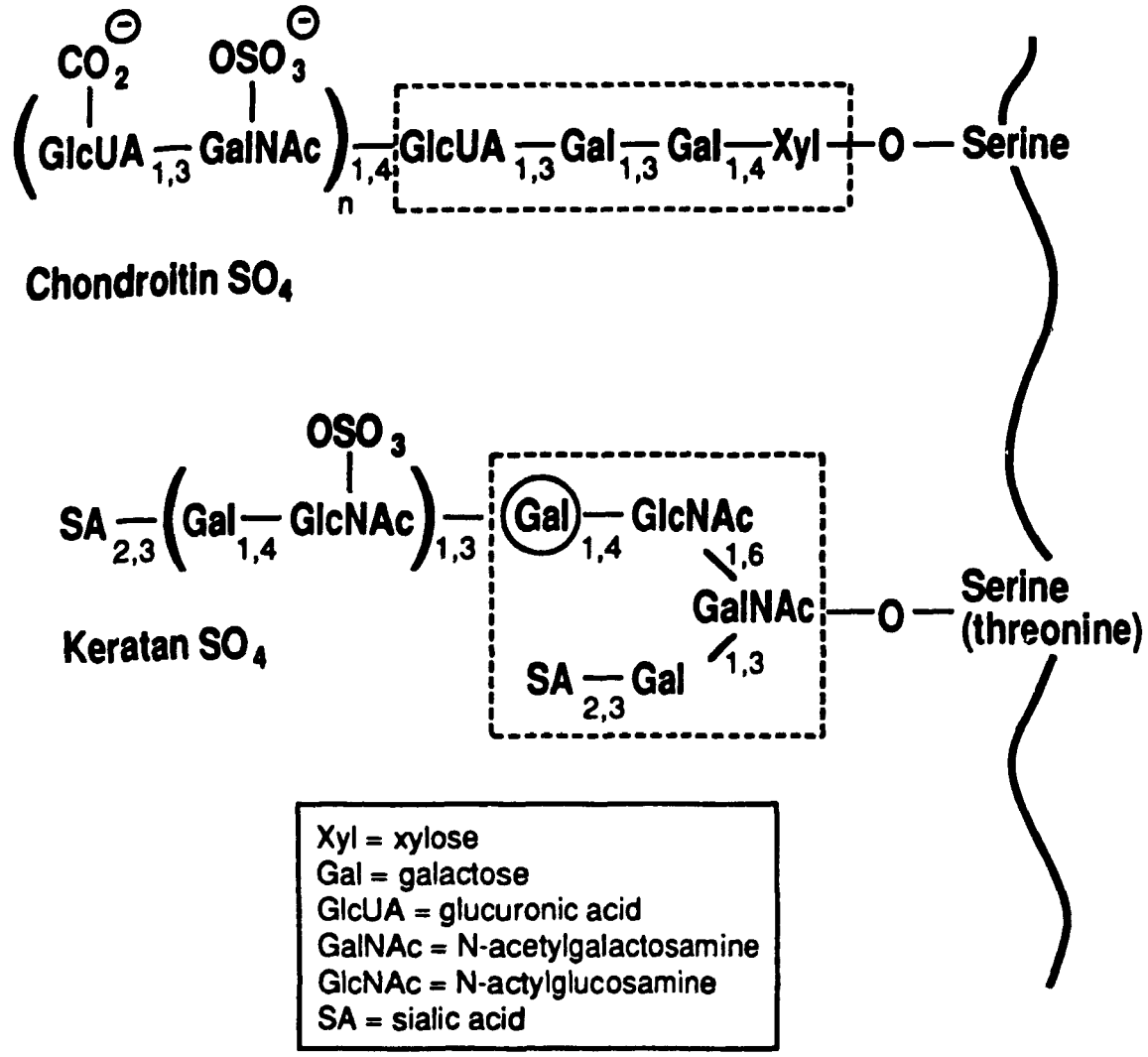


Figure 2 : Structure of the Linkage Region of Glycosaminoglycan Chains to Proteoglycan Core Protein

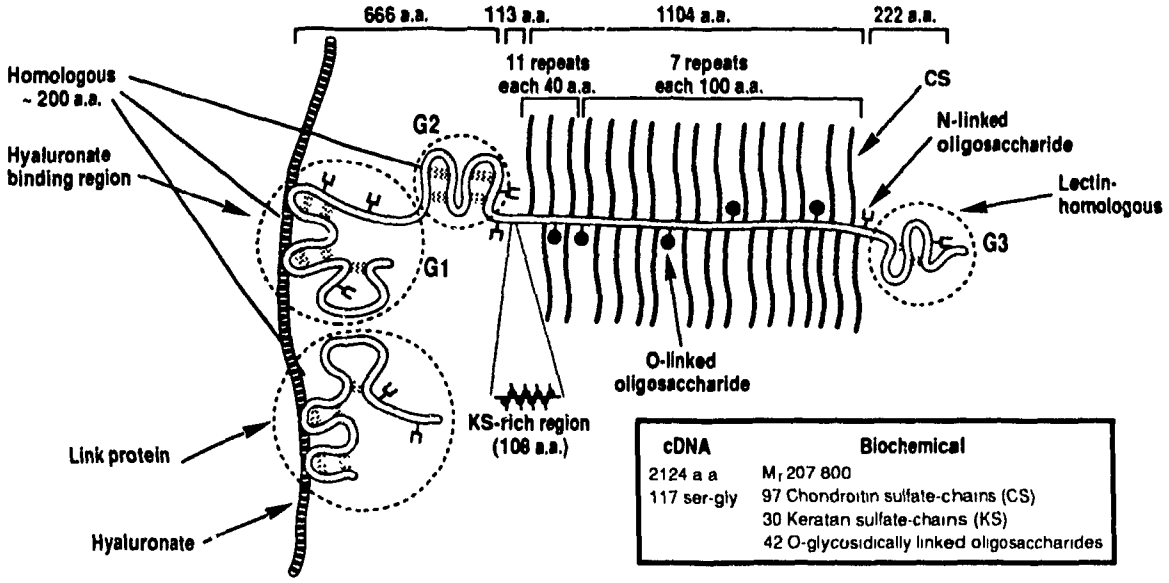


Figure 3 : Schematic Representation of a Proteoglycan Monomer Based on the Rat Chondrosarcoma Cartilage Proteoglycan Monomer

From the amino acid sequence of the corresponding region, G1 is considered to contain a double loop and a single loop which are located at the amino-terminus (40,114). G1 was shown to have a hyaluronic acid-binding region (110,121). Despite the high homology between double loops of G2 and G1 and link protein (40,114), G2 did not show any binding property with hyaluronic acid, proteoglycan monomers and other matrix molecules (47,110). Thus, its possible function in the matrix remains unknown. The third globule (G3) is homologous with a hepatic lectin and can bind to galactose and fucose with a relatively low affinity (58), providing the possibility of this domain to interact with some other, as yet unidentified, matrix molecule. At the end of the E2 region close to G2, a peptide which corresponds to the keratan sulfate-enriched region is absent in rat chondrosarcoma proteoglycan (40) but is found in bovine cartilage proteoglycan (16). This peptide consists of 23 tandemly repeated hexapeptides which are highly conserved. Almost all serine and threonine residues within this peptide are O-glycosidically substituted with carbohydrate chains (6). This region is followed by the major part of the core protein, chondroitin sulfate-enriched region, consisting of two distinguishable domains based on the different sizes and numbers of their repeating amino acids (40). These two domains contain a total of 117 serine-glycine residues, about 100 of which provide the attachment sites for chondroitin sulfate. The first one contains 11 repeats of 40 amino acids. Each repeating unit carries 4 serine-glycine residues with a random distribution. The second one contains 7 repeats of 100 amino acids, each of which has more regular and segmental distribution of serine-glycine residues thereby forming clusters of chondroitin sulfate chains. A short domain, adjacent to G3, contains 6 serine-glycine residues and two N-linked glycosylation sites. This domain is not highly conserved between species. There is short additional domain in humans,

which is homologous to an epidermal growth factor-like sequence seen in other proteins (11).

The majority of proteoglycan monomers can specifically interact with hyaluronic acid to form aggregates (61,67,123,145). Each aggregate usually contains 20-50 proteoglycan monomers that are bound to a single hyaluronic acid chain. However, aggregates which contain extremely large numbers of monomers have been reported in bovine epiphyseal cartilage (25). Aggregates are extremely large molecular assemblies in the range of 50-150S depending on the length of hyaluronic acid and the number of monomers bound to it (63). The specific and non-covalent interaction between the hyaluronic acid-binding region of the core protein and hyaluronic acid enables monomers to form aggregates with hyaluronic acid (70). It requires a minimum decasaccharides of hyaluronic acid (62,68) and is greatly stabilized in the presence of link protein (63,163), a double loop of which is homologous to the corresponding site of hyaluronic acid-binding region of the core protein (40,114). This strong supramolecular interaction plays an important role in anchoring monomers to hyaluronic acid in vivo.

In free solution, the proteoglycan molecules are fully expanded and can ensnare 50 times their volume of water per dry weight (66). This is caused by the hydrophilic property of glycosaminoglycan side chains which bear the highly anionic charge groups as discussed previously. In cartilage matrix, the concentration of proteoglycan is approximately 10% of the wet weight, which represent 10 ml/g (dry weight) or 20% of the fully expanded domain in free solution (69). It has been, therefore, considered that in the matrix, proteoglycan exerts a swelling pressure on the inextensible collagen fibrillar network and thus provides the cartilage with the functional property of compressive stiffness or

resiliency to mechanical loading (86).

The maintenance of the water compartment in the tissue by proteoglycan has another role in allowing chondrocytes to access water, soluble nutrients, electrolytes and small proteins, such as growth factors, essential for their survival.

1.3. CARTILAGE PROTEOGLYCANS AND THE GROWTH PLATE

Matrix macromolecules including collagen and several non-collagenous proteins have been investigated in relation to their potential role in the initiation and growth of hydroxyapatite in calcifying tissues (19). Cartilage aggregating proteoglycan is one of them. Atomic elemental studies of the growth plate demonstrated the spatial co-distribution of calcium and sulfur separate from localized concentrations of phosphorus in precalcifying matrix of the growth plate using electron spectroscopic imaging (8). These elemental distributions change subsequently into a virtual superposition of the three at early mineral deposition sites. A similar accumulation of sulfur associated with early mineral deposition was observed with a dye-binding method by (154). These results suggest the presence of sulfur-containing matrix macromolecules, probably proteoglycans, at these foci, offering evidence for the potential involvement of this molecule in the initiation of mineral deposition. Rosette-like structures of concentrated proteoglycans were observed in calcifying sites in the cartilage matrix fixed with dye prior to demineralization (155). Similar structures stained with proteoglycan-specific antibodies were also produced artificially in precalcifying zones of the growth plate when calcium in the tissue was extracted with EDTA in the presence of proteinase inhibitors (129). The focal concentrations of proteoglycans are thought to give rise to nucleation sites required for mineral growth (123,134). This hypothesis is supported by the

evidence that polysulfated proteoglycans function as an ion-exchanger, first capturing calcium which is then displaced by increased $[PO_4]$. This raises the focal $[Ca]$ and $[PO_4]$ leading to precipitation of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) crystals (77). Others have also reported that cooperative actions of sulfates and structured carboxylates, in which the former concentrate calcium, induce oriented-calcite nucleations, implicating proteoglycans in hydroxyapatite crystal nucleation and growth (1).

In contrast to these observations, in vitro studies employing free solution systems have suggested that proteoglycan monomers and, especially aggregates, inhibit hydroxyapatite formation from calcium phosphate solutions by their binding capacity to calcium (16,35). In these systems, it appears unlikely to create desired conditions for crystalline hydroxyapatite formation where proteoglycans capturing calcium are focally condensed and inorganic phosphate is supplied continuously, which would be achieved in in vivo situations.

Large proteoglycans in the growth plate have been studied particularly in order to understand what happens to these molecules during the processes of matrix calcification. Some have reported by biochemical analyses that there is a loss of these molecules from the matrix as it calcifies (18,49,99). Others found no evidence of such a loss by immunohistochemical methods using antibodies against proteoglycans or quantitative electron probe microanalysis for sulfur in situ (65,124,125). Recent biochemical studies, in this laboratory, of matrix molecules of the different zones have revealed that the content of proteoglycans per wet weight tissue decreased progressively from the proliferative zone to the upper hypertrophic zone. However, it increases gradually when expressed per unit matrix volume rather than total tissue volume (3). It has been reported that proteoglycans are degraded reducing the sizes of their monomers and aggregates

as indicated by the shorter hyaluronic acid filament lengths and fewer monomers per aggregate (10,28) and aggregatability with hyaluronic acid (28,33) according to one school of thought. This would decrease their potential to inhibit hydroxyapatite deposition, and therefore permit cartilage calcification. Recently, biochemical and immunochemical analyses of these molecules have revealed no evidence of any changes in monomer sizes and their aggregatability at the initiaiton of calcification, whereas in well calcified zones, a slight decrease in size of the monomer was identified (104).

Related to the possible degradation of these structural components, there have been several reports describing proteoglycanase activities in the growth plate in vitro (28,43,44). Later, synthesis of stromelysin, which is believed to cleave proteoglycans by growth plate chondrocytes (particularly cells in the upper hypertrophic zone) was demonstrated immunologically (23). These studies need to be substantiated with respect to these enzymatic activities in situ.

1.4 THE HUMAN SKELETAL DYSPLASIAS

The human skeletal dysplasias are a highly heterogenous family of more than 100 distinct genetic disorders whose common clinical characteristics are disproportionate short stature (dwarfism) and/or skeletal deformities. These conditions result from impaired and abnormal growth or development of cartilage and/or bone. Until the 1960s, most of them were considered to have either achondroplasia (those with short limbs) or Morquio disease (those with a short trunk) (143). Recently, the enormous heterogeneity of individual disorders within these conditions has been recognized and a classification and nomenclature have been proposed. It is in common usage (International nomenclature of constitutional diseases of bone, 1978, 1983) based on their clinical and radiological characteristics, the classification primarily divides this

highly complex family of the constitutional diseases of the skeleton into five major groups: the osteochondrodysplasias (abnormalities of cartilage and/or bone growth and development), the dysostoses (malformation of individual bones, singly or in combination), the idiopathic osteolysis (a group of disorders associated with multifocal resorption of bone), the miscellaneous disorders with osseous involvement, and a group of skeletal disorders associated with primary metabolic abnormalities due to chromosomal aberrations.

The osteochondrodysplasias can be subdivided into three groups: the chondrodysplasias (defects of growth and/or spine); a group with disorganized development of cartilage and fibrous components of the skeleton; and a group with abnormalities of density of cortical diaphyseal structure and/or metaphyseal modelling. While the classification is clinically based and straight forward to use, the nomenclature is still complexed and confusing. This is because it is based upon either the affected parts of skeletons, the appearance of the deformities and clinical courses, eponyms, or the pathogenesis of the conditions. Although some of them were named on the basis of their pathogenesis, the names achondroplasia and achondrogenesis, for example, do not appear to accurately reflect their pathogenesis. Revision of the nomenclature to discriminate and define these individual diseases awaits the elucidation of their specific pathogenesis and biochemical defects.

Until two decades ago, investigations of the skeletal dysplasias were only restricted to clinical and radiographic studies (140). Since then, morphological studies of cartilage and bone tissues have been made to make more accurate diagnosis of the specific disorders, thereby indicating their biological abnormalities (143,157,160). Recent progress has been made in biochemical studies of structural matrix elements of cartilage and bone, particularly

dominant macromolecules such as large aggregating proteoglycan, and type I and type II collagens. These studies have improved our understanding of the structural assembly and organization of these matrix molecules in situ. Furthermore, remarkable developments in molecular biology have made it possible to analyze the structures of these collagen. These, together with the indicated biological abnormalities in particular forms of these human conditions, have attracted much attention in attempts to determine biochemical defects in these matrix molecules or specific mutations in the genes that encode the molecules responsible for certain types of skeletal dysplasias.

