Observations on the adult sternoclavicular joint and the neonatal costochondral junction in acquired and genetic disorders

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Abstract

The precise aetiopathogenesis of human osteoarthritis is still a matter of debate and controversy despite the fact that knowledge of many aspects of the disorder has dramatically increased in the past several decades. Human diarthrodial joints are complex structures and it has become very clear that different joints show variations in the prevalence, degree and intraarticular anatomical locations of the osteoarthritic process. The present studies were undertaken to determine the features of osteoarthritis exhibited by the human sternoclavicular joint. This joint is superficial in the body and readily accessible. It is sufficiently small to allow a relatively easy complete examination and is complex by virtue of its surface topologies and its intraarticular disc. Although the joint is, strictly speaking, not weight-bearing, in the human all movements of the upper limb result in transmission of forces across the joint.

An unselected series of right and left sternoclavicular joints was collected at post-mortem examination. Whole and partial joint preparations were made for histological, histochemical, immunohistological and lectin histochemical studies. A particular use was made of large format photomicrographs to enable the observation and annotation of the features of osteoarthritis. A full range of features from normality to severe osteoarthritis was present in the series. Histochemical techniques were applied to detect the presence and distribution of glycosaminoglycans and collagen in different parts of the whole normal joint and those affected by osteoarthritis. Changes in disease were detected and recorded with a general reduction in glycosaminoglycans and collagen distribution. The histopathology of the osteoarthritis was graded by the histological-histochemical grading system as proposed by Mankin et al (1971). Mild to moderate changes were combined in one grade because differentiation between them was very difficult. According to this modified grading system 18 cases were normal, 44 had severe osteoarthritis and 58 cases showed mild to moderate osteoarthritic changes. The presence of clones of chondrocytes was the most common osteoarthritic change in sternoclavicular joints (76 cases) suggesting an attempted repair process.

Lectin histochemistry was applied by the use of an extended panel of lectins to produce glycoprofiles of the cartilages in normal and diseased joints. This approach builds a detailed picture of the presence and distribution of the smaller molecular weight glycans in the cartilage. There is very little information about these potentially important structures in normal and osteoarthritic tissues. Differences in glycosylation patterns were observed and recorded. Chondrocytes of normal and osteoarthritic articular cartilage

showed similar patterns of expression of the N-glycans especially high mannose and complex residues. However there were differences in the intensity of staining between normal and osteoarthritic cartilage indicating that either the quantity of glycans expressed or their accessibility were different. The matrix of the articular cartilage of the sternoclavicular joints showed reactions with a number of the lectins (HHA, PSA, LCA, e-PHA, UEA-I, MAA, ECA, PNA, DBA and MPA) due to the presence of high mannose and complex N-glycans with terminal mannose, fucose (terminal and core), terminal N-acetylglucosamine and N- or O-glycans with terminal β galactose. However, the intensity of the lectin staining was different between normal and osteoarthritic cartilage. A higher intensity of lectin staining was seen in mild to moderate osteoarthritis compared to normal articular cartilage, perhaps because of attempted repair processes. Severe osteoarthritic cartilage showed less intensity of staining overall and this might be because of higher degradative enzyme activity. N-acetylgalactosamine (α1,3 linked as shown by DBA) was present in normal articular cartilage but absent from osteoarthritic cartilage. Moreover, N or O-glycans with β-galacotyl termini (as shown by ECA) were present in osteoarthritic cartilage and absent in normal cartilage especially middle and deep zones. Therefore, DBA and ECA are potential good markers in distinguishing normal from osteoarthritic articular cartilage.

A particular feature of the aged and osteoarthritic sternoclavicular joint was the high occurrence of osteoarticular amyloidosis. Immunohistochemical techniques were used to analyse the chemical composition of the amyloid deposits. β_2-Microglobulin and P-component were present in all samples and there was no association with renal failure or any form of dialysis. In addition, a mixed chemical composition with the presence of λ and κ light chains was observed. Such mixed deposits are unusual. Twenty cases were negative for amyloid deposits, but 100 cases were positive. Ninety-five cases showed amyloid deposits in the disc, 91 in clavicular cartilage and 73 in sternal cartilage. No exact relationship was found between osteoarthritis and amyloidosis. However, the heaviest deposits were found in the older subjects with severe osteoarthritis. The occurrence of amyloid deposition showed a relationship with advanced age.

In a second arm of the project a series of foetal/neonatal costochondral junctions was studied by histological, histochemical and lectin histochemical methods. The costochondral junction in this age group is a non-weight-bearing growth plate undergoing the process of endochondral ossification. The series consisted of normal costochondral junctions and a smaller number of abnormal samples derived from genetic and other disorders. The histological and standard histochemical studies confirmed a zonal

architecture in normal costochondral junctions and structural abnormalities in some of the abnormal cases. Histological staining did not demonstrate differences in the distribution of glycosaminoglycans and collagen between normal and abnormal cases and between abnormals. Therefore, detailed glycoprofiles were determined by lectin histochemical staining to reveal the type and distribution of glycans in the growth plates. There were differences in the various zones in normal and abnormal and between abnormal cases. Chondrocytes of the normal growth plates showed reactions with the lectins HHA, PSA, e-PHA, CTA, ECA, PNA, WFA and DSA, which means they expressed high mannose and some subsets of complex N-glycans, and N- or O-glycans with terminal β-galactose and N-acetyllactosamine whilst chondrocytes of the abnormal growth plates showed no or weak reactions with these lectins. Normal matrix reacts with PSA, e-PHA, CTA, ECA, WFA and DSA especially in the proliferative and hypertrophic zones. Some differences between chondrocytes and matrix are likely to be due to post translation changes. Abnormal growth plates showed no reaction with DSA and had a stronger reaction than the normal ones with e-PHA. Therefore, DSA could be used as a marker of normal growth plate and strong e-PHA staining as a marker of abnormal growth plate.

The lectin histochemical studies showed that the chondrocytes of growth plate expressed more glycans than the chondrocytes of articular cartilage. However, the matrices of the growth plates contained less glycans than the matrices of the articular cartilage. The superficial zone of the osteoarthritic articular cartilage showed similarities with the proliferative and hypertrophic zones of the growth plate matrix. Compared to articular cartilage the growth plates showed intense reactions with WFA whereas articular cartilage was negative, and they had weaker reactions with the lectins that bind to high mannose and complex N-glycans. These results demonstrated differences between the matrices of articular cartilage and the growth plate. However, their matrices showed similarities in the presence of β-galactosyl termini and there was no sign of α1,3-linked N-acetylgalactosamine residues in either.