Most forms of osteogenesis imperfecta, characterized by brittle bone and abnormal membranous ossification, are now known to result from various mutations in type I collagen genes (31,135). Kniest dysplasia was shown to probably involve the suspected abnormal processing of type II procollagen, characterized by the abnormal intracellular cleavage of procollagen and the retention of removed C-propeptide from the rest of this molecule. It suggests that type II collagen fibril assembly in the absence of the C-propeptide leads to the formation of aberrant collagen fibrils and a disorganized cartilage matrix (130). Biochemical studies of spondyloepiphyseal dysplasias have demonstrated that some of them are associated with a structural defect in type II collagen, which result from delay of the triple helix formation and consequent overmodification of this collagen (113). More recently, an individual and a family with this disorder were shown to carry abnormal type II collagen genes with a single exon deletion and an internal tandem duplication, respectively (96,165). Another example of association of aberrant type II collagen with another form of chondrodysplasias was demonstrated by the immunohistochemical and biochemical analysis of an infant with a lethal form of

type II achondrogenesis-hypochondrogenesis (55,56). Further analysis revealed that this infant bore a point mutation in the type II collagen gene (171). Genetic linkage studies of the families with the Stickler syndrome have also shown that this disease is linked to a specific type II collagen gene allele (48,92). More recently, a family with primary generalized osteoarthritis associated with mild chondrodysplasia was also shown to have close genetic linkage to a specific type II collagen gene allele (93). However, this family did not have a single particular abnormality in cartilage proteoglycan responsible for the cause of the disease (84). These investigations of particular forms of human chondrodysplasias have clearly suggested that various types of mutations at different sites within the gene that encodes the type II collagen can result in diverse aberrant gene products and consequent mutation-specific phenotypes of these conditions.

Besides the human investigations, there are animal models including various species of mammals and birds which are available for the study of some of these human conditions (24,108). The biological analyses of these animal models including histological, genetic and biochemical studies are considered to be helpful for the understanding of the biological basis of the human skeletal dysplasias, by comparing disease-specific features of the animals to those of humans. Because of the high degree of inbreeding and background uniformity, the murine models have been extensively studied. They show a high heterogeneity of affected molecules, genetic codes, and phenotypes of diseases as humans (24,142). Genetic studies of mice may also facilitate the determination of specific loci in human chromosomes, which may enable us to identify polymorphisms for linkage analyses (32).

Of particular interest are certain animal models, including those invoiving mice and chickens, which exhibit abnormal synthesis of cartilage-specific

proteoglycan in relation to a particular form of human chondrodysplasia. Pseudoachondroplasia has been also suggested to have an abnormality involving proteoglycan (discussed in next section). In murine models, two autosomal recessive forms of chondrodysplasias have been identified within this group: brachymorphic (bm/bm) and cartilage matrix deficiency (cmd/cmd) mice. The brachymorphic mutant exhibits dwarfism, a short tail and a rounded snout. The mutant cartilage matrix contains normal collagen fibrils, but the proteoglycan aggregate granules are smaller and are present in reduced numbers, particularly in the hypertrophic zone of the growth cartilage (117). The mutant cartilage was shown to synthesize normal levels of glycosaminoglycans but the proteoglycan was abnormal with reduced sulfate incorporation when labelled with $^{35}SO_4^{2-}$ (118). Further biochemical studies focusing on the sulfation pathway have revealed that this is due to a defect in 3'-phosphoadenosine 5'-phosphosulfate formation. This functions as a sulfate donor in the sulfation of glycosaminoglycan chains (162). In contrast to the comparatively mild, nonlethal phenotype of the brachymorphic mice, the cartilage matrix deficiency mouse is a newborn lethal chondrodysplasia characterized by a short trunk, limbs and tail as well as a blunt snout, a protruding tongue due to a small jaw, and a cleft palate. The morphology of the growth cartilage displays closely packed chondrocytes with a loss of their normal columnar arrangement and reduced extracellular matrix (144). There is an absence of the synthesis of proteoglycan core protein in the cartilage, where a normal content of type II collagen is found (87). This defective synthesis of proteoglycan core protein is limited to the cartilage-specific large proteoglycan (21). There is a homozygous recessive mutant of dwarfism in chickens, named nanomelia characterized by short limbs and a parrot-like beak (94). As in cmd/cmd mice, this is characterized by the defective

synthesis of cartilage-specific proteoglycan core protein (7). Further studies have revealed that this deficiency occurs at the level of the transcription of the gene encoding proteoglycan core protein. Comparative analyses of normal and the mutant genomic DNA revealed that there is no major deletion in the core protein gene, suggesting that the mutation may affect a protein which regulates the transcription of the gene (161). In addition, to naturally occuring mutations, some drugs are capable of causing phocomelia or micromelia as a result of teratogenic side effects. An effect on cartilage-specific proteoglycan was shown in azaserine-induced chick micromelia. Administration of this drug to chick embryos produced micromelial limbs, in which the deficient synthesis of a particular form of cartilage-characteristic proteoglycan with a reduced number of chondroitin sulfate chains was seen (74). These investigations strongly suggest that abnormalities in the synthesis and assembly of cartilage-specific proteoglycans can result in the phenotypic expression of certain forms of skeleta. dysplasias.

1.5 PSEUDOACHONDROPLASIA

Pseudoachondroplasia is a relatively common form of the human chondrodysplasias. It was first recognized in 1959 by Maroteaux and Lamy (102). The disorder is distinguishable from the most common form of these conditions, achondroplasia, by the late onset of clinical manifestations, a lack of involvement of the skull and face, and characteristic roentgenographic appearances of epiphyses, metaphyses, and vertebrae (102). Although mild and severe forms of autosomal dominant and recessive inheritance have been described (59), it is still unclear whether there are subtypes of this disorder on the basis of clinical and radiographic features (141, 181). Furthermore, as has been suggested for some patients with achondroplasia and an autosomal dominant

type of osteogenesis imperfecta, gonadal mosaicism may account for unexpectedly high segregation ratios in some pedigrees with this disease which were thought to be autosomal recessive traits (60). Clinical manifestations of the disease are short stature with a relatively long trunk, marked short limbs with various deformities, unstable joints, and precocious osteoarthritis, particularly in knees and hips. Roentgenographic changes include delayed development of epiphyses, and flared and ragged metaphyses of vertebrae, pelvis, and limb bones, suggesting abnormal endochondral ossification.

Historically, many investigations have been made to attempt to elucidate the molecular pathobiology of this disorder. Light microscopic examinations of cartilage have shown reduced staining of the matrix with cationic dye for proteogylycan and a disorganization characterized by the absence of hypertrophy of some cells, disruption of endochondral ossification, and clumping of the aberrant cells with inclusion bodies (122,157,160). The cells appear to contain inclusion bodies in the rough endoplasmic reticulum detected by electron microscopy (37). Gel-electrophoretic studies of proteoglycans have suggested that there is one population of proteoglycans absent from the cartilage matrix (158). Recent immunohistochemical studies of cartilage have revealed that intracellular material that accumulates reacts with antibodies against the core protein of the large cartilage proteoglycans (159, 160). Biochemical analysis of the cartilage proteoglycans has demonstrated a decreased ratio of chondroitin 4-sulfate to chondroitin 6-sulfate and an increased content of keratan sulfate (122). More recently, an individual with this disease was shown to have no abnormalities in newly synthesized proteoglycans labelled with $^{35}SO_4^{2-}$ in terms of their sulfate incorporation, monomer sizes and electrophoretic mobilities, chain length of chondroitin sulfate, and composition of chondroitin sulfate (13).

Taken together, these previous observations suggest that the disease appears to be characterized by the normal intracellular processing of cartilage proteoglycan involving the imperfect intracellular trafficking of proteoglycan core protein and the alteration of sulfation position on chondroitin sulfate chains.

1.6 AIMS OF THE THESIS

As reviewed in this chapter, pseudoachondroplasia is not fully elucidated in the light of the primary molecular defect responsible for the phenotypic expression of the disease. Determination of the defect may permit cause-oriented treatment of the disease and improve our understanding of normal molecular mechanisms in the growth plate. Investigations have been made as follows:

1. Comparative studies of large aggregating proteoglycans extracted from rib cartilages of patients with this disease as well as normal controls using biochemical and immunochemical techniques with respect to (a) the contents of proteoglycans and epitopes of specific antibodies, (b) the hydrodynamic sizes of proteoglycan monomers and their immunological characterization, (c) the chain length of chondroitin sulfate, (d) the composition of chondroitin sulfate, and (e) the reaggregatability of these proteoglycans with added hyaluronic acid.
2. Comparative quantitative analyses of another matrix molecule, hyaluronic acid.

CHAPTER 2

MATERIALS & METHODS

2.1 PREPARATION OF SAMPLES

2.1.1 Source of tissue

Rib cartilages from four patients of increasing ages (4,9,12 and 31 year old patients) were obtained at biopsy and separated into three groups: young (age 4 years), intermediate (age 9 and 12 years) and adult (age 31 years). Diagnoses of the disease were made on the basis of the presence of the characteristic clinical features (see section 1.5.) by Dr. David L. Rimoin (Cedars-Sinai Medical Center, Los Angeles, California), who is an expert on clinical and morphological studies of these patients and a project director of the investigations on the human skeletal dysplasias. Cartilages were immediately freeze-dried and stored at -20°C until examined. The same tissues from age-matched normal controls (4,11 and 30 years old) who had no past history indicative of skeletal abnormalities were isolated within 12 h post-mortum at autopsy and then stored at -20°C until examination. It has been known that, within this period, no catalytic changes of cartilage proteoglycan occur (12). Samples from patients were provided by Dr. David L. Rimoin.

2.2. ANALYSIS OF PROTEOGLYCANS

2.2.1 Extraction of proteoglycans

Samples from patients were rehydrated for 2 h at 4°C with phosphate buffered saline, pH 7.4 (PBS) and wet weights were determined. The tissue was then frozen sectioned to 20 μm thickness using a cryostat. The same procedure was employed for normal cartilages except for the rehydration. Sliced cartilages were treated first with 5 volumes of PBS containing 1% 3-[(3-cholamidepropyl)-

dimethylammonio]-1 propanesulfonate (CHAPS) (Calbiochem Corp., La Jolla, CA) for 1 h at room temperature. CHAPS is a detergent which solubilizes lipid components in cell membranes and enables one to extract intracellular materials efficiently prior to extraction with guanidine hydrochloride (30). Cartilages were then extracted with 25 volumes of extraction buffer to give a final 30 volumes of 4 M guanidine hydrochloride, 0.1 M sodium chloride, pH 6.0 containing protease inhibitors (1 mM concentrations each of ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, and phenylmethylsulfonyl fluoride (PMSF), and 5 µg/ml of pepstatin) for 48 h at 4°C with stirring (146). After samples were centrifuged for 15 min at 1600 g, supernatants were collected as total cartilage extracts and stored at -20°C. Pellets were stored at -20°C as tissue residues prior to measurement of uronic acid contents in both extracts and residues by using the carbazole method (15) as described below (to determine proteoglycan extractability).

2.2.2. Uronic acid assay

The dialyzed samples were diluted with distilled water to give the assay volume of 500 µl followed by the addition of 3 ml of 0.025 M sodium tetraborate in concentrated sulfuric acid. After vortexing vigorously, test tubes were cooled in ice cold water for 10 min and then heated at 100°C for 10 min. Then the samples were cooled again at 0°C for 10 min and 100 µl of 0.125% carbazole in absolute ethanol was added. The reaction mixture was heated at 100°C for 15 min and cooled briefly at 0°C. The absorbance was measured at 530 nm with a spectrophotometer (Gilford, model 250). The contents of uronic acid in the samples were determined with reference to the standard curves by assaying the varying concentrations of glucuronic acid in the same assay.

2.2.3 Sepharose CL-2B gel chromatography

In order to determine the hydrodynamic sizes of proteoglycan monomers, 500 µl volumes of cartilage extracts were diluted twice with dissociative buffer (4 M guanidium hydrochloride, 0.1 M sodium acetate and 0.5 mg/ml sodium azide, pH 6.0) and chromatographed on Sepharose CL-2B (Pharmacia Fine Chemicals, Uppsala, Sweden), packed in an Econo column (110 x 1.25 cm, BioRad, Montreal) at a flow rate of 6 ml/h in dissociative buffer. 1 ml fractions were collected.

To determine the aggregatability of proteoglycans with hyaluronic acid, 500 µl of cartilage extracts were dialyzed against 1 liter of associative buffer (0.2 M sodium acetate, 0.5 mg/ml sodium azide, pH 5.5) to remove guanidine hydrochloride, which allowed proteoglycan monomers to reaggregate with hyaluronic acid. Samples were then chromatographed on Sepharose CL-2B under associative conditions. Prior to the dialysis, hyaluronic acid (high molecular weight, human umbilical cord, a generous gift by Dr. P.J. Roughley, Genetics Unit, Shriners Hospital for Crippled Children, Montreal) was added to the extracts at the concentration of 10% weight/weight of proteoglycans in the extracts to obtain maximal reaggregation of proteoglycans. The proteoglycan contents were evaluated as being four times those of uronic acid contents according to previous data (150). The dialyzed extracts were centrifuged for 10 min at 1,000 g to remove visible precipitations and diluted twice with associative buffer and then chromatographed on a Sepharose CL-2B column (110 x 1.25 cm) with an elution rate of 6 ml/h of associative buffer. 1 ml fractions were collected.

To standardize each gel chromatogram, these two columns were calibrated. For the dissociative column, its void and total volumes were determined by the elution profiles of blue dextran 2000 (Pharmacia Fine Chemicals) and

glucuronolactone (Sigma Chemical Co.), respectively. For the associative column, human fetal proteoglycan aggregates and glucuronolactone were used to determine its void and total volumes, respectively. Contents of blue dextran in fractions were measured by its ultraviolet absorbance at 280 nm. Contents of proteoglycan and glucuronolactone were measured by uronic acid assay. All column profiles of both dissociative and associative studies were plotted against K_{av} value of each fraction calculated from the following formula:

$$Kav = \frac{\text{fraction volume - void volume}}{\text{total volume - void volume}}$$

2.2.4. Chondroitinase ABC digestion and sodium dodecyl sulfate (SDS) treatment

In order to determine total contents of various proteoglycan epitopes in radioimmunoassays discussed later, 50 µl of each associative sample was serially diluted (50 - 30,200 times dilution) with associative buffer and then digested with or without an equal volume of 0.2 M Tris/acetate, pH 7.6, containing 0.04 units/ml chondroitinase ABC (ICN, ImmunoBiologicals, Costa Mesa, CA, U.S.A.) for 6 h at 37°C to create proteoglycan-specific epitopes. The samples were then treated with a half volume of 0.1 M Tris/acetate, pH 7.5 containing 0.125% (w/v) sodium dodecyl sulfate (SDS) to give a final concentration of 0.025% (w/v). They were heated at 80°C for 15 min. This redissociated the monomers and hyaluronic acid. Previous studies indicated that this treatment prevents proteoglycan monomers from reaggregating with hyaluronic acid. This causes masking of epitopes, resulting in poor reaction of antibodies with specific epitopes in radioimmunoassays (136).

All dissociative column fractions were first microdialyzed against 4 liters of associative buffer for 48 h at 4°C to remove guanidine hydrochloride using a microdialysis unit (Bethesda Research Laboratories Inc., Gaithersburg, MD) and

Spectrapor, 3500 molecular weight cut-off dialysis membrane (Sepctrum Medical Industries, Los Angeles, CA). The same fixed volume of all the dialyzed fractions was digested with chondroitinase ABC and heat-treated with SDS in the same method as described above.

All associative fractions were also treated in the same manner as dissociative fractions except that microdialysis was not necessary.

2.2.5. Immunoassays of proteoglycan

Iodination of proteoglycan

Purified human fetal and adult cartilage proteoglycan monomers were iodinated by employing the chloramine-T method. Under optimum conditions, the reaction of cationic iodine (I^+), with tyrosine residues, is maximized and thereby maximum incorporation of iodine into protein can be obtained (17). Two hundred micrograms of D1 preparations of either fetal or adult proteoglycan were dissolved in 100 µl of radiolabelling buffer (50 mM Tris/HCl, 150 mM sodium chloride, pH 7.5). Ten microliters of 0.4 M potassium phosphate buffer containing ^{125}I-sodium iodine (0.5 mCi, Amersham) and 10 µl chloramine-T (600 µg/ml) were added to the solution and then gently vortexed for exactly 2 min. The reaction was stopped by addition of 100 µl sodium metabisulfite (1.2 mg/ml) and then chromatographed on Sephadex G-25 (Pharmacia Fine Chemicals) (bed volume 10 ml) together with 200 µl sodium iodide (10 mg/ml in radiolabelling buffer) in 100 mM Tris/acetate, 100 mM sodium acetate, 50 µg/ml sodium azide, pH 7.3 to separate the radiolabelled proteoglycan from unbound radioactive iodine. Prior to loading the radiolabelled materials, non-specific binding to the column was prevented by the application of 400 µg of bovine serum albumin (RIA grade, Sigma). Radioactivity in each fraction (approximately 0.5 ml) was monitored and first peak fractions (over 500,000 cpm/10 µl of fraction) eluting at

the void volume of the column were pooled. The radiolabeled proteoglycan was digested with chondroitinase ABC (0.02 units/ml) for 6 h at 37°C in the presence of proteinase inhibitors (1 mM each phenylmethylsulfonyl fluoride, iodoacetamide, and EDTA and 5 µg/ml pepstatin A) and then dialysed overnight against 2 liters of PBS at 4°C to remove free oligosaccharides created by the digestion. An equal volume of radioimmunoassay buffer (next section) was added to the dialyzed sample.

Assay

Proteoglycan contents in Sepharose CL-2B column fractions (starting from several fractions before the void volume up to just after the total volume of the columns) as well as in total cartilage extracts were measured employing solution phase inhibition radioimmunoassays. The buffer used throughout for assays consisted of 7.5 mM potassium dihydrogen phosphate, 150 mM disodium hydrogen phosphate, pH 8.1 containing 1 mg/ml BSA, 5 mg/ml sodium deoxycholate, 0.25% (v/v) Nonidet P-40 and 500 µg/ml sodium azide. Fifty microliters of native or chondroitinase ABC digested proteoglycan which had been treated with SDS (in triplicate) was first incubated in a glass test tube with 50 µl of optimally diluted proteoglycan-specific antibody (next section) which previously had been shown to give 40-50% binding of labelled antigen for 1 h at 37°C. This was followed by addition of 50 µl of ^{125}I-proteoglycan antigen (10,000 cpm) and incubated for 1 h at 37°C. The subsequent procedures for the recovery of the immune complexes varied according to the type of immunoglobulin or antiserum. In the case of mouse IgG antibodies, the separation of bound from free antigen was done by addition of 50 µl of three times diluted protein A bearing Staphylococcus aureus (10% in suspension in distilled water) (Zymed, Cedarlane Laboratories, Hornby, Ontario) that has high affinity for these antibodies at the pH of the assay. The

mixture was incubated for 30 min at room temperature. Mouse IgM antibodies were first combined with either 50 µl of optimally diluted rabbit or pig antiserum (raised against mouse IgM) overnight at 4°C and then incubated with protein A in the same way as mouse IgG. Immunoprecipitates were harvested by removal of the supernatant after addition of 1 ml of buffer and centrifugation for 20 min at 2400 g. The radioactivities of the pellets were counted with a β-counter (1270 Rack Gamma II, LKB, Wallac).

The background radioactivity was measured in the absence of antibodies at the same time of each assay. This was subtracted from the given counts. The percent inhibition of binding yielded by an unknown amount of proteoglycan in a sample was calculated relative to bound labelled antigen in the absence of non-labelled competing antigen by the following formula:

$$\% \text{ inhibition} = 100 - 100 \times \frac{\text{cpm bound in presence of inhibitor}}{\text{cpm bound in absence of inhibitor}}$$

The epitope contents of proteoglycan in samples were referred to the standard inhibition curves constructed using known amounts of the appropriate species of standard proteoglycan, depending on the antibody used, at the same time of each assay. Thirteen serial doubling dilutions of each standard proteoglycan starting from 100 µg/ml were always made of the same D1 preparation of either human adult or fetal cartilage proteoglycan with or without chondroitinase ABC digestion (according to the assay) followed by SDS treatment. Standard curves ranged from 100% down to 0% inhibition of binding. Samples to be assayed were diluted so that the % inhibition values after appropriate dilutions fell within the linear portions of the curves, from which their epitope contents were obtained with minimum errors. Contents of epitopes were related to equivalents of intact standard proteoglycans.

Antibodies used in these studies of proteoglycan were mouse monoclonal

antibodies, namely 3-B-3 (IgM) (ICN ImmunoBiologicals, Costa Mesa, CA, U.S.A.). 1-B-5 (IgG_1 kappa) (ICN ImmunoBiologicals), 6B (IgG_1), AN9P1 (IgG_{2a}), 846 (IgM). Mouse monoclonals 6B and AN9P1 were developed by Mrs. Carolyn Webber in this laboratory and raised against chondroitinase ABC-digested human fetal proteoglycan and native human adult proteoglycan, respectively. Monoclonal antibody 846 was generated in this laboratory by Dr. Tibor T. Glant raised against native human fetal proteoglycan. Rabbit antiserum 483 was raised by Dr. A.R. Poole against the hyaluronic acid-binding region (G1) isolated from the rat chondrosarcoma cartilage proteoglycan.

Previous characterization of the antibodies and further characterization of 6B and 3-B-3

a. Antibody 6B: The binding studies of 6B showed that the antibody did not bind to intact proteoglycan but reacted only with chondroitinase ABC-digested human fetal proteoglycan (not adult). The binding of 6B to ^{125}I-human fetal proteoglycan (chondroitinase ABC-treated) was specifically inhibited by the addition of either the enzyme-digested proteoglycan or unsaturated disaccharides of chondroitin 4-sulfate, suggesting that the antibody reacts not only with chondroitin 4-sulfate "stubs" that remain attached to a core protein after the enzymic treatment but also with the free disaccharides (172). For further characterization of the antibody, the following experiments were designed to determine which one of the two recognizable antigens the antibody preferentially reacts with when both are present in the same solutions. A standard inhibition assay was performed using human fetal proteoglycan either with or without dialysis against associative buffer to remove generated oligosaccharides after chondroitinase ABC digestion as described above. Each concentration was plotted on the basis of proteoglycan protein content measured

by the Lowry method.

b. Antibody AN9P1: AN9P1 was characterized in detail elsewhere (132). This antibody primarily reacts with intact keratan sulfate chains attached to the core protein of proteoglycan monomer.

c. Antibody 846: Studies of 846 raised against intact human fetal proteoglycan monomers were reported previously revealing the unique feature of this antibody (54). The epitope recognized by this antibody is preferentially expressed on cartilage proteoglycans of fetal ages and almost absent in the adult. A loss of binding of the antibody to native fetal proteoglycan was obtained by either papain digestion or chondroitinase ABC treatment of the proteoglycan. These studies indicate that the antibody recognizes a carbohydrate epitope in intact chondroitin sulfate chains attached to the core protein and that reactivity is dependent on the attachment of these chains to core protein.

d. Antibody 3-B-3: Characterization of 3-B-3 was described elsewhere (38). This mouse monoclonal antibody was produced by the immunization with chondroitinase ABC-digested rat chondrosarcoma proteoglycan. A specific epitope of 3-B-3 was determined to be the unsaturated glucuronic acid residue linked to N-acetylgalactosamine created by chondroitinase ABC digestion of chondroitin 6-sulfate. It also recognizes intact chondroitin 6-sulfate. This antibody was also shown to have minor reactivity with unsulfated chondroitin after the enzymatic digestion.

It was investigated further with respect to whether it reacts only with unsaturated chondroitin 6-sulfate stubs that remain attached to the core protein or whether free chondroitin 6-sulfate oligosaccharides created after the enzymatic digestion are also recognized in the assays. Inhibition assays were done using human adult proteoglycan with or without dialysis to remove

oligosaccharides after the enzymatic digestion. Each concentration was plotted against proteoglycan protein content quantitated by the Lowry method.

e. Antibody 1-B-5: This is an antibody raised against chondroitinase ABC digested rat chondrosarcoma proteoglycan (38). A specific epitope of 1-B-5 was determined to be the unsaturated glucuronic acid residue linked to N-acetylgalactosamine created by chondroitinase ABC digestion of unsulfated chondroitin.

This antibody reacted best with chondroitinase ABC-digested bovine fetal proteoglycan in enzyme linked immunosorbent assays among proteoglycans used (bovine fetal, rat chondrosarcoma, human fetal and adult) (data not shown). A D1 preparation of bovine fetal proteoglycan was iodinated and chondroitinase ABC digested in the same way as other proteoglycan described above. Standard proteoglycan (bovine fetal) as well as cartilage extracts were digested with the enzyme and heat-treated with SDS in the same way as described above.

Since the antibody did not bind well to protein A, the reaction mixtures of immune complexes were incubated with rabbit antiserum against mouse IgG overnight at 4°C and then precipitated by the protein A method.

f. Antiserum R483: This is a rabbit antiserum raised against an isolated G1 domain of the rat chondrosarcoma proteoglycan in the same manner as described previously for R103 (177). This antibody reacted best with reduced and alkylated human adult proteoglycan. It also reacted with chondroitinase ABC and SDS treated proteoglycan without reduction and alkylation, but to a lesser degree.

A D1 preparation of human adult proteoglycan was first reduced and alkylated with dithiothreitol and iodoacetamide, respectively (176). The proteoglycan was then iodinated and digested with chondroitinase ABC in the same way as above. Standard proteoglycan as well as cartilage extracts were

digested with the enzyme and heat-treated with SDS. In this assay, immune complexes were precipitated by overnight incubation at 4°C with a pig anti-rabbit $(Fab')_2$ antiserum (prepared in this laboratory) together with normal rabbit serum.

Table 1 summarizes the assay procedures for each antibody, Figure 4 shows standard inhibition curves, and Figure 5 shows the recognition sites for each antibody.

2.2.6. Determination of the chain length of chondroitin sulfate

Three hundred microliters of cartilage extracts were dialyzed against 0.2 M sodium acetate buffer, pH 5.0, overnight at 4°C. Papain (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the same buffer was added to the dialyzed material at a concentration of 10 µg/mg proteoglycan, at the beginning of the digestion and again after 4 h. After incubation for 24h at 37°C, the enzyme was inactivated by the addition of iodoacetamide to a final concentration of 10 mM (132).

The digested proteoglycan was directly applied to a column of Sepharose CL-6B (Pharmacia Fine Chemicals) (110 x 1.25 cm, BioRad). Chromatography was performed by downward elution in 0.5 M sodium acetate, pH 5.5, at 6 ml/hr. 1 ml fractions were collected. Contents of chondroitin sulfate in fractions starting before the void volume up to just after the total volume of the column were measured by uronic acid assay. The void volume and total volumes of the column were determined from the elution profiles of blue dextran 2000 and glucuronolactone, respectively, as described above.

2.2.7. Examination of the position of sulfation on chondroitin sulfate chains

One hundred microliters of total extracts were dialyzed against distilled water overnight at 4°C. Proteoglycan in the dialyzed extracts was digested with chondroitinase ABC at a final concentration of 0.25 units/ml for 2 h at 40°C by

ANTIBODY & RECOGNITION SITE	STANDARD AND SAMPLE TREATMENTS	RADIOLABELED PROTEOGLYCANS	STANDARD PROTEOGLYCAN	PRECIPITATION STEP
3-B-3 Mouse IgM recognises C6S stubs left on core protein after chase ABC treatment	Chase ABC & SDS	^{125}I-HAPG / Chase ABC	HAPG (D1)	R191 (rabbit anti-mouse IgM) & protein A
846 Mouse IgM recognises CS related epitope expressed maximally on HFPG	SDS	^{125}I-HFPG / native	HFPG (D1)	R191 (rabbit anti-mouse IgM) & protein A
6B Mouse IgG_1 recognises C4S stubs left on core protein after chase ABC treatment	Chase ABC & SDS	^{125}I-HFPG / Chase ABC	HFPG (D1)	Protein A
1-B-5 Mouse IgG_1 recognizes COS stubs left on core protein after Chase ABC treatment	Chase ABC & SDS	^{125}I-BFPG / Chase ABC	BFPG (D1)	R844 (rabbit anti-mouse IgG) & Protein A
AN9P1 Mouse IgG_{2a} recognises a KS-rich PG, native	SDS	^{125}I-HAPG / native	HAPG (D1)	Protein A
R483 Rabbit antiserum to rat chondrosarcoma HABR	Chase ABC & SDS	^{125}I-HAPG / reduced & alkylated	HAPG (D1)	Pig anti-rabbit $F(ab')_2$ + NRS

Table 1 : Summary of Radioimmunoassay Procedures for the Antibodies

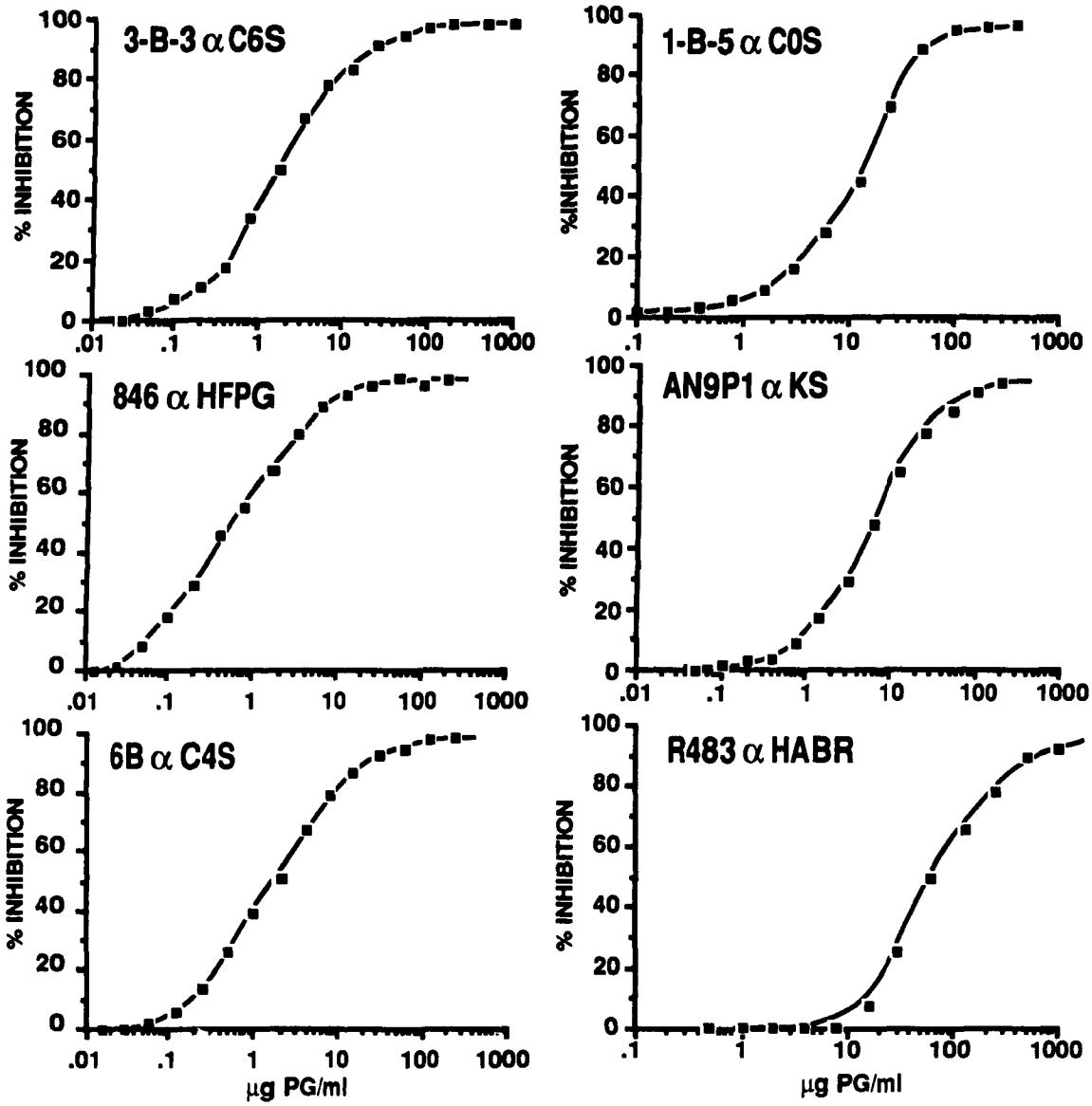
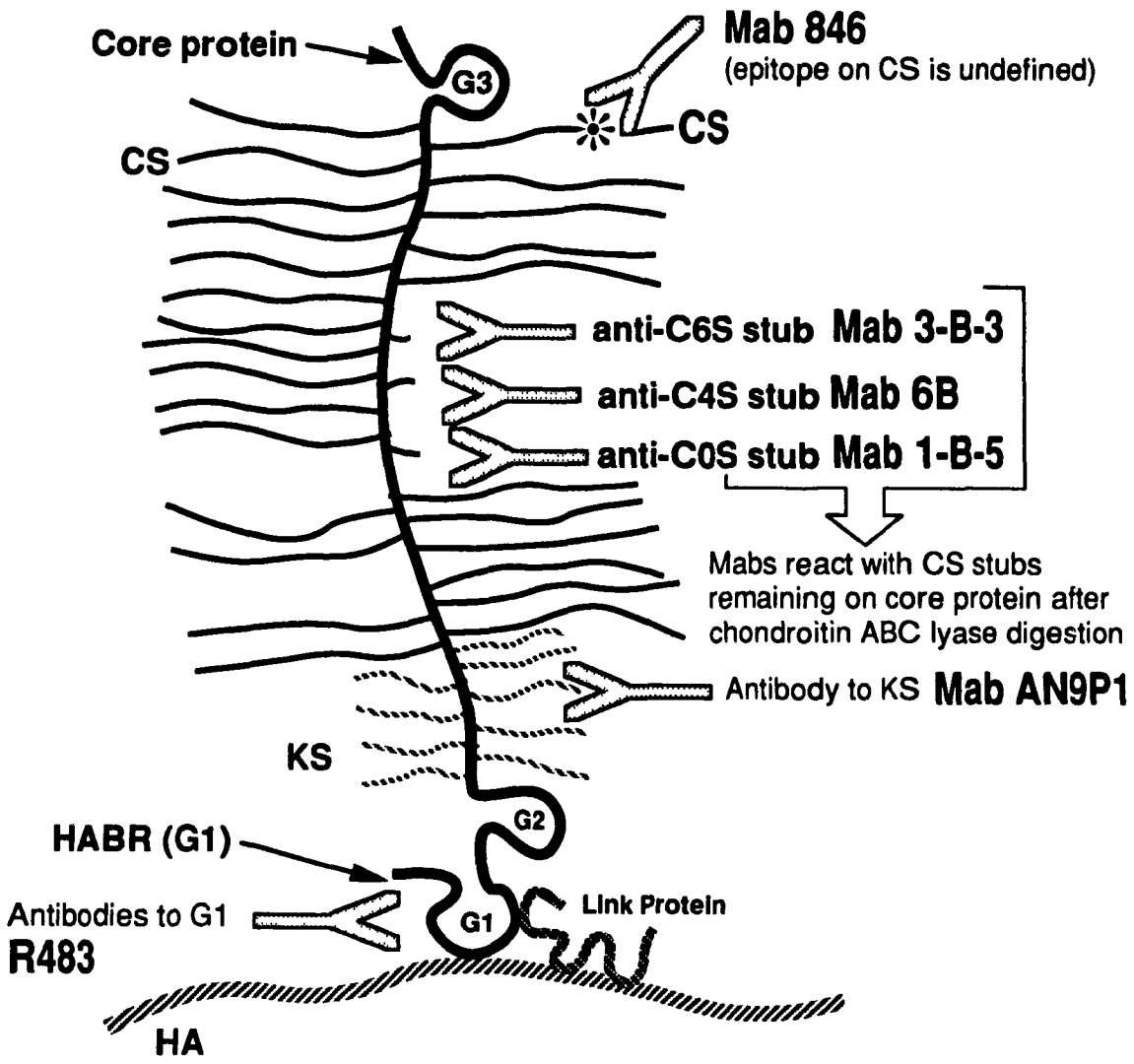