The lectin histochemical studies in particular showed that chondrocytes in normal and diseased articular cartilage and in normal growth plate cartilage were variously active in terms of carbohydrate metabolism. Whilst there were similarities there were subtle and complex differences revealed which underscores the sophistication of the applied technique. The particular activities of the cells seem to be related to their physical location within the cartilaginous structures and to be reflected in the composition of their matrices.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Publications/presentations arising from this work

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Ghasemi N, McClure SF, McClure J (2001): Osteo-arthrosis of the sternoclavicular joint. (Presentation to the 34th Annual and 9th International Convention of the Islamic Medical Society of North America, Teheran, July 2001.)

Ghasemi N, McClure J, Stoddart RW, McClure SF (2001): Immunohistological and lectin histochemical studies of sternoclavicular amyloidosis. (Presentation to the Annual European Congress of Rheumatology "EULAR 2001", Prague, June 2001.)

List of abbreviations

AA	Amyloid A
AAA	*Anguilla anguilla* agglutinin
AB	Alcian blue
AC	Articular cartilage
ACR	Alkaline Congo red
APES	3-Aminopropyl-triethoxysilane
Asn	Asparagine
CCJ	Costochondral junction
CS	Chondroitin sulphate
CTA	*Erythrina corallodendron* agglutinin
DA	Dermatan sulphate
DAB	Diaminobenzidine-tetrahydrochloride
DMSO	Dimethylsulphoxide
DBA	*Dolichos biflorus* agglutinin
EDTA	Ethylenediamine-tetraacetic acid
DSA	*Datura stramonium* agglutinin
ECA	*Erythrina cristagalli* agglutinin
E-PHA	*Phaseolus vulgaris* haemagglutinin
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	N-acetylgalactosamine (2-deoxy,2-acetamido galactose)
Glc	Glucose
GlcNAc	N-acetylglucosamine (2-deoxy,2-acetamido glucose)
GP	Growth plate
H&E	Haematoxylin and eosin
HA	Hyaluronan
HHA	*Hippeastrum* hybrid agglutinin
HPA	*Helix pomatia* agglutinin
HS	Heparan sulphate
IL	Interleukin
IMS	Industrial methylated spirit
KS	Keratan sulphate
LCA	*Lens culinaris* agglutinin
L-PHA	*Phaseolus vulgaris* leukagglutinin
MAA	*Maackia amurensis* agglutinin
Man	Mannose
β_2-M	β_2-microglobulin
MMP	Matrix metalloproteinase
MPA	*Maclura pomifera* agglutinin
NeuNAc	N-Acetylneuraminic acid
OA	Osteoarthritis
PG	Proteoglycan
PNA (AHA)	*Arachis hypogaea* agglutinin
PSA	*Pisum sativum* agglutinin
PSR	Picro sirius red
SAA	Serum amyloid A
SBA	*Glysine max* agglutinin
SCJ	Sternoclavicular joint
Ser	Serine
TB	Toluidine blue

TBS	TRIS buffered saline
TGF	Transforming growth factor
Thr	Threonine
TNF	Tumour necrosis factor
TTR	Transthyretin
UEA-I	*Ulex europaeus*-I agglutinin
VVA	*Vicia villosa* agglutinin
WFA	*Wisteria floribunda* agglutinin

Chapter 1

Introduction and Review

In this review of the literature the topics considered are in the following order: features of cartilage structure and function, the classification and structure of joints, the skeletal dysplasias, osteoarthritis and amyloidosis.

Section 1

1.1 Cartilage

1.1.1 Introduction

Cartilage has existed in vertebrates since their origin, slightly more than a half-billion years ago, and it may have an earlier invertebrate inheritance. Although cartilage is a distinctively vertebrate tissue (it is central to the evolution of the vertebrate skeletal system) invertebrates also have endoskeletal cartilage of mesenchymal and mesodermal origin. Cartilage, as a tissue, has evolved from connective tissue cells, the secretion of which have a primarily mechanical function (Berrill 1971). Cartilage was formed originally to give structure and support to other cells, tissues, or organs and to progress growth; therefore it is important for the shape of the organism. Increased size of an organism causes problems with mobility and the replacement of cartilage by harder more rigid bone helps to solve some of these problems. Cartilage is also an initial embryonic adaptation for the orderly development of an ossified endoskeleton. Thus, both in the evolutionary history of vertebrates and in embryonic development, cartilage, as a skeletal tissue, precede bone. Limb origins in mammalian embryos are predominantly cartilaginous. Therefore, the ratio of cartilage to the total body mass, in early stages of fetal development, is very high. This decreases with time and development, as most of the cartilage becomes calcified and is finally replaced by bone so that in adult mammals there is very little calcified cartilage left (Fell 1925).

Initial studies of cartilage tissue suggested that it was characterised by gross physical properties such as hardness, flexibility and resistance to pressure and bending forces. With such criteria in mind, crab claw, tendon, and even some other non-cartilaginous structures were mistakenly thought to be cartilage. With the new cell theory of Schleiden (1838) and Schwann (1839) and with increasing use of light microscopy, new criteria were developed for the characterization of cartilaginous tissues. According to these criteria, cartilage was a tissue composed of cells or groups of cells suspended in a more or less rigid matrix, with varying ratios of cells to matrix which was synthesised by the cells. In addition, perichondrium, a connective tissue, surrounded the cartilaginous organ. Using these criteria, many biologists of the

nineteenth and early twentieth centuries considered that both invertebrates and vertebrates possessed true cartilage.

Studies of cartilage chemistry developed more slowly than those of its histology. Insufficient knowledge of invertebrate cartilage chemistry and a lack of an obvious evolutionary link between invertebrate and vertebrate "cartilage" led to the conclusion that the invertebrate tissue was not true cartilage. True cartilages were the precursors of the vertebrate bony axial skeleton, and some other cartilages such as those of larynx, bronchial tree, ear, nose, etc. and should be distinguished from cartilage-like tissue in both invertebrates and vertebrates, which termed chondroid and chordoid. The distinctions between these three classes of tissue were based, at times, upon rather small differences in histology and staining properties.