Figure 4 : Standard Inhibition Curves of Different Antibodies



CS : chondroitin sulfate
KS : keratan sulfate
HABR : hyaluronic acid binding region
G1, G2, G3 : globular domains
G1 binds to hyaluronic acid

Figure 5 : Diagramatic Representation of Recognition Sites of the Antibodies in an Aggregating Proteoglycan Monomer

the addition of 100 µl of the buffer (0.2 M sodium acetate, 0.2 M Tris/HCl, pH 7.3) containing the desired concentration of the enzyme. Eight hundred microliters of ethanol were added to the digested mixture leaving overnight at 4°C. Unsaturated disaccharides were recovered from the supernatant, which was evaporated to dryness and resuspended in 300 µl of the HPLC elution buffer.

The composition of unsaturated disaccharides in samples was determined using high performance liquid chromatography (HPLC) on a LiChrosorb-NH_2 column (µBONDAPAK, Waters). Twenty five microliters of samples were chromatographed at a flow rate of 1 ml/min of the elution buffer (10 mM sodium acetate, 20 mM sodium sulfate, pH 5.0). For calibration of the column, 25 µl of standard sample containing 1.0 µg of each unsaturated chondroitin 4-, 6-, and 0-sulfate disaccharides (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) was used. Disaccharides were monitored by their UV absorbance at 230 nm. The heights as well as areas of the peaks corresponding to standard disaccharides were measured representing contents of each disaccharide.

2.3. COMMERCIAL HYALURONIC ACID RADIOASSAY

To identify the hyaluronic acid content in total extracts, a commercial hyaluronic acid radioassay was used according to the manufacturer's instructions (20) (Pharmacia Canada). Samples for the assays were heat-treated with SDS in the same way as proteoglycan. This treatment did not interfere with hyaluronic acid reactivity in this assay.

CHAPTER 3

RESULTS

3.1 CHARACTERIZATION OF ANTIBODIES 6B AND 3-B-3

3.1.1 Effect of oligosaccharides on epitope content of 6B

The chondroitinase ABC-treated standard proteoglycan with (in the absence of oligosaccharides) or without dialysis (in the presence of oligosaccharides) exhibited different curves that had identical slopes (Fig. 6). The former inhibited the binding of 6B to ^{125}I-proteoglycan approximately 3 times more efficiently than the latter, comparing the two concentrations required for 50% inhibition. This indicated that oligosaccharides can partly inhibit the reactivity of this antibody with chondroitin 4-sulfate stubs that remain attached to the core protein after the enzyme digestion.

3.1.2 Effect of oligosaccharides on epitope content of 3-B-3

The two sets of standard proteoglycan with or without dialysis after chondroitinase ABC digestion exhibited essentially identical curves when plotted against each proteoglycan protein content (Fig. 7). This indicates that oligosaccharides do not affect the reactivity of this antibody to chondroitin 6-sulfate stubs that remain linked to the core protein after the enzyme digestion.

3.2 ANALYSES OF PROTEOGLYCAN

3.2.1 Percentage Extraction of Proteoglycan

Examinations of tissue residues after extraction with 4M guanidine hydrochloride revealed that on the basis of uronic acid contents good extractability of proteoglycan (over 90%) was invariably obtained both for normal controls and patients of young and intermediate age groups (Figure 8). There appear to be no significant effects on extractability induced by both disease and age within this range. On the other hand, the adult age group

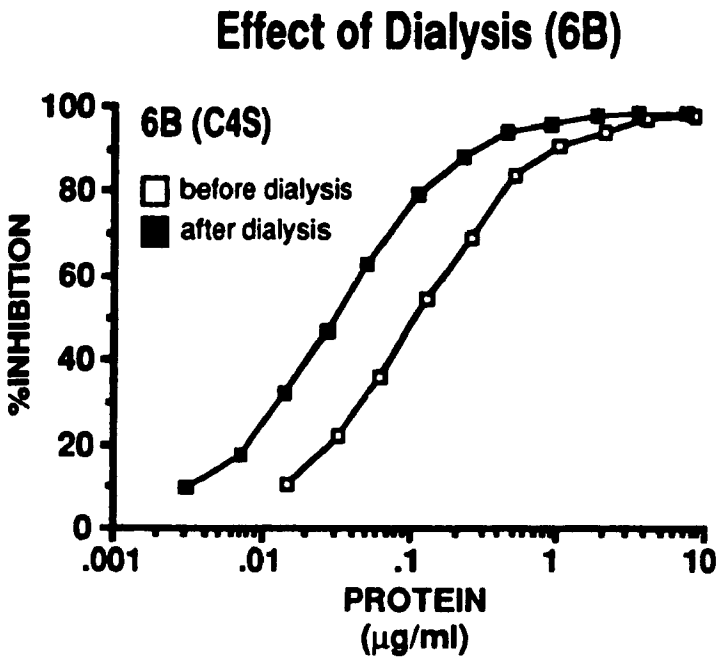


Figure 6 : Effect of Oligosaccharides on Reactivity of Antibody 6B to Epitope on Proteoglycan Core Protein

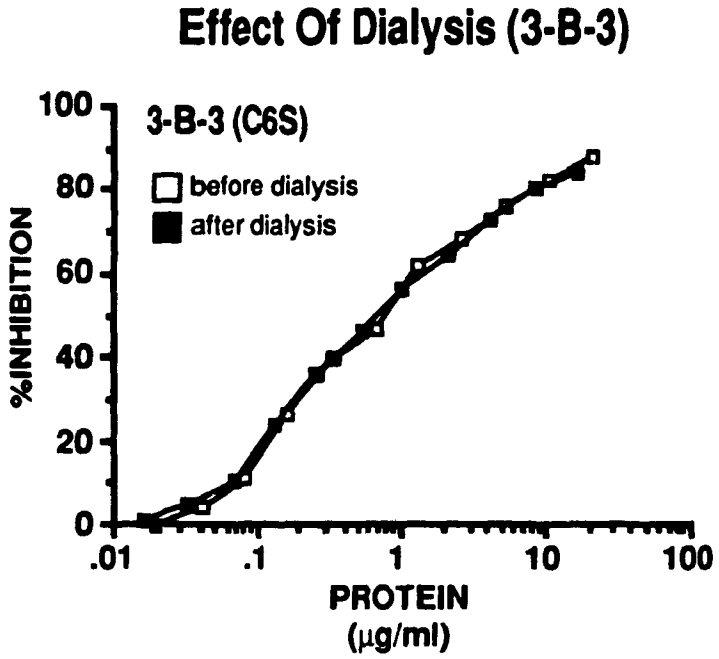


Figure 7 : Effect of Oligosaccharides on Reactivity of Antibody 3-B-3 to Epitope on Proteoglycan Core Protein

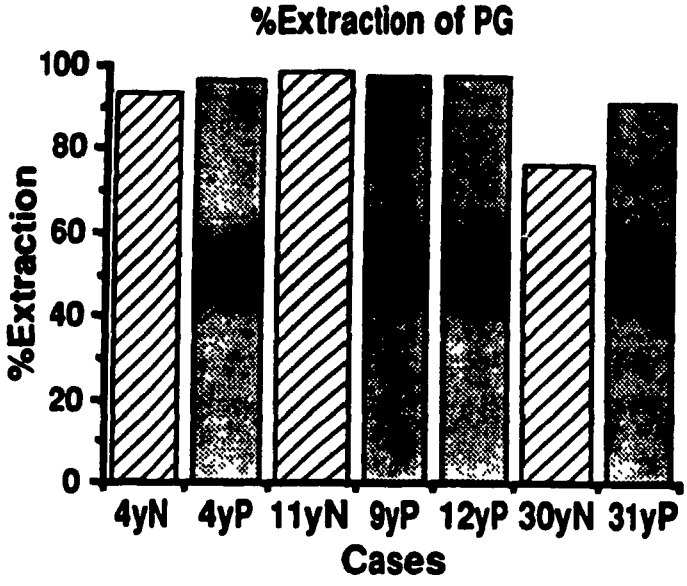


Figure 8 : Extractability of Proteoglycans

demonstrated less extractability in normal compared with the patient cartilage.

3.2.2 Total Content of Uronic Acid

Uronic acid contents were similar in all cases except a normal case of an intermediate age group which had a higher content when expressed on the basis of tissue wet weight (Fig. 9).

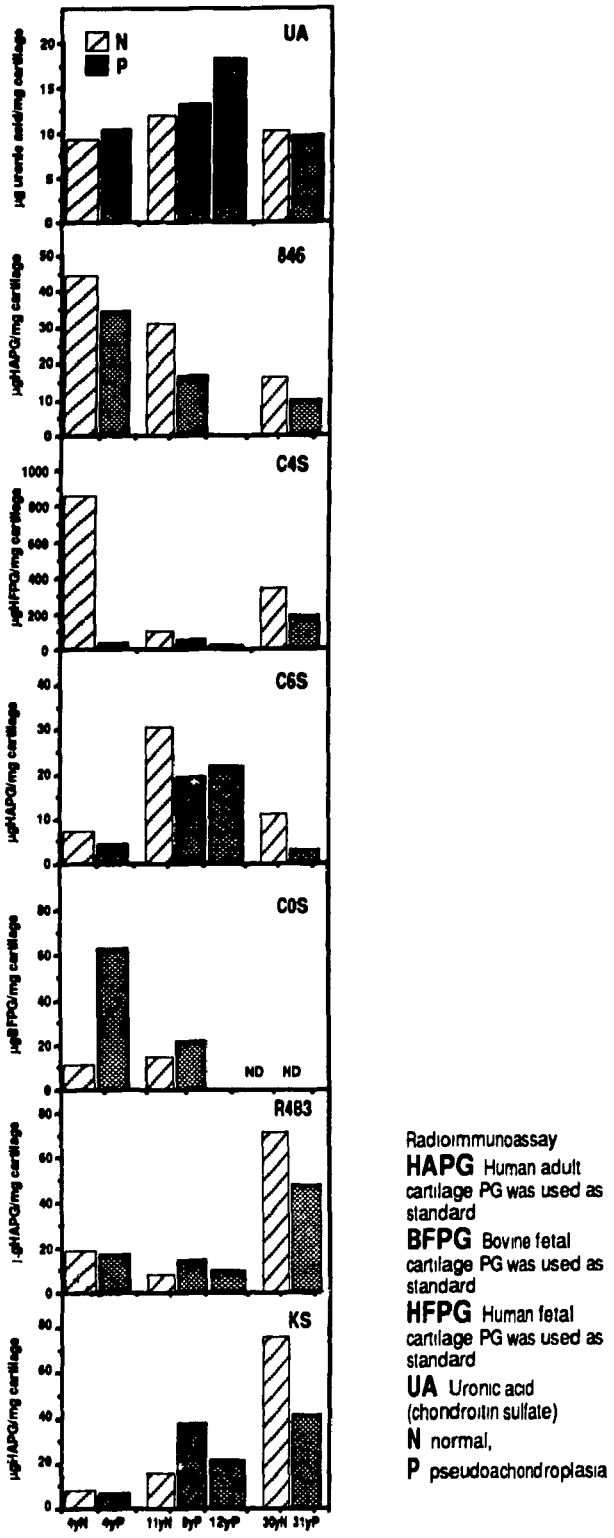
3.2.3 Total Contents of Proteoglycan-Specific Epitopes

Total contents of proteoglycan-specific epitopes are shown in Figure 9.

a. 6B (chondroitin 4-sulfate epitope)

Total contents of epitope 6B in the extracts demonstrate a decrease in the content of this epitope in all cartilages with this disease compared with those of the age-matched normal controls. This is particularly marked in the patient of the young age where a decrease of more than ten fold was observed. In normal cases of different ages, the content of the 4 year-old case is the highest among these age pairs is reduced at the intermediate age (11 years old) and then is increased in adult age (30 years old). The epitope content remains consistently low in the cartilages of young patients ranging from 4 up to 12 years old and thereafter is increased in parallel to the normal increase seen within the same range of ageing.

Since the further characterization study of 6B indicates that free oligosaccharides can reduce the reactivity of this antibody with chondroitin 4-sulfate epitope on the core protein, some of total extracts after chondroitinase ABC digestion was dialyzed to remove the free oligosaccharides and reassayed for the epitope contents. The dialysis, however, essentially did not affect the relative values of the contents of all samples examined (data not shown).



Radioimmunoassay

HAPG Human adult cartilage PG was used as standard

BFPG Bovine fetal cartilage PG was used as standard

HFPG Human fetal cartilage PG was used as standard

UA Uronic acid (chondroitin sulfate)

N normal,

P pseudoachondroplasia

Figure 9 : Total Contents of Uronic Acid and Proteoglycan Epitopes

b. 3-B-3 (chondroitin 6-sulfate epitope)

The contents of 3-B-3 in the cartilages of patients are consistently lower. Profiles of these contents of patients with respect to age-related changes resemble those of normal cases, where cases of the intermediate age contain the highest amounts. Since dialysis of chondroitinase ABC-treated standard proteoglycan did not affect its inhibition curve, dialysis of the sample extracts was not performed in the assays for 3-B-3 epitope.

c. 1-B-5 (unsulfated chondroitin epitope)

There is an increase in this epitope in patient cartilages of young and intermediate age pairs, particularly in the young case, while no detectable content of the epitope was seen in the cartilages of the adult pair. In patient cartilages, the content progressively decreases with increasing age. Normal cases, however, show any particular changes related to ageing process.

d. 846 (chondroitin sulfate-related epitope)

The contents of this epitope are lower in patients of all ages. Profiles of the contents of patients in terms of ageing-associated changes are identical to those of normal cases, both of which exhibit a progressive decrease with increasing age.

e. AN9P1 (keratan sulfate epitope)

This epitope does not show any consistent differences in contents between controls and patients. In comparison with controls, whereas there is no considerable difference in a young patient cartilage, there is an increase in cartilage of the patient of intermediate age and a decrease in that of cartilage of the patient of an adult age. The contents increase up to an intermediate age group with the disease and then remains relatively unchanged in the adult patient. There are continuing increases in the contents of normal controls with

ageing.

f. R483 (hyaluronic acid-binding region epitope)

The contents of this epitope in patients are similar to those of normal controls up to an intermediate age but there are marked increases in the contents in both the patient and control of an adult age with the lower content in this patient.

3.2.4 Monomer Sizes of Proteoglycans

1. Comparison of patients and age-matched controls

Since most of the column profiles are relatively symmetrical on either side of their center lines, Kav values of profiles were determined as those of points which divided peaks into two. Examples of K_{av} values of all column profiles are shown in Table 2 for keratan sulfate (AN9P1). These values were plotted for each case (Fig. 10). In profiles which are not symmetrical or have multiple peaks, approximate K_{av} values were determined and demonstrated with brackets in Table 2.

Dissociative studies of whole populations of proteoglycans in cartilage extracts have revealed three distinct populations of proteoglycans in normal controls of all ages, which can be recognized based on their different hydrodynamic sizes (Fig. 10). Figures 11-13 show column profiles of patients and controls of different ages. These are characterized by the abundance of epitope 3-B-3 (chondroitin 6-sulfate linkage regions) and 846 (fetal type carbohydrate epitope), of epitope 6B (chondroitin 4-sulfate linkage regions), and of epitope AN9P1 (keratan sulfate) in order from the largest to the smallest proteoglycans. In contrast to those of normal controls, these three populations were recognized only in the 31 year-old patient among patients of all ages. In patients of 4 and 12 years old, chondroitin 4-sulfate epitope is identified as a

	KS (small)	KS (large)
4yN	0.53	(0.23, 0.35)
4yP	0.55	(0.2, 0.4)
11yN	0.55	(0.25)
12yP	0.55	(0.18)
30yN	0.59	Absent
31yP	0.60	Absent

Table 2 : Kav Values for Each Epitope of Dissociative Column Profiles

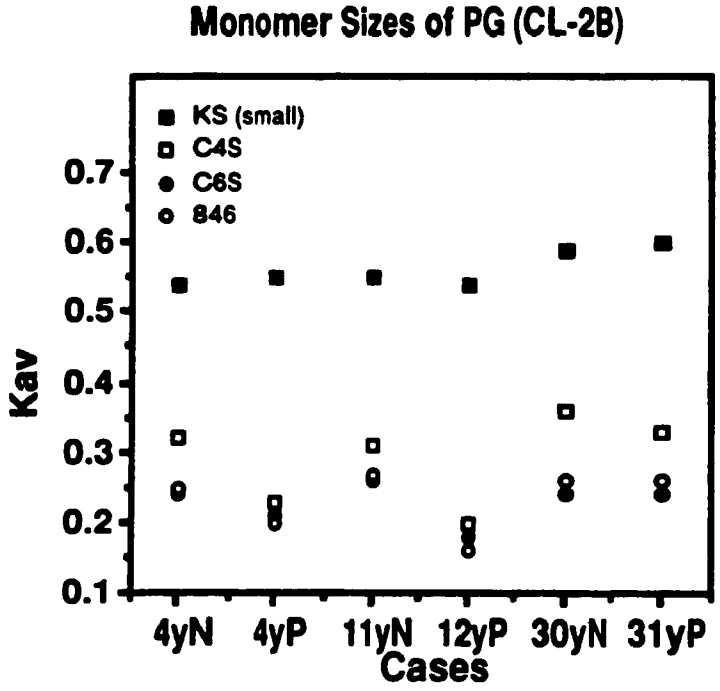


Figure 10 : Monomer Sizes of Proteoglycan Populations

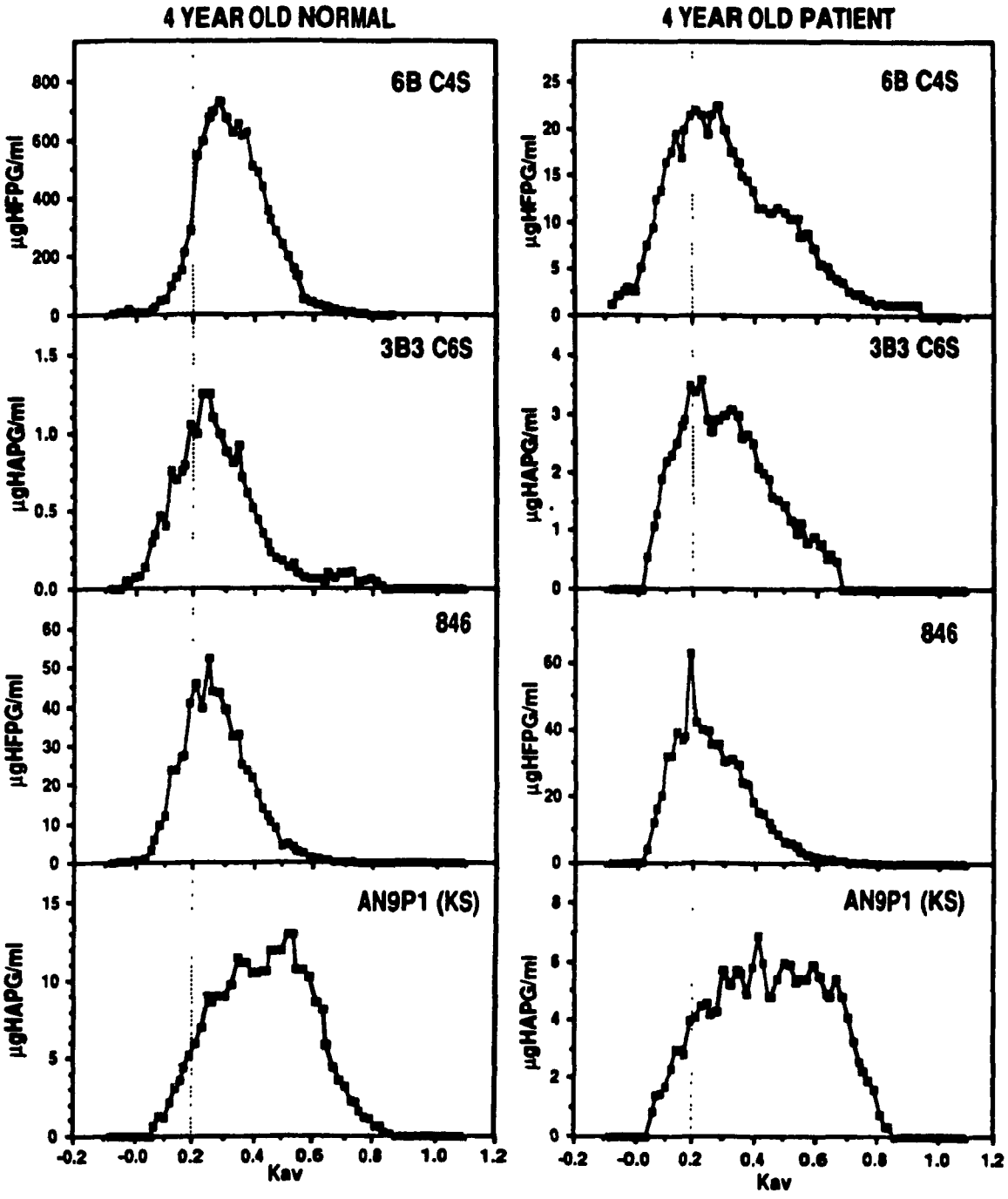


Figure 11 : Examination of Proteoglycan Monomer Sizes in a 4 Year-Old Normal and a 4 Year-Old Patient

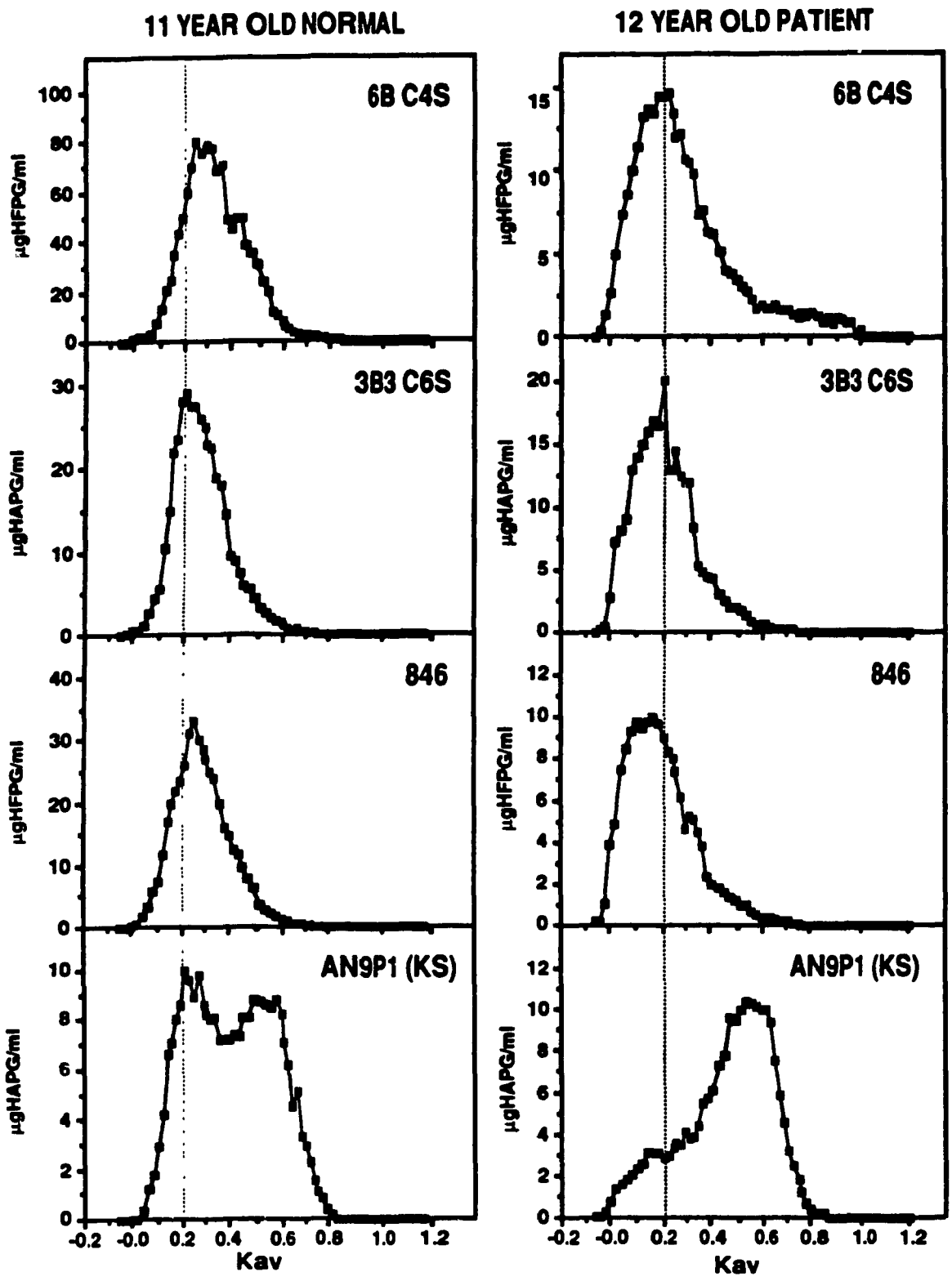


Figure 12 : Examination of Proteoglycan Monomer Sizes in an 11 Year-Old Normal and a 12 Year-Old Patient

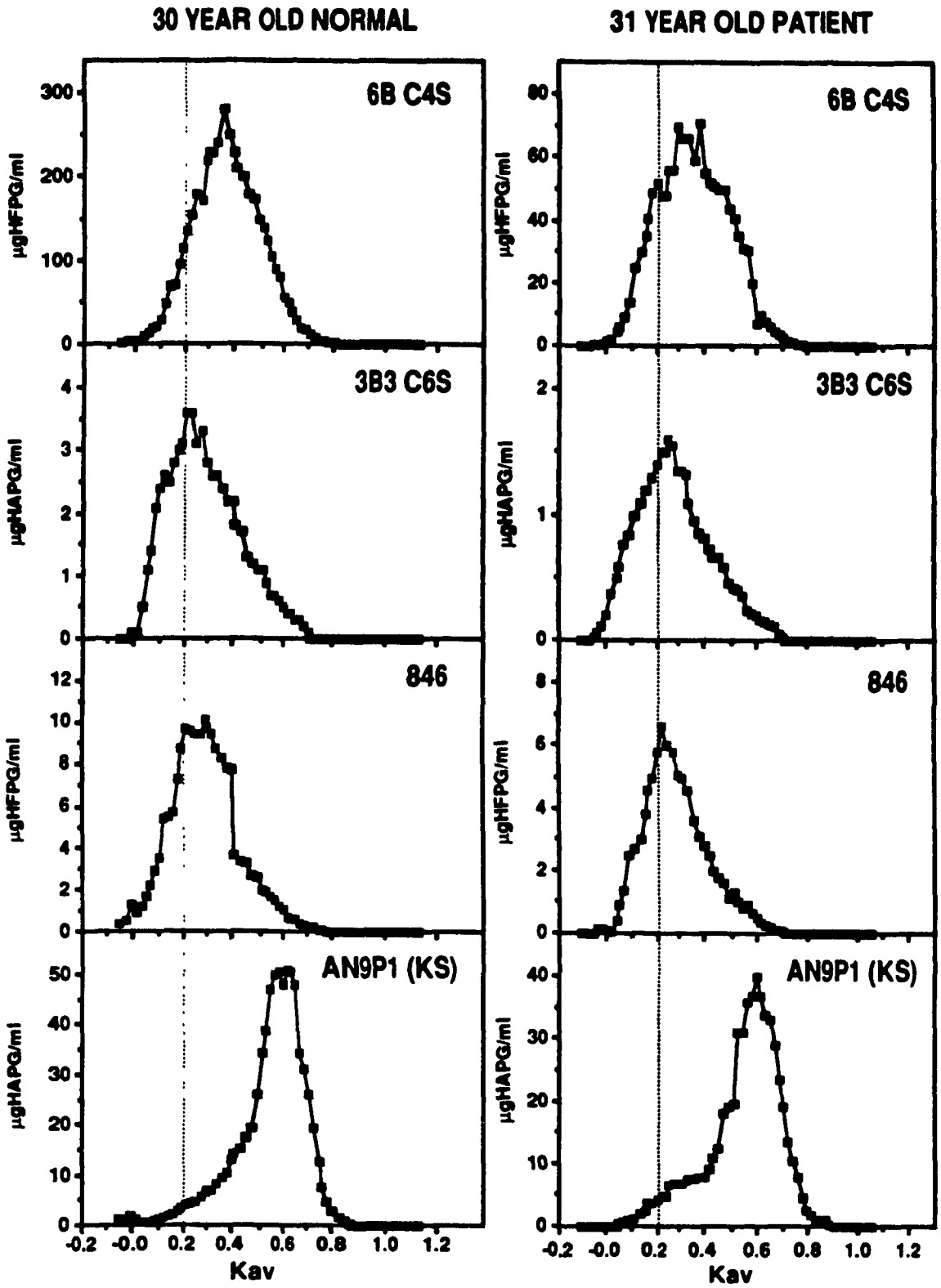


Figure 13 : Examination of Proteoglycan Monomer Sizes in a 30 Year-Old Normal and a 31 Year-Old Patient

subcomponent of the largest population, and therefore only two populations are distinguishable in each case. The larger one of these two seen in younger patients can be characterized by the abundance of all chondroitin sulfate epitopes (chondroitin 4-(6B), 6-sulfate (3-B-3) and epitope 846 and the smaller one by the abundance of keratan sulfate epitope (AN9P1). This larger species of proteoglycan in the younger patients is larger than the other corresponding population in controls. But this was not observed in the adult age pair, where the larger species were of similar molecular sizes. The largest species of the three seen in all normal controls and in the patient of 31 years old have an almost identical K_{av} value of about 0.25.