Cartilage is usually endoskeletal but can also be exoskeletal. Physically, cartilages are gristle-like in texture, quite rigid, and resistant to forces of compression, tension, and shearing (Broom and Silyn-Roberts 1989). As a skeletal support structure, cartilage helps in movement and in resistance to the effects of gravity. Histologically, it is a form of connective tissue composed of polymorphic cells dispersed in a highly hydrated, inflexible, metachromatic colloidal gel matrix. Chemically, cartilage is characterised by its high content of collagen, glycosaminoglycan, hyaluronan, and water. The general functions of cartilage are as follows.

1-The protection and support of related non-skeletal tissues and organs.

2-The formation of articulations between skeletal elements.

3-The facilitation of certain dynamic processes related to skeletal growth and remodelling.

1.1.2. Differentiation and development

In the developing embryo, differentiation of cartilage (chondrogenesis) is the earliest morphogenetic event in the overall process leading to the formation of the skeleton providing structure, enhanced motility and protection to the developing vertebrate animal (Fell 1925). Chondrogenesis begins with cytodifferentiation. The morphogenetic phase is characterised by migration of mesenchymal cells, cell division, and cell-cell interaction. The cytodifferentiation phase is characterised by synthesis and secretion of highly sulphated cartilage proteoglycan and collagen (Barry et al 2001).

The cells from the mesodermal lateral plate provide the skeletal elements of the embryonic limb (Fell 1925). These cells move into the limb field and undergo a phenomenon termed pre-cartilaginous condensation, which is one of the earliest

morphological events in skeletogenesis. This is a transient stage that provides the scaffold for the formation of the endochondral skeletal elements and is a distinct and important phase of normal skeletal development (Fell 1925). This highly organised transformation needs the precise arrangement of cell-cell, cell-matrix and growth factor-mediated signalling events, which finally result in the regulation of gene transcription and function. Mesenchymal cell condensation is mediated by extracellular matrix (Dessau et al 1980). This facilitates the necessary cell-cell interaction, which leads to changes in the composition of the extracellular matrix and the differentiation of mesenchymal cells into chondrocytes. Cartilage development depends on interactions with the extracellular environment. The state of differentiation can be assessed by the type of extracellular matrix molecules synthesised (Solursh 1989). Two signalling molecules, Indian hedgehog (Ihh) and parathyroid hormone-related peptide (Phrp) stimulate chondrocytes in the centre of cartilaginous templates to proliferate and then continue through stages of maturation and hypertrophy. Invading osteoblasts replace the chondrocytes in the region of hypertrophy and the tissue is replaced by bone and bone marrow. This process is typified by the formation of the growth plates at the distal ends of the long bones that separate the epiphysis from the diaphysis.

During condensation, communication between neighbouring cells via adhesion and extracellular matrix molecules is critical in order to establish both temporal and spatial regulation of chondrogenesis in the developing limb. The extracellular matrix not only functions in adhesion and during condensation (Solursh et al 1984), but also serves to regulate the effects of growth factors and morphogens through presentation of molecules complementary to their cognate receptors (Gomes et al 1996). Furthermore, extracellular matrix molecules function in the transmission of signals from the surrounding environment of the cell to the cytoskeleton, cytoplasm and nucleus (Mow et al 1999). Hence, the extracellular matrix is a versatile regulator of chondrogenesis, mediated through various functions and mechanisms during mesenchymal condensation and differentiation (Lopez-Casillas et al 1994).

1.1.3. The definition of cartilage

Cartilage is a specific connective tissue, which plays a central role in the formation of the vertebrate skeleton. It is usually a non-calcified, compact structure and has distinctive physiochemical properties. These characteristics of cartilage give rise to its physiological properties. It contains a large amount of collagen, rather less

proteoglycan and relatively smaller numbers of dedicated, differentiated cells (Heinegard and Oldberg 1989).

Cartilage lacks most of the characteristics of other body tissues: it has no nerves, no blood vessels and no lymphatic system. It is recognised not only by the properties of its cells (chondrocytes), but also by what the cells secrete. This matrix includes water, collagen, hyaluronan, proteoglycans, and other glycoproteins (Maroudas 1979). Chondrocytes occupy about 2% of the total tissue volume. Three quarters of the extracellular matrix of cartilage is water (Mow et al 1984). Collagen occupies about 70% of the dry weight of cartilage matrix, proteoglycans 20% and other proteins the remaining 10% (Lohmander 1988).

In addition to supportive and protective functions cartilage participates in fracture repair (Tatsuyama et al 2000). It provides a structural component of joints allowing articulations, permitting body movement and locomotion. Cartilage is essential for the growth of long bones both before and after birth (Caplan 1985).

1.1.4. Cartilage function

Cartilage is able to resist load by the major matrix macromolecules, the proteoglycans and type II collagen, and matrix fluid (Heinegard and Sommarin 1987). The major cartilage macromolecule, aggrecan, interacts with water by the negative charges of glycosaminoglycans (GAGs) (Hardingham and Muir 1972). The macromolecules are compressed and the fluid is exuded under load. When pressure increases, either hydraulic or osmotic, it causes water and ions to flow through the porous-permeable solid phase (Maroudas 1979). The collagen network is thought to determine the extent of instantaneous deformation in compression. However, no correlation exists between the compression resistance and collagen content. The proteoglycans (PGs) are responsible for providing stiffness for the tissue in compression (O'Connor et al 1988). The highly loaded regions of articular cartilage generally have a greater PG content and they are stiffer in compression than the less loaded regions (Mow et al 1992).

Compressive forces generate tensile stress within the material being compressed. The networks of collagen fibres in the extracellular matrix are the primary determinants of the tensile behaviour of cartilage and, therefore, the orientation of the collagen network influences their tensile behaviour (Askew and Mow 1978).

Shear stress occurs when forces are applied parallel to the cartilage surface (Cohen et al 1998). In shear stress, the volume and hydrodynamic pressure do not change. This shows the intrinsic viscoelastic properties of the cartilage solid matrix without the

effects of interstitial fluid flow. The collagen network plays an active mechanical role in contributing to the shear stiffness and proteoglycan does not provide much resistance to shear (Mow et al 1992).

1.1.5. Cartilage matrix

The particular anatomical and physiological functions of tissues make the presence of an extracellular matrix essential to protect cells and to bind them together. The quantity of the matrix and its organisation depend on the exact cellular functions. However, the matrix controls the arrangement and organisation of the cells during their development.

Like most matrices, cartilage matrix is highly hydrated. Water constitutes 80% of the wet weight of the normal cartilage (Mow et al 1984) and many cartilage functions depend upon interactions between water and other macromolecules (Walker et al 1968). The amount of extracellular matrix in a tissue varies widely. Components of the extracellular matrix provide the framework and give physical protection from external forces. Important tissue specificities are achieved by the various functions of the extracellular matrix (Urban 1994).

It is believed that the primary function of the components of the cartilaginous matrix, a resilient amorphous gel, is something more than an immobile scaffolding for tissue (Lai et al 1991). It directly controls essential cellular functions by affecting metabolism, cell shape, cell migration and development. It also reacts to varying pressure loads and helps the weight-bearing capacity of cartilage through its high degree of hydration and the movement of water (Walker et al 1968). Thus cartilaginous matrix has an active rather than a passive role.

Stability of the matrix over time depends, in part, on the metabolic state of the chondrocytes; since a change in activity of chondrocytes may alter the type of collagen expressed and influence the release of matrix-degrading enzymes (Muir 1995).

1.1.5.1. Collagen

In mammalian tissues collagen represents approximately 30% (by weight) of body protein. Collagen fibrils are thin but strong. They reinforce tissues and provide tensile strength, provide form during embryonic and fetal development, cooperate with other proteins to build tissue and organs, separate cell layers and provide filtration barriers between spaces (Eyre 1980). They are too narrow to be seen by light microscopy, being only 10nm to 100nm in diameter. The diameter of the microfibrillar unit varies with the species, the age of the individual, and the depth of tissue (Byers 1993). Fibrillar collagen is birefringent and can be studied using polarised light and phase contrast microscopy, but

these techniques lack the resolution to identify individual fibres and give only their general orientation (Clark 1985).

The collagens are a family of extracellular proteins, which have different chemical structures, morphologies, distributions, functions and pathologies. Even with wide variations between their structures, all collagens consist of triple helices of polypeptide chains coiled into a unique type of rigid helical structure. The triple helix assembles into collagen fibrils, fibrils aggregate to form fibres and fibres associate into bundles (Junqueira and Montes 1983).

Most collagens are synthesised only by particular differentiated cell types, although a single cell type may synthesise several collagens. Articular cartilage chondrocytes, for example, can synthesise collagens of types II, IX, X, XI and VI, but not collagens of types I or III. The collagen gene family has more than 25 members dispersed to more than 12 chromosomes (Vuorio and Decrombrugghe 1990). The proteins encoded by the genes share the following feature: they form trimers that contain a triple-helical domain characterised by the repeating amino acid motif glycine (Gly)-X-Y (Byers 1993).

1.1.5.2. Cartilage collagen

Most collagens have a tissue-limited distribution. Cartilage matrix consists of 40% to 70% collagen, by dry weight, varying with the source. The collagens of hyaline cartilage are principally type II, which accounts for about 80-90%of the total; types VI, IX, and XI compose the remaining. However, fibrocartilage collagens include at least types I, II, V, VI, IX and XI (Eyre et al 1987).

The experimental reconstitution (after pepsin digestion) of cartilage collagens suggested that collagens II, IX, and XI were necessary in the real quantity of 8:1:1 (molar ratio) to form combinations similar to the thin primary fibrils of immature cartilage (Hagg et al 1998).

Interaction of the collagen and proteoglycans is necessary for the biomechanical properties of cartilage which provides a highly hydrated cushioning tissue in regions of weight-bearing stress (Kaab et al 1998). The main role of collagen is to make stable tissue scaffolding and a reinforcing and restraining meshwork for cells (Byers 1993, Hedlund et al 1999).

1.1.5.2.1. Type II collagen

Type II collagen is specific for cartilaginous tissue, and is the major collagen of all hyaline cartilage. It is synthesised as a procollagen and is assembled into units to form fibrils. It has three identical α1-polypeptide chains, amino acids and carboxy extension peptides, which are removed before it is integrated into fibrils (Slutskii 1984, Eyre et al

1991). The specificity of biosynthesis is high, therefore the form of type II collagen is a definitive marker of cartilage differentiation (Slutskii 1984). Turnover of type II collagen is extremely slow, with a half-life of more than 100 years (Knudson and Knudson 2001) (Figure 1.1.).

The fibrils of type II collagen are thin (average diameter 25nm). The oriented populations of the collagen type II fibrils become evident when studied by the picro sirius red polarizing method (Junqueira et al 1979a). Sirius red stains collagen by reacting, through its sulphonic acid groups, with basic groups present in the collagen molecule (Junqueira et al 1979b). However, the interaction of collagen molecules with the ground substances causes a different birefringence and a varying colour.

Type II collagen fibrils in various cartilages show very different diameters. However, in all hyaline cartilages, except articular cartilage, thin fibrils form a loose meshwork with a regular diameter (Junqueira and Montes 1983). Type II collagen has a more highly hydrated fibril in comparison with collagen type I (Grynpas et al 1980) and this may be because it has many glycosylated hydroxylysine residues along the triple helix. Type II collagens interact with other matrix macromolecules and these cause a unique fibril organisation and distinctive matrix properties (Eyre and Wu 1995).

Distribution of the type II collagen is constant and no notable variations in distribution have been seen in human articular cartilage with age (Duance and Wotton 1991).

1.1.5.2.2. Type IX collagen

The type IX collagen molecule contains three collagenous domains and four noncollagenous domains. It is assembled from three genetically distinct chains to make a single chain. It carries a single chondroitin sulphate chain and, therefore, it is also a proteoglycan (Huber et al 1988).