2. Age-related changes in epitope-characteristic monomer sizes

6B-characteristic proteoglycan monomers

Normally, this single species is always recognized as being distinctive and of an intermediate size (Figs. 10,14). It is comparatively constant in its size up to the 11 year-old control and then decreases in the size in the 30 year-old case. On the other hand, the size of this species increases slightly, judged from a decrease in a K_{av} value from 0.23 to 0.20 obtained in the patients of 4 and 12 years old, respectively. Thereafter, it decreases considerably as seen in the 31 year-old patient.

3-B-3 and 846-characteristic monomers

These two epitopes are always co-distributed in the largest population in all cases and are relatively absent from other populations of smaller sizes (Figs. 10,15,16). This species are relatively invariable in size in normal cases regardless of age (K_{av}: approximately 0.25). In contrast to normals, the molecular size of this species in patients changes with age. It increases from 4 to 12 years old, reaching a maximum, and then decreases significantly as

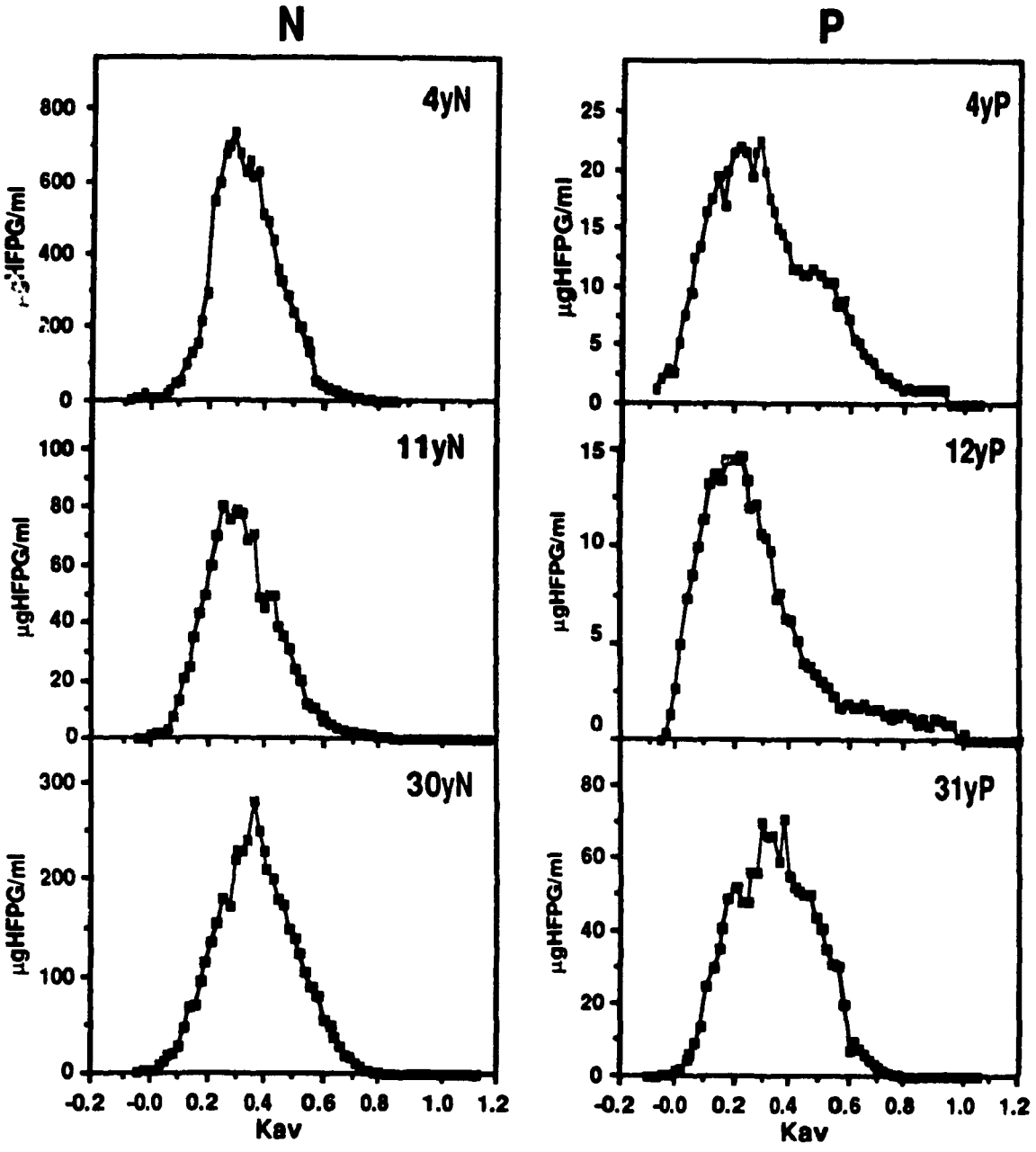


Figure 14 : Age-Related Changes in Size of a 6B-Epitope Rich Monomer

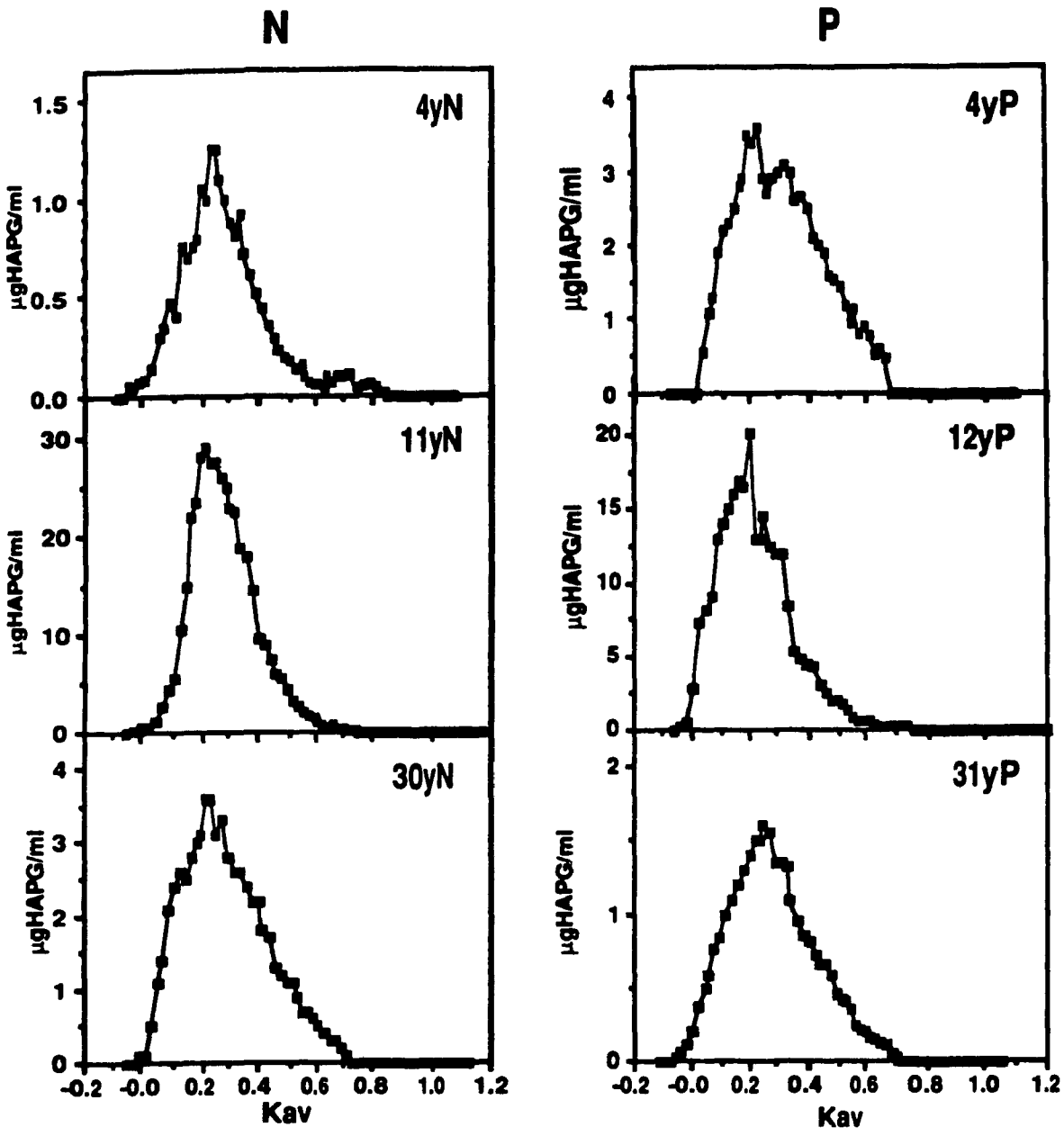


Figure 15 : Age-Related Changes in Size of a 3-B-3-Epitope Rich Monomer

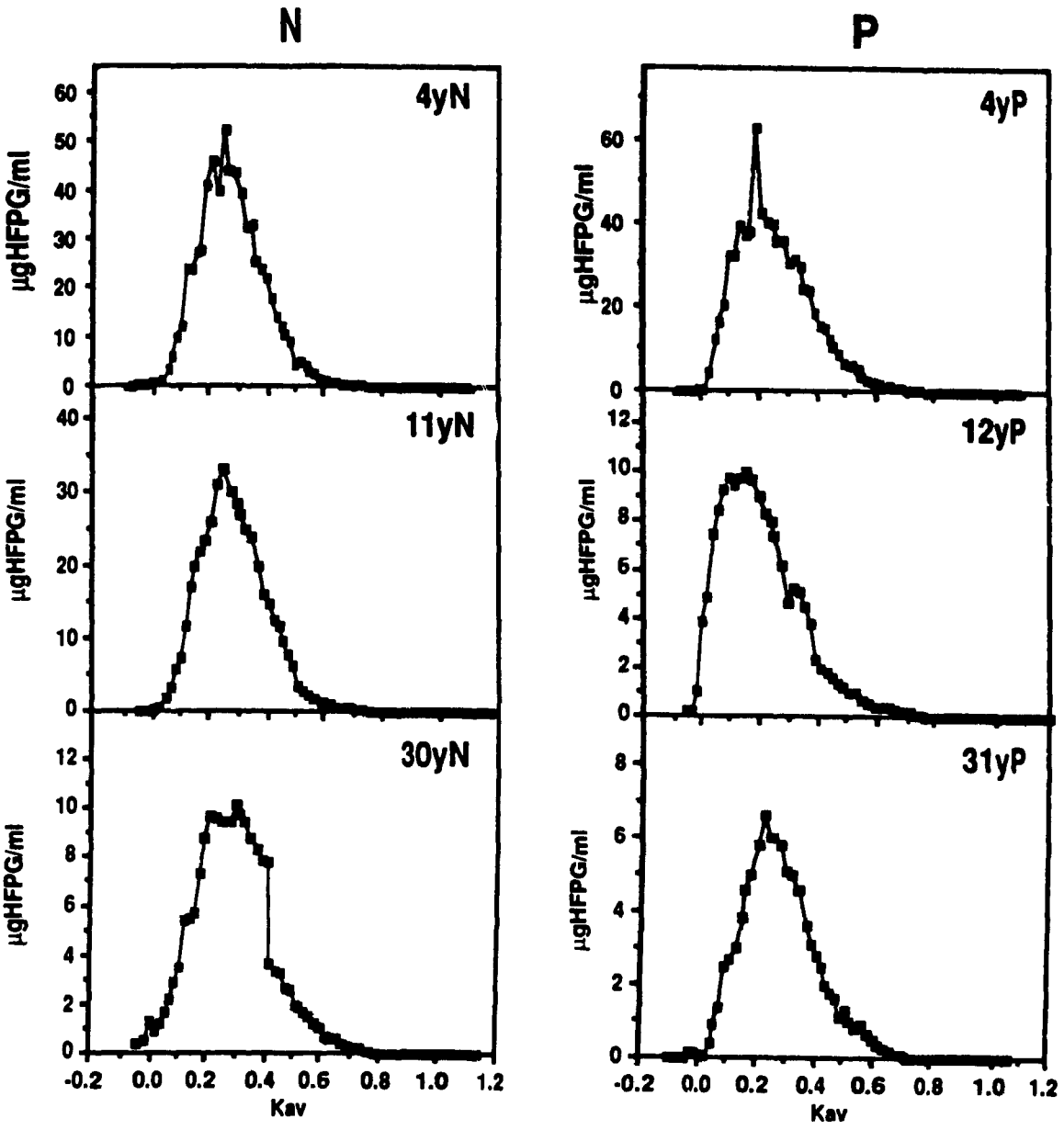


Figure 16 : Age-Related Changes in Size of a 846-Epitope Rich Monomer

observed in the patient of 31 years old with the level equal to that of the control.

AN9P1-characteristic monomers

The subpopulations bearing this epitope change considerably with ageing as depicted in Fig. 10 and 17. In some cases, it is difficult to identify the accurate peak positions of subpopulations, particularly when the profiles have small multiple peaks or exhibit fluctuations about the peaks. There appear to be at least two subpopulations in each case of the 4 year-old pair. In the normal control, two populations can be seen at K_{av} value of around 0.53, 0.35, and possibly another one around 0.23. In the patient, there are three subpopulations around K_{av} of 0.2, 0.4, and 0.5 to 0.6. In the intermediate age pair, two subpopulations are recognized. The 11 year-old normal control has one at K_{av} of 0.55 and the other at 0.25. The 12 year-old exhibits one at 0.55 and possibly, the other one being recognized as a small shoulder at 0.18. In contrast to these immature individuals, both of the adult cases show profiles basically without any distinct subpopulations and no apparent peak was detected in the positions of the peaks which are abundant of chondroitin sulfate epitopes. The smallest subpopulations seen in the young cases and the populations in the adult cases are well separated from other populations of chondroitin sulfate proteoglycan and do not appear to change their molecular sizes within this range of ages. This was a consistent finding for keratan sulfate-specific monomers regardless of the disease or ageing.

3.2.5 The Chain Length of Chondroitin Sulfate

Since all column profiles are quite symmetrical on either side of their center lines, Kav values of the peaks were determined in the same way as dissociative column profiles. Kav values obtained for patients are 0.52, 0.49, and

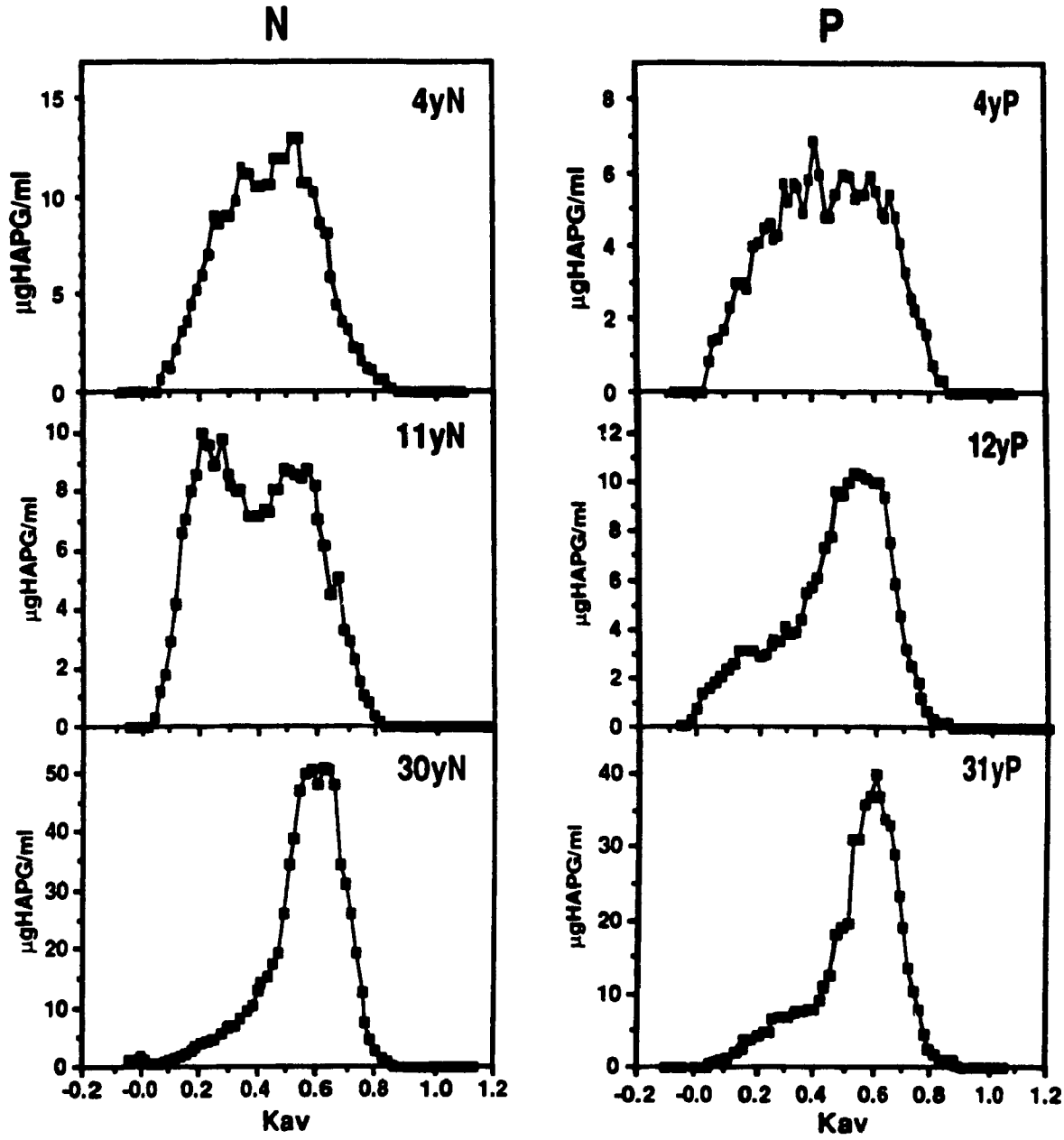


Figure 17 : Age-Related Changes in Size of AN9P1-Epitope Rich Monomer

0.51, and for controls 0.56, 0.55, and 0.60 in order of increasing age (Fig. 18). Chondroitin sulfate chains are longer in patients of all ages. While the chain lengths of patients are constant within this range of ages, those of controls remain the same from young up to an intermediate age and are, thereafter, reduced at 30 years of age.