Type IX collagen locates regularly on the surface of type II collagen fibrils, is cross-linked to the type II collagen fibrils and projects from the surface (Eyre et al 1987). These projections can interact with the polyanionic glycosaminoglycan chains of the proteoglycan aggregate present between the fibrils in cartilage (Duance and Wotton 1991, Mayne 1989). They can cross-link between type II molecules located in two separate fibrils and interact with other matrix components. Hence, they are important in stabilizing the cartilage matrix, and loss of them might lead to swelling of the matrix and the early onset of osteoarthritis (Duance and Wotton 1991). They can also link to each other in cartilage. Type IX represents 10% (w/w) of the total collagen in embryonic cartilage and only 1-2% (w/w) of the total collagen in adult articular cartilage (Eyre and Wu 1995), the amount decreasing relative to type II collagen with increasing cartilage maturity. This

may have important consequences in predisposing cartilage to degradative changes (Duance and Wotton 1991).

1.1.5.2.3. Type X collagen

Type X collagen is synthesised only by hypertrophic chondrocytes and is transiently expressed at sites of materialisation of cartilage and at epiphyseal growth plates (Kielty et al 1985). However, collagen type X is one of the normal components of human articular cartilage (Schmid and Linsenmayer 1985). It is also present in association with clusters of hypertrophic chondrocytes in osteoarthrotic articular cartilage (Rucklidge et al 1996). It is found in the pericellular domain of the tidemark chondrocytes of adult human cartilage (Rucklidge et al 1996).

1.1.5.2.4. Type XI collagen

Type XI collagen was first isolated from the pericellular regions of human costosternal and femoral head cartilages (Burgeson and Hollister 1979). The molecule of this collagen is synthesised as a procollagen (Clark and Richards 1985). It is present in the same fibrils as type II collagen (Vaughan et al 1988). It has $\alpha 1$ and $\alpha 2$ chains of type V collagen and $\alpha 3$ chains like type II collagen (Mayne 1989). The latter might facilitate the incorporation of type XI collagen molecules into fibrils of type II collagen (Eyre and Wu 1995).

Type XI collagen is essential for the control of lateral fibril growth. The bulky N-terminal domain of type XI collagen covers eight nearby type II collagen molecules and prevents further growth in diameter (~ 20nm). However, collagen II/XI mixtures come together into larger banded fibrils in cartilage matrix (Blascke et al 2000). Type XI collagen retains an additional binding site at the amino-terminal ends of $\alpha 1$ and $\alpha 2$ chains as a functional element throughout the lifetime of the fibril. By contrast, type II collagen is fully developed as a functional unit (Vaughan et al 2001).

There is no difference in distribution of type XI collagen with age, though less staining (immunofluorescence) appeared in the older samples. This may be because of age-dependent variation in the accessibility of the epitopes recognised by antibody (Duance and Wotton 1991).

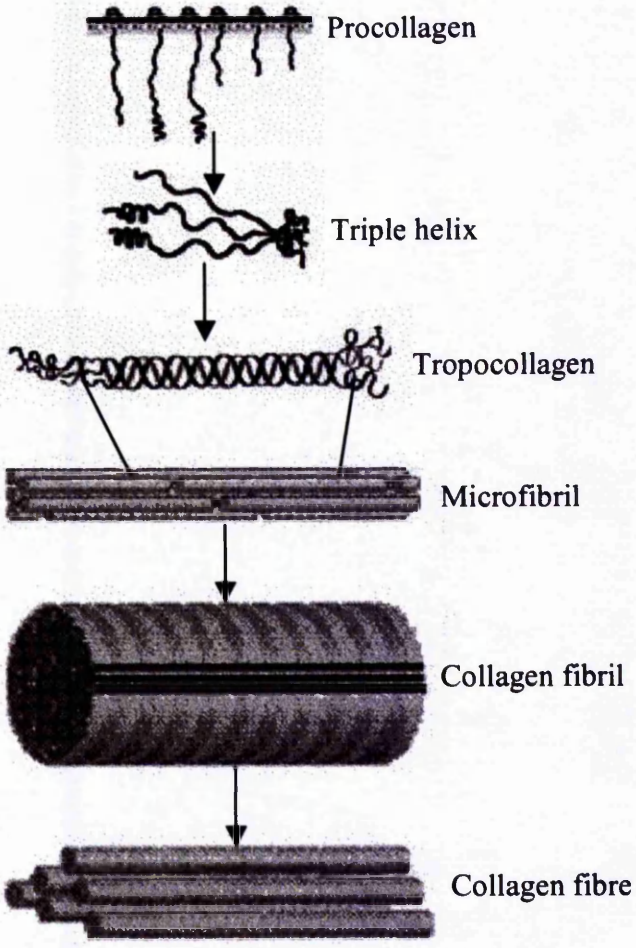


Figure 1.1. The normal processes for collagen fibre production
(Adapted from www.detserv1.dl.ac.uk/Herlad/xray_dif_collagen.htm).

1.1.5.2.5. Type VI collagen

Type VI collagen has been found in small amounts in articular cartilage (Poole 1997), but not in other cartilages (Eyre et al 1987). It has been demonstrated in the extracellular matrix of most connective tissues (Mayne 1989). It is concentrated pericellularly around the chondrocytes of all cartilage zones. In the middle and deep zones, type VI collagen is also present in the interterritorial matrix (Pullig et al 1999). It forms a branched network around collagen fibrils and on the surfaces of cells in both embryonic and adult tissue (Duance and Wotton 1991).

1.1.5.3. Proteoglycans

Three decades ago proteoglycans (PG) were extracted from cartilage as molecular units (Muir 1990). The molecular weight of proteoglycans is high, and they form the major group of macromolecules in extracellular matrix. PGs represent a diverse family of macromolecules, which contain protein linked with at least one glycosaminoglycan (GAG) chain such as chondroitin sulphate, keratan sulphate, heparan sulphate and dermatan sulphate. They are structurally diverse in their core proteins and glycosaminoglycan chains. GAGs have repeating disaccharide units at the time of synthesis, most of which are modified by 5-epimerisation of uronic acid residues, a variable pattern of addition of sulphate ester groups, a variable de-acetylation and N-sulphamido-group formation and by the addition of saccharides as branches. True GAGs are linked to core protein via tetrasaccharide glucose-galactose-galactose-xylose by serine or threonine residues. PGs also contain O and N-linked oligosaccharides and they are synthesised by most eukaryotic cells and are present in almost all mammalian tissues (Muir 1990).

Various PGs were found in various parts of tissues depending on their individual biological properties. Their characteristics and functions are ordered by their structure, anionic GAG component and core protein and this variability allows PGs to have different properties to meet different biological needs (Hardingham and Fosang 1992). They can also interact with other macromolecules such as collagen, which is important for the more specific biomechanical functions of tissues. The range of biological effects of PGs is being increasingly recognised. The function of PGs can be generally categorised into space-filling and specific interactions. They are known to affect cellular growth and differentiation, to bind growth factors and to change their potency and affinity for receptors. They are very important in cartilage, where the PG concentration is higher than in any other tissue (Yanagishita 1993).

1.1.5.4. Cartilage proteoglycan

Cartilage is one of the richest sources of PG among mammalian connective tissues. Because of this high concentration, cartilages have been widely used as a source of PG for biochemical analysis. Cartilage PG consists of core protein, to which is added up to 100 chondroitin sulphate chains and a variable number of keratan sulphate chains, during intracellular post-translational synthesis (Hardingham et al 1991). Various types of PGs have been isolated from cartilage (Hascall 1986). They may be subdivided into three major groups: large aggregating PG, large non-aggregating PG, and small PG (Lohmander 1988).

The proportions of these different types may vary between different locations within the same joint, different layers of the tissue, different joints and with age.

The PGs give important characteristics to cartilaginous tissue, which are (a) compressive stiffness (b) Donnan osmotic pressure and (c) regulation of tissue hydration. Interactions take place between the PG molecules, the fluid and various electrostatic charges. PGs are hydrophilic and interact with water by the negative charges of GAGs. There are electrostatic attractions between the negative charges of the GAGs and the positive charges of the collagen molecules. When compressive load is applied to cartilage, the fluid tries to move out of the tissue. PG molecules produce repulsive forces and osmotic pressure so they attempt to restrain water fluid. The compressive properties of cartilage are a direct result of these properties. Exudation of fluid is at first rapid and gradually diminishes until compression stops (Mow et al 1992). This serves to maintain a high degree of hydration in cartilage.

1.1.5.4.1. Aggrecan

Aggrecan is the brief name of the large aggregating proteoglycan, which is one of the main structural macromolecules of cartilage extracellular matrix. An aggrecan monomer consists of a core protein backbone attached to chondroitin and keratan sulphate chains and N and O-linked oligosaccharide chains. Core protein, typically, has a molecular weight of 230 kD and consists of three globular domains, G_1, G_2 and G_3, and two interglobular domains. Interglobular domains are the sites of keratan sulphate and chondroitin sulphate attachment and are located between G_2 and G_3 and in a short region between G_1 and G_2 (Hardingham et al 1991). Chondroitin sulphate chains are mainly found in the C terminal domain (90%) with some keratan sulphate, while the most keratan sulphate chains are sited near the N terminus. Each core protein typically carries 80-100 chondroitin sulphate chains, about 30 keratan sulphate chains, about 50 O-linked mucin-

type oligosaccharides and 5-10 N-linked oligosaccharides (Caplan 1985, Sandy et al 1991).

A single molecule of hyaluronan (see page 31) interacts with up to 100 aggrecan monomers to form an aggregate of very high molecular weight. The first globular domain at the N terminus of the aggrecan core protein termed G_1, or the hyaluronan-binding region, is substituted with N-linked oligosaccharides and is involved in the binding of aggrecan to hyaluronan and link protein. This immobilizes the PG in the tissue (Sandy et al 1991). The G_1 domain contains three looped subunits, A, B, and B′. Both B and B′ loops have a disulphide-bridged double loop structure, called tandem repeats, which work as an effective location for the interaction with hyaluronan (Watanabe et al 1997). Loop A has structural characteristics common to members of the immunoglobulin family and it acts in the interaction between aggrecan and the link protein (Barry et al 1995). A further globular domain between the G_1 domain and the keratan sulphate-rich region on the core protein is termed G_2 and shares sequence homology with G_1. However, it is not concerned in the binding with either link protein or hyaluronan, and its function has not yet been identified (Morgelin et al 1994). The third globular domain, termed G_3, is located at the C terminus of the core protein, and consists of three modules. These are the epidermal growth factor-like module, the C-type lectin-like module, and a region (or module) resembling the amino-acid sequences of complement-regulatory proteins. The lectin-like module binds to fucose and galactose, whilst the G_3 domain may interact with tenacin. The functions of the different components of the G_3 domain have not been completely determined. With ageing, the proportion of aggrecan without the G_3 domain increases, probably because of proteolytic degeneration of the matrix (Watanabe et al 1997, Knudson and Knudson 2001) (Figure 1.2.).

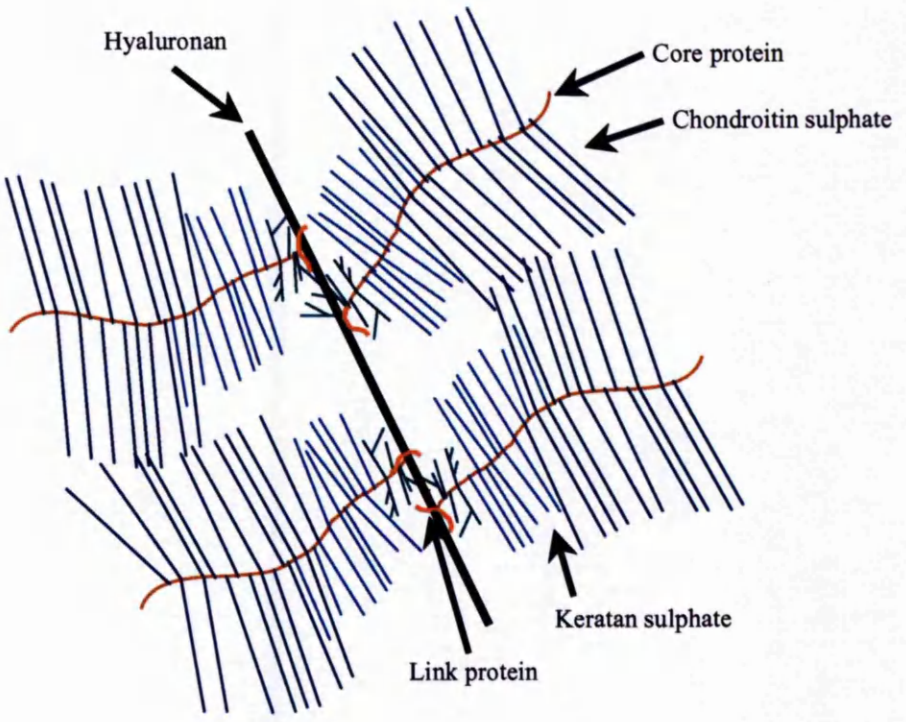


Figure 1.2. The structure of part of the aggrecan molecule

1.1.5.4.2. Non-aggregating proteoglycans

Among other PGs identified in cartilage are the small leucine-rich PGs, decorin, biglycan, fibromodulin, lumican, and epiphycan, the cell surface PGs, syndecans and glypican, and the basement membrane PG, perlecan (Knudson and Knudson 2001).