3.2.6 The Composition of Chondroitin Sulfate Chains

4-Sulfation on Chondroitin Sulfate Chains

The analyses have revealed a decrease in 4-sulfation on chondroitin sulfate chains in cartilages of the patients from 4 up to 9 years old and no change in that of the patient of 31 years old (Fig. 19). There are progressive decreases in this sulfation with increasing ages in cartilages of both patients and controls.

6-Sulfation on Chondroitin Sulfate Chains

The degrees of 6-sulfation on chondroitin sulfate chains in the tissues of patients of all ages did not appear to differ significantly from those of controls (Fig. 19). In the light of age-related changes, a decrease in the cartilage of the 31 year-old patient was seen in contrast to cartilages of the younger patients. The degree of this sulfation in controls is unchanged up to 9 years old and then reduced in the adult.

The Ratios of 4- to 6-Sulfation on Chondroitin Sulfate Chains

The ratios of 4- to 6-sulfation in each case were calculated (Fig. 19). These ratios are lower in samples of the patients of 4 and 9 years old and remain reduced but similar to normal in the patient of the adult age. The patients profiles in terms of age-associated changes are similar to those of 4-sulfation but with a minimum change of a slight increase in this ratio in the adult. The normal profiles show continuing decrease in the ratio with increasing age.

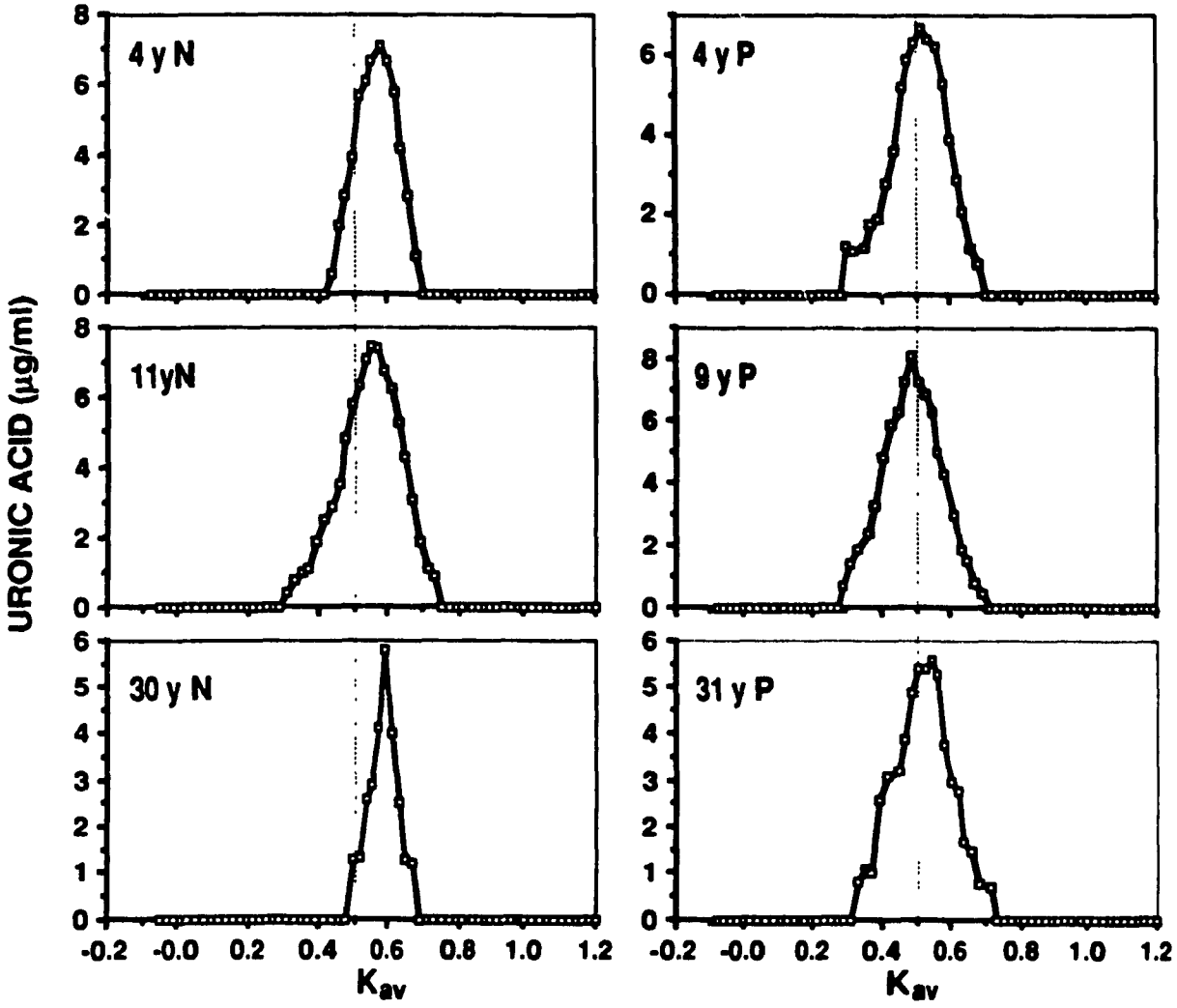


Figure 18 : Determination of the Chain Length of Chondroitin Sulfate

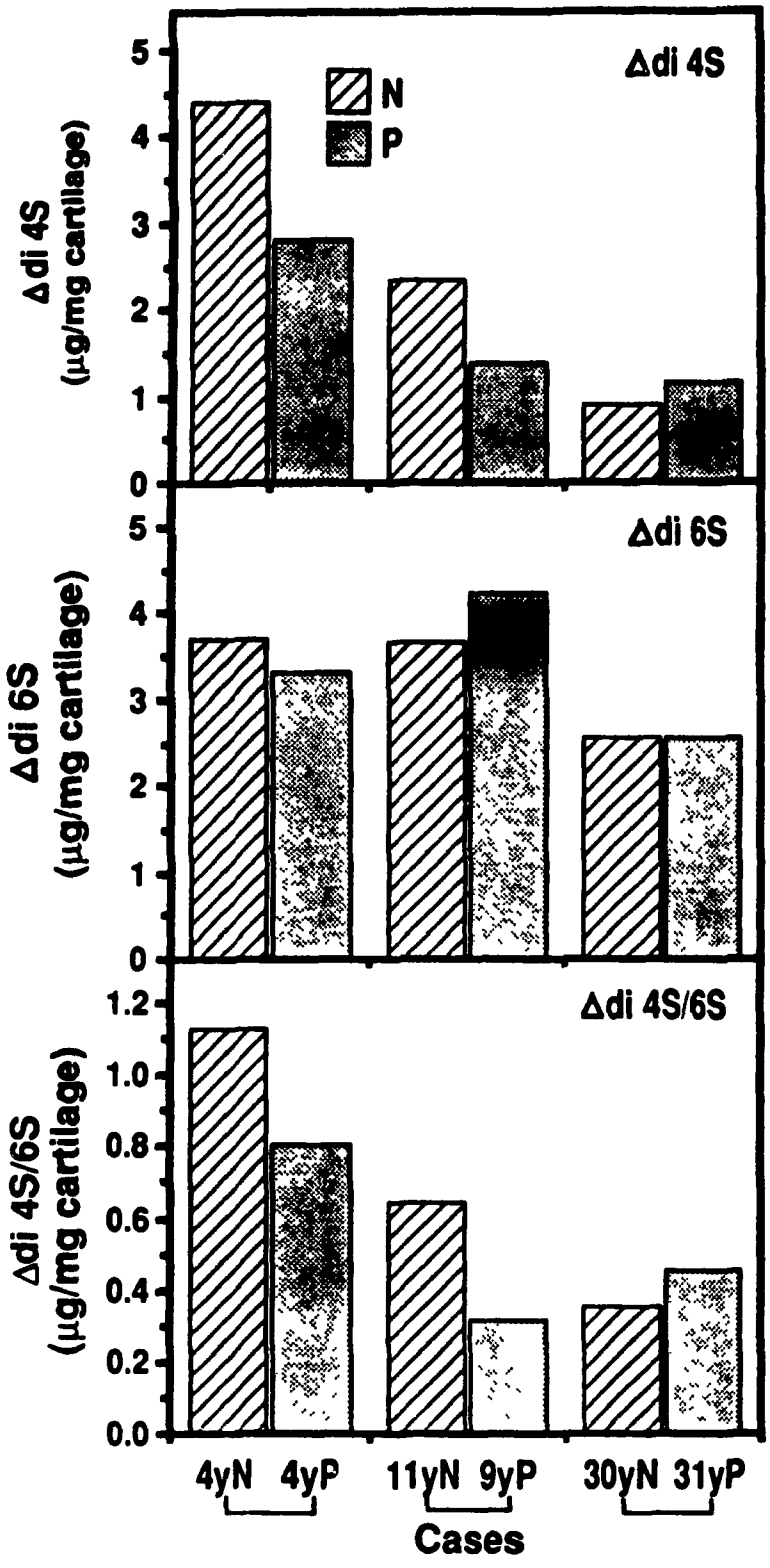


Figure 19 : Examination of Composition of Chondroitin Sulfate

3.2.7. Aggregatability of Proteoglycans

Aggregatability of proteoglycans that were dissociatively extracted was examined for each epitope. Since aggregated proteoglycans are too large to be included by Sepharose CL-2B gel, they can be separated from non-aggregated monomers as proteoglycans that eluted at the void volume of the associative column. In this work, all the material eluted up to $K_{av} = 0.1$ was considered as the aggregated proteoglycans (shaded area) (Fig. 20). The percent aggregation of each epitope is shown in Table 3.

When compared with normal, similar percent aggregation of all epitopes seemed to be obtained in patients at all ages except for the case of epitope 6B in the 12 year old patient which showed considerably higher percent aggregation.

3.3. TOTAL CONTENT OF HYALURONIC ACID

The content of this glycosaminoglycan showed an increase in patients at all ages (Fig. 21). There was a minimum level in the 4 year old control. The content then increased in the adult control. The profile of patients showed a similar pattern to normal. The content decreased from the 4 year old patient to the 9 year old patient and then increased in the adult patient where the maximum level was detected.

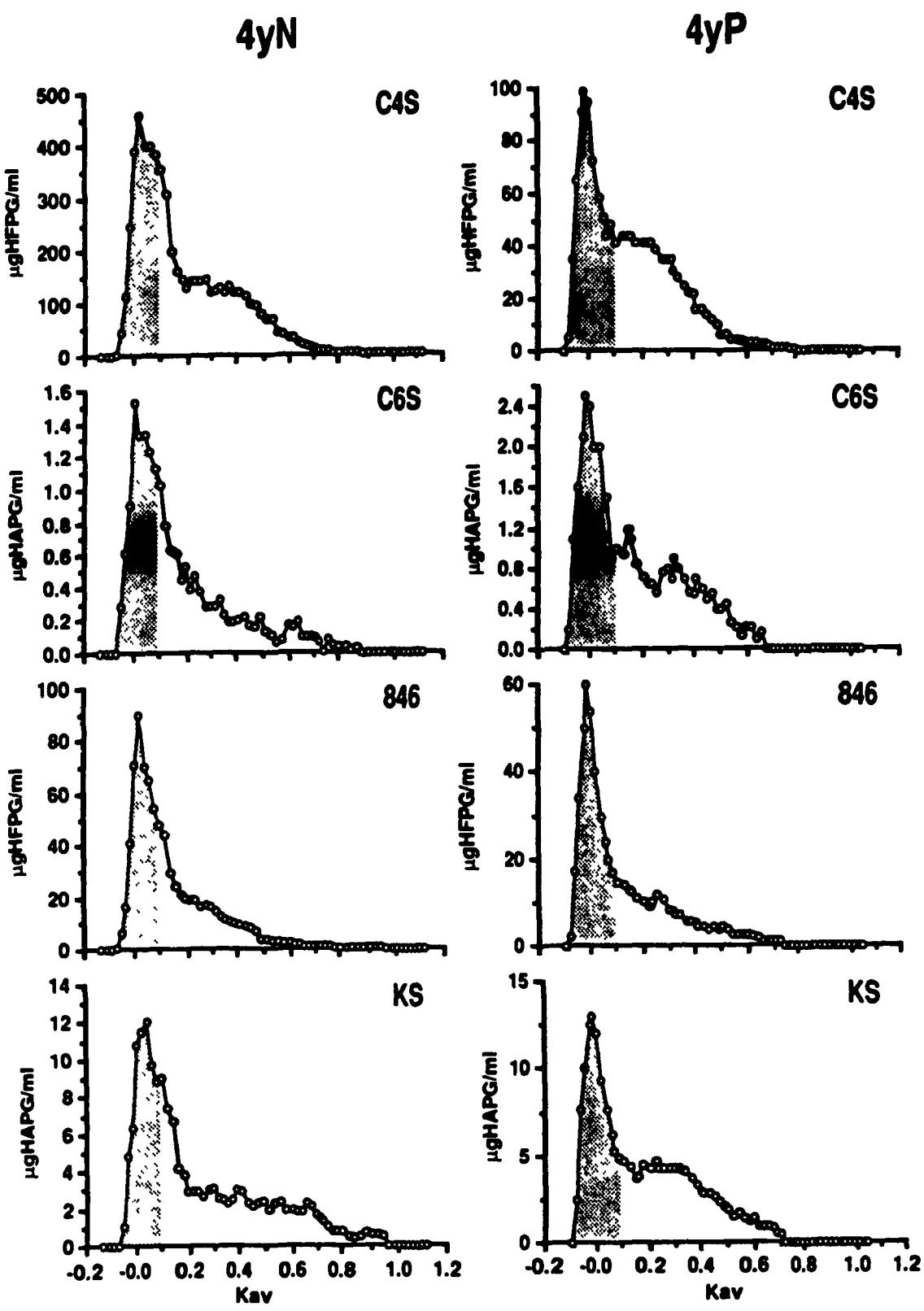


Figure 20 : Analysis of Aggregation of Proteoglycans in a 4 Year-Old Normal and a 4 Year-Old Patient

Cases	C6S	C4S	KS	846
4yN	55.0	48.7	45.0	59.6
4yP	49.0	46.1	54.0	70.0
11yN	58.5	39.0	48.2	46.7
12yP	59.2	63.0	58.9	64.2
30yN	42.0	40.6	33.3	50.8
31yP	42.3	45.4	47.6	45.0

Table 3 : Percent Aggregation of Proteoglycans

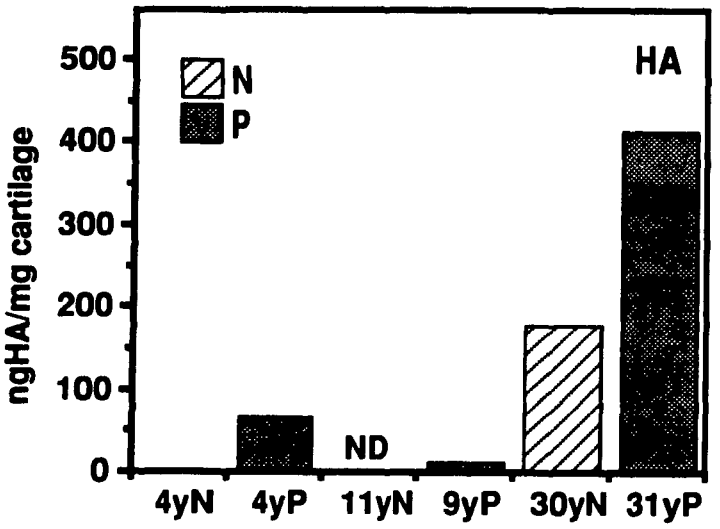


Figure 21 : Total Content of Hyaluronic Acid.
Those of 4yN and 11yN are 3ng/mg and non-detectable. The 9yP is 12.9ng/mg.