The small leucine-rich PGs can be divided into three classes. Class I includes decorin and biglycan, both of which are found in cartilage (Iozzo 1999). Biglycan normally carries two GAG chains; decorin carries only a single chain. Human biglycan and decorin contain N and O-linked oligosaccharide attachment sites (Kresse et al 1993). Epiphyseal cartilage and articular cartilage stain for decorin and biglycan (Bianco et al 1990). In articular cartilage, biglycan is found in the pericellular matrix (Poole et al 1996), and decorin is absent (Knudson and Knudson 2001). Decorin is present in interterritorial matrix in the deeper zones of the adult articular cartilage (Miosge et al 1994). The superficial layer of articular cartilage consists of decorin (Poole et al 1996) and the amount of decorin is found to decrease with depth in the layer of the cartilage (Miosge et al 1994).

The horseshoe-shape of decorin supports its interaction with collagen fibrils as a decorating PG. It decorates type II collagen fibrils in cartilage, but has no effect on fibre diameter (Hagg et al 1998). Biglycan was found to be able to interact with collagen type II, and the interaction seemed to be quite strong (Vynios et al 2001).

Class II, leucine-rich PGs include fibromodulin, lumican, and the protein known as PRELP (proline arginine-rich end leucine-rich repeat protein). Fibromodulin and lumican have been found in interterritorial matrix and decorate the surface of collagen fibres (Svensson et al 2000). Therefore, they adjust fibril diameter (Ezura et al 2000). Fibromodulin is deposited closer to the cell than decorin and it may have a primary role in collagen fibrillogenesis (Saamanen et al 2001). Both may play an important role in interactions with growth factors (Gomes et al 1996) and they can be detected in growth plate, but lumican could not be detected throughout the growth plate (Alini and Roughley 2001). Fibromodulin and lumican were increased with age in articular cartilage (Knudson and Knudson 2001) and transform from proteoglycan to glycoprotein as a result of either a synthetic or a degenerative event (Melching and Roughley 1999). The amino acid composition and sequence of PRELP and its four potential N-linked glycosylation sites are similar to the structure of fibromodulin, but it has a high content of proline and arginine residues. PRELP shows a limited distribution, and is expressed at high concentration in cartilage (Bengtsson et al 1995).

Class III leucin-rich PG, known also as epiphycan, is a dermatan sulphate (DS) PG (Johnson et al 1997). DS or chondroitin sulphate B consists of hexuronic acid and N-acetylgalactosamine and it can also contain glucuronic acid. Epiphycan is expressed in growth plate and articular cartilage and its function may be to arrange the matrix of the growth plate (Johnson et al 1999).

Syndecan and glypican are cell surface PGs, which are expressed by chondrocytes. Syndecan may carry two or more heparan sulphate chains, alone or in combination with chondroitin sulphate (see page 30) (Solursh et al 1990). Low-level expression of glypican has been found in cartilage, but the most abundant small PG was one type of syndecan. Syndecan behaves as a matrix receptor, which binds both extracellular matrix components and basic fibroblast growth factor (Bernfield and Sanderson 1990).

Perlecan is a heparan sulphate (HS) PG, originally identified in basement membrane (Yanagishita 1993). HS consists of glucuronic acid and N-acetylglucosamine and contains numerous variations of sulphate and L-epimerisation. The functions of perlecan include interaction with extracellular matrix protein (Arikawa-Hirasawa et al 1999) and growth factors (Mongiat et al 2000), which influence normal cartilage development. It

regulates cell and matrix adhesion and protects extracellular matrix of the cartilage from degradation (Costell et al 1999). In addition, it provides a sufficient signal to trigger stem cells to the chondrogenic differentiation pathway (French et al 2002).

1.1.5.5. Glycosaminoglycans

1.1.5.5.1. Chondroitin sulphate

Chondroitin sulphate (CS) is the most abundant GAG in cartilage. Muller in 1837 obtained 'chondrin', a colourless, amorphous, nitrogenous substance similar to gelatine, by autoclaving cartilage in water. Partially purified CS was first isolated by Krukenberg in 1884, using extraction with alkali, which splits the protein from the polysaccharide. Now CS is extracted from cartilage using an enzyme, such as chondroitinase ABC.

The CS family of GAGs consists of unbranched polymers of 40-100 repeating disaccharide units, each containing glucuronic acid and N-acetylgalactosamine (Mitchell and Hardingham 1982). CS chains are attached to core protein via the xylose-galactose-galactose-glucose tetrasaccharides. The sugar xylose binds O-glycosidically to the serine residue of the successive serine-glycine residue (Shibata et al 1992). Most of the hexosamine component is sulphated at C4 yielding chondroitin sulphate A, or at C6 yielding chondroitin sulphate C (Yanagishita 1993). Both forms are present in cartilage, but chondroitin 6-sulphate predominates with increasing age (Mourao et al 1976). Normally each unit has two negative charges, which are responsible for the hydrophilic and space filling properties of the PG (Lohmander 1988).

The CS-rich region of core protein, containing up to 100 CS chains, is found at the C-terminal part and constitutes more than 80% of the molecular mass. The CS is linked via O-glycosidic bonds between serine in the peptide chain and a xylose residue at a reducing end of the GAG chain. Proteolytic treatment does not damage this linkage, but it is destroyed by alkali as the result of β-elimination (Stockwell 1979, Hedlund et al 1999).

1.1.5.5.2. Keratan sulphate

Keratan sulphate (KS) was first isolated from the cornea, and owes its name to the anatomical structure where it was found. Skeletal KS is basically similar to corneal KS. In cartilage, it was first detected by chemical analysis of human intervertebral disc.