CHAPTER 4

DISCUSSION

4.1. EXTRACTABILITY OF PROTEOGLYCANS

The high degree of extractability found in young normals and the reduced extractability in the normal adult are consistent with previous reports of age-related changes in articular cartilage proteoglycans. This is probably related to increased cross-linking of the collagen fibrils with aging (146). High extractability of proteoglycan was also reported in the growth plate (10). The increased extractability observed in the adult patient compared to that in the age-matched control may reflect less cross-linking of the collagen fibrils or a different interaction of proteoglycan molecules or hyaluronic acid with the collagen network.

4.2. TOTAL CONTENTS OF SUBSTRUCTURES OF PROTEOGLYCANS

The total content of uronic acid represents the total of chondroitin sulfate and hyaluronic acid since the minimum repeating disaccharide unit of these glycosaminoglycans contains glucuronic acid. The total content of extractable hyaluronic acid is, at most, 2% of the total chondroitin sulfate and hyaluronic acid in the 31 year old patient in which the maximum content of hyalurnic acid eas detected. The content of uronic acid is similar for all cases but not for the 12 year old patient where it was elevated. It is considered that there are three variables that may determine the total content of chondroitin sulfate in the extracts: number of proteoglycan molecules, number of chondroitin sulfate chains per molecule, and their length.

Direct analyses to determine the total numbers of chondroitin sulfate chains using three different antibodies against linkage regions of chondroitin 4-(6B), 6-sulfate (3-B-3) and non-sulfated chondroitin (1-B-5) to core protein failed

to clearly demonstrate differences between patients and age-matched controls. Although the young patient cartilage exhibited a lower content of epitopes of both 6B and 3-B-3 than normal, the content of epitope 1-B-5 was higher in this and other patients. This was particularly well expressed in the 4 year old patient, where there was a marked decrease in the content of 6B and a corresponding increase in the content of 1-B-5. Because two types (adult and fetal) of proteoglycans were used in the assays for these three epitopes (depending upon the specific reactivity of these proteoglycans with these antibodies), the sum of the three epitopes for each case is not representative of the total number of chondroitin sulfate chains (linkage regions).

It is of interest that there is an inverse relationship between the content of 3-B-3 and 6B in normal cases with respect to age-related changes. This suggests switching between 4- and 6- sulfation on the linkage regions of chondroitin sulfate chains, which are related to ageing. With development, 6-sulfation predominates at the young age and then drops significantly in the adult. Associated with this, 4-sulfation, which is dominant in the young, is reduced markedly at the intermediate age and then increases in the adult. On the other hand, in patients, this inversed relationship can be observed only between patients of the intermediate and adult ages, thereby identifying the 4 year old patient as an exceptional case. This is due to the great decrease in the number of chondroitin 4-sulfate linkage regions, which is accompanied by the pronounced increase in the number of non-sulfated chondroitin linkage regions. Considering that growth curves of patients with this disease show their growth retardation two years later on (75), these observations seen in the 4 year old patient appear to be the earliest event and the most characteristic and representative change in proteoglycan with this disease in terms of linkage regions of chondroitin sulfate

chains to the core protein. However, only oen patient of this age group was available for study.

The content of epitope 846 is lower in patients at all ages. These differences may be related to fewer chondroitin sulfate chains (discussed later) or some structural changes on the chains affecting the epitope.

It has been established, by gel electrophoresis and chromatographic methods, that aggregating cartilage proteoglycans can be separated into several populations with different sizes and gel-electrophoretic mobilities (146,174). These populations are now considered to reflect proteolytic degradation of proteoglycan molecules (121). In human adult articular cartilage, there is a progressive increase in the amount of keratan sulfate-rich proteoglycan populations of smaller sizes with ageing (174). This suggests that the keratan sulfate-rich population is derived from the larger chondroitin sulfate-rich proteoglycan population by proteolytic cleavage. Moreover, there is evidence indicating that the hyaluronic acid-binding regions essentially devoid of chondroitin sulfate and keratan sulfate also accumulate in ageing human articular cartilage (147). These observations suggest that keratan sulfate and the hyaluronic acid-binding regions are retained in the cartilage by interacting with hyaluronic acid and that the contents of these two substructures of proteoglycan are indicative of the catabolism of the molecules.

The total content of epitope AN9P1 (keratan sulfate) increased with age in normals. In contrast, although the cartilages from patients of the intermediate age contained larger amounts of keratan sulfate, the content of the adult patient did not increase from the intermediate age as the control did. This may suggest that changes in the metabolism of keratan sulfate (decreased synthesis of keratan sulfate or increased degradation of keratan sulfate-rich proteoglycan)

occur within this range of age in pseudoachondroplastic cartilage.

Although total content of epitope R483 (the hyaluronic acid-binding region) in patients of the young and the intermediate age is unchanged compared with controls, a decreased content was detected in the adult patient. This decrease may reflect a reduced concentration of proteoglycans in the tissue from the patient which resulted from the long term synthesis of these molecules at a reduced rate. As is described in the Introduction, there are large intracellular accumulations of proteoglycan core protein within chondrocytes. Histochemical studies have shown that this intracellular material was not metachromatic, indicating that it is free of glycosaminoglycan chains (159,160). It is therefore considered that the intracellular accumulation is present in pre-Golgi organelles in the processes of its synthesis since the attachment of glycosaminoglycans does not occur as a pre-Golgi event (51,89,101,137). Thus, the similar content of this epitope in the extracts of young patients (containing intracellular material) in comparison with controls may represent a decreased concentration of proteoglycan molecules in the extracellular matrix, probably derived from reduced synthesis of these molecules.

4.3. CHAIN LENGTH OF CHONDROITIN SULFATE

Direct analysis of the chain length of chondroitin sulfate derived from papain-digested proteoglycan using a chromatographic technique revealed longer chains in patients at all ages. This, together with the result of essentially unchanged content of uronic acid, indirectly indicates that there are probably fewer but longer chondroitin sulfate chains in the extracts from patients.

There have been several reports concerning the factors which determine the chain length of chondroitin sulfate. Treatment of cultured chondrocytes with cycloheximide, an inhibitor of protein synthesis, induced synthesis of

proteoglycans which contain longer chondroitin sulfate chains without changes in sulfation (83,88). Other studies demonstrated the effect of β-D-xyloside treatment, a competing acceptor for initiation of synthesis of chondroitin sulfate with endogenous proteoglycan core protein acceptor, on the synthesis of chondroitin sulfate by chondrocytes (100,109). This agent, at a proper concentration, maximally stimulated the synthesis of core protein-free chondroitin sulfate chains. However, the free and core protein-bound sulfate chains were shorter than chains attached to core protein in the absence of the agent. These observations suggest that chondroitin sulfate chain length is dependent on the ratio between available core protein and the metabolic precursors or chain-elongating enzymes. It is therefore possible, as suggested by Poole (1986), that longer chondroitin sulfate chains may result from a reduced rate of translocation of core protein through the Golgi apparatus. This may be reflected by the presence of core protein in the rough endoplasmic reticulum.

4.4. POSITION OF SULFATION ALONG CHONDROITIN SULFATE CHAINS

Composition analyses of chondroitin sulfate chains demonstrated that there is reduced 4-sulfation and unchanged 6-sulfation along chondroitin sulfate chains in this disease.

4.5. SIZES OF PROTEOGLYCAN POPULATIONS

Chromatographic studies of monomer sizes revealed larger proteoglycan monomers in the cartilages of the 4 and 12 year old patients. This probably results from the longer chondroitin sulfate chains although these are less sulfated. This increase in the size of monomers was not found in the adult patient. It may be because of the increased undersulfation (fewer sulfation groups) found in the adult.

In relation to different populations of proteoglycans, the largest chondroitin sulfate-rich proteoglycans were for the first time resolved into two species. The larger one was characterized by the abundance of chondroitin 6-sulfate linkage regions and 846, and the smaller one by chondroitin 4-sulfate linkage regions. This may be because the proteolytic degradation of monomers produces these proteoglycan populations of different sizes which are still capable of aggregation and are retained in the cartilage. It also may indicate regional organization of chondroitin sulfate structure on these proteoglycans. Because of their association with the largest species of proteoglycan, these large molecules may possess a core protein segment to which chondroitin sulfate chains bearing epitope 846 and 3-B-3 are attached which is located near the G3 domain (C-terminus) of the core protein. The segment to which chondroitin sulfate chains bearing 6B are attached is probably further from the G3 domain since it is concentrated in the smaller of the two large populations.

While the chondroitin 4-sulfate-rich population is recognized as a distinct species of an intermediate size in normal controls at all ages, there is no distinct population rich in only chondroitin 4-sulfate epitope which can be separated from the largest species in the 4 and 12 year old patients. It may represent decreased degradation of proteoglycan molecules or disease-induced structural changes in the molecules.

These observations thus indicate that proteoglycan monomers in pseudoachondroplasia are characterized by longer chondroitin sulfate chains with progressive undersulfation and reduced 4-sulfation with ageing resulting in larger proteoglycan molecules (which were not observed in the adult). These chondroitin sulfate chains are attached to the core protein by fewer chondroitin 4- and 6-sulfate (especially chondroitin 4-sulfate) linkage regions and more unsulfated

chondroitin linkage regions.

4.6. AGGREGATION OF PROTEOGLYCANS

There were no marked differences in aggregatability of substructures of proteoglycans detected by this immunochemical method between patients and normal controls. Proteolytic degradation of monomers is considered to produce fragmented molecules which are not capable of aggregation and thereby disappear from the cartilage matrix. This may reflect no change in the rate of degradation of these molecules in this disease.

4.7. TOTAL CONTENT OF HYALURONIC ACID

There is a marked increase in the content of extractable hyaluronic acid in the cartilages of patients at all ages. This content increases with age in patient as well as normal controls. These age-related changes are in agreement with a previous report of human articular cartilage (173). An increased content of these extractable molecules seen in patients may reflect changes in the collagen organization or increased synthesis of these molecules, suggesting different molecular organization in matrix.

Comparing our observations with previous reports, some of our results are consistent with previous findings and some are not.

The increase in the total content of keratan sulfate in the patients of the intermediate age is similar to the previous observations of a patient of the same age by Pedrini-Mille et al. (1984). They also found a decreased ratio of chondroitin 4-sulfate to chondroitin 6-sulfate, which is in agreement with our results. Although undersulfation was not detected in purified proteoglycan monomers, an increased content of unsulfated disaccharides was found in cartilage extracts after chondroitinase ABC digestion, indicative of increased content of unsaturated chondroitin sulfate since hyaluronic acid amounts to no

more than 2% of the total CS and HA contents. Beck et al. (1988) reported no changes in the molecular weight of monomers which were smaller (K_{av} = 0.4 on Sepharose CL-2B gel) than those in this study (K_{av} = 0.2), the length of chondroitin sulfate chains and composition of chondroitin sulfate of $^{35}SO_4$-labelled proteoglycans from iliac cartilage of a 3 year old patient. These may arise from the difference between two tissues (rib cartilage and iliac cartilage) or differences in the proteoglycan populations studied (the whole populations versus newly synthesized molecules). In their study, gel-electrophoresis of $^{35}SO_4$-labelled proteoglycans from the patient yielded only one band in the control. This also suggests that three populations of proteoglycans recognized in normal controls at all ages and the adult patients in this study are produced as a result of proteolytic degradation of one population of newly synthesized proteoglycan. In an earlier study, Stanescu (1975, 1982) demonstrated, by gel electrophoresis of whole populations of proteoglycans, that one population of proteoglycans is absent from the matrix in a 7 year old patient with this disease. This is consistent with our dissociative studies which also showed an absence of one population of proteoglycan (chondroitin 4-sulfate-rich proteoglycan) in the 4 and 12 year old patient. These observations therefore suggest that there may be abnormal catabolism of proteoglycans at a reduced rate in pseudoachondroplastic cartilage, if these different populations arise from the largest population.

CHAPTER 5

SUMMARY & CONCLUSIONS

These studies employing immunochemical analyses with different antibodies against various substructures of cartilage proteoglycans enabled an investigation of the content, size, structure and aggregatability of these molecules in very small samples. From the data obtained, it is possible to state that rib cartilages of patients with pseudoachondroplasia exhibited the following differences from normal site- and age-matched cartilage:

1. Compared with normal control cartilages, the percentage of proteoglycans extracted from pseudoachondroplastic cartilage with 4 M guanidine hydrochloride is unchanged in the young cartilages (4,9 and 12 years old) but higher in the adult cartilage (31 years old).
2. The proteoglycans from the cartilages of patients with this disease have longer chondroitin sulfate chains. This was deduced by the direct analyses of the chain length of chondroitin sulfate.
3. These chondroitin sulfate chains contain a decreased amount of chondroitin 4-sulfate disaccharides, no change in 6-sulfate disaccharides and a corresponding increased number of unsulfated chondroitin disaccharides. These chains bear less epitope 846 which is mostly found in fetal cartilage. They are attached to core protein by fewer chondroitin 4- and 6-sulfate linkage regions and more unsulfated linkage regions.
4. Three different proteoglycan populations are recognized in normal controls at all ages as well as in the adult patient. The largest population is abundant in epitope 3-B-3 (chondroitin 6-sulfate linkage region) and 846 (fetal type chondroitin sulfate epitope). The species of intermediate size is

abundant in epitope 6B (chondroitin 4-sulfate linkage regions). The smallest one is abundant in AN9P1 (keratan sulfate), which appears to be a degradation product of the larger one. In the 4 and 12 year old patients, the chondroitin 4-sulfate epitope is recognized as a component of the largest population: therefore only two populations were identified in these patients.

5. The largest proteoglycan population seen in the cartilage of the 4 and 12 year old patients is larger than that in the normal controls.
6. The contents of the hyaluronic acid-binding regions and keratan sulfate are decreased in the adult patient. There is an increase in the content of keratan sulfate in the patients of intermediate age.
7. Aggregation of proteoglycans is unchanged in this disease except for the 12 year old patient which showed a significant increase in percent aggregation of chondroitin 4-sulfate epitope.
8. The content of extractable hyaluronic acid is higher in patients at all ages.

Based on these observations, it is apparent that there is evidence for abnormal metabolism of cartilage proteoglycans in pseudoachondroplasia with respect to biosynthesis and catabolism of these molecules. The abnormal biosynthesis of proteoglycans causes structural changes in these molecules. These are characterized by longer chondroitin sulfate chains with reduced 4-sulfation and increased undersulfation, producing larger proteoglycan molecules. The longer chondroitin sulfate chains of proteoglycans, together with the retention of proteoglycan core protein in the rough endoplasmic reticulum, which was previously shown by another group (159,160), may suggest decelerated translocation of this protein through the Golgi apparatus which could result in increased chain length. An absence of one population of proteoglycans of an

intermediate size may reflect reduced degradation of these abnormal molecules.

Whether these changes in proteoglycans are associated with changes in the moleculear organization of the extracellular matrix and how these changes are related to incomplete endochondral ossification leading to growth retardation in pseudoachondroplasia remains to be established.

CHAPTER 6

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