KS is made of the same repeating disaccharide unit. It contains galactose and N-acetylglucosamine and lacks uronic acid. KS is linked to core protein via asparagine by N-acetylglucosamine or via serine/threonine by N-acetylgalactosamine. The glucosamine residue is normally sulphated at C6, but this may be absent, or extra sulphate groups may be present on C6 of the galactose residues (Stockwell 1979). KS can carry branches of sugars such as L-fucose.

The linkage of KS to the core protein, in the N-terminal region near to the G_2 domain, is via a glycosidic bond between serine or threonine and N-acetylgalactosamine. This linkage sequence is the same as that of the mucin-type O-glycan (Lohmander 1988). However, in bovine articular cartilage, KS may be either N or O-linked (Barry et al 1995).

In mammalian tissue, two forms of KS are found, KSI and KSII. In the large PGs of cartilage KSII is present, while in small PGs such as fibromodulin and lumican, and in cornea, KSI is present. KSII is attached through an oligosaccharide structure that is linked O-glycosidically to the hydroxyl group of serine or threonine. KSI is attached N-glycosidically to the asparagine residues of the core protein (Choi and Meyer 1975). Two types of KSII are present in cartilage: type A occurs in articular cartilage and intervertebral disc, and contains fucose and N-acetylneuraminic acid, while type B occurs in trachea and nasal cartilage, and lacks fucose and N-acetylneuraminic acid.

1.1.5.5.3. Hyaluronan

Hyaluronan (HA) was first isolated from the vitreous body of the eyeball, and its name was derived from this structure (Osterlin 1969). It is found in synovial fluid, articular cartilage, vitreous body and skin. HA is not a typical GAG, and differs from KS and CS in several ways. It is not sulphated, it is much longer, it is not covalently linked to protein, and is synthesised in a different way from the true GAGs.

The biosynthesis of HA differs markedly from that of the other GAGs. It occurs at the plasma membrane (Prehm 1984) and it does not require the presence of core protein as a primer. Exceptionally among eukaryotic glycans the polymer grows at the reducing end of the chain. During synthesis, the carbohydrate assembled inside or at the plasma membrane is pushed out into the extracellular space (Nishida et al 1999). Hyaluronan molecules bound to core protein may play an important role in both the formation and maintenance of the aggrecan-rich rim that surrounds each chondrocyte (Hua et al 1993). Interleukin-1 has a strong and reproducible stimulatory effect on the synthesis and extracellular release of HA (D'Souza et al 2000).

HA is a repeating disaccharide, consisting of alternating residues of glucuronic acid and N-acetylglucosamine. It is a linear, unbranched, flexible, long-chain polymer. HA is an important factor in the aggregation of proteoglycans into complexes, which are necessary for the structure and stability of the cartilage matrix.

One HA molecule associates with about 100 PGs. It is highly negatively charged. Therefore basic amino acid-rich regions of the link protein have a tendency to interact with HA (Perkins et al 1989). Each PG interacts with a segment of the HA by a single

binding site on the core protein, but little is known of the hyaluronan-link protein linkage (Watanabe et al 1997).

1.1.5.6. Oligosaccharides

Proteoglycans carry covalently attached carbohydrates, other than GAGs, in the form of both N-linked and O-linked oligosaccharides, depending on the amino acid residues to which they are linked. O-linked oligosaccharides are linked to the hydroxyl groups of serine and threonine side chains. When they contain N-acetylgalactosamine linked to serine or threonine they are termed mucin-type glycans (Misra et al 2001). About 50 such O-linked oligosaccharides were found near the N-terminal of core protein. The basic structure of these oligosaccharides is similar to that of the linkage region of KS to protein (Lohmander 1988).

N-linked oligosaccharides contain the amino sugar N-acetylglucosamine linked to the side chain nitrogen of asparagine. Hundreds of different glycan structures have been isolated from glycoproteins. This huge diversity of structure is caused by fairly few different structural elements. All N-glycans contain a common pentasaccharide core sequence that contains three mannose and two N-acetylglucosamine residues. The variety of N-linked oligosaccharides is due to additional sugar, such as xylose, fucose, or N-acetylglucosamine, attached to the common core. Further N-glycan variety is created by extension of the cores with outer chains, or antennae, of varying lengths and branching patterns. The three types of N-linked oligosaccharides are high-mannose, hybrid and complex (Elbein 1984). In the high mannose type, additional mannosyl residues are linked to the mannose of the core. In the complex type, other sugars are added to the terminal mannose of the core or to the N-acetylglucosamine components. The hybrid type has both additional mannose and other sugars. The 5-10 N-linked oligosaccharides in proteoglycans are clustered in the aminoterminal domain of the core protein. They resemble the mannose-containing oligosaccharides that occur in many types of glycoprotein (Gopal and Ballou 1987).

1.1.5.7. Link protein

Link protein (LP) is usually purified very easily from cartilage. It binds to both HA and core protein, which stabilises the aggrecan. Three types of LP have been isolated from various types of cartilage of different ages and their structures are closely related. LP_1, with two N-linked oligosaccharides has the highest molecular weight, LP_2, with one N-linked oligosaccharide, is smaller than LP_1, and the smallest, which accumulates with age, is LP_3 (Neame and Barry 1993). A human LP, potentially, has two N-linked oligosaccharides, one is at amino acid 6 and the other is at amino acid 41. It is always

glycosylated at amino acid 6 (Watanabe et al 1997) and glycosylation at amino acid 41 is variable. The gene for human LP has been recognised on chromosome 5, and does not appear to correlate with any known genetic disease loci in this region (Neame and Barry 1993).

The function of LP is to stabilise the PG aggregate and it may also have a role in protecting the part of the PG monomer which binds to HA (the G_1 domain) from proteolytic degradation. There are almost 100 PG monomers per aggregate and it is thought that there is a 1:1 ratio of LP with monomers. When LP is present, the globular LP and the hyaluronan binding region of the G_1 domain form a solid display along the HA chain (Lohmander 1988, Neame and Barry 1993). Link protein represents about 0.05% of the net weight of cartilage and it is thought of as a cartilage macromolecule (Neame and Barry 1993).

1.1.5.8. Water

Each repeating disaccharide unit of KS and CS carries two negative electric charges (Malmonge and Arruda 2000). Water is a small electric dipole, with positive charges at the hydrogen atoms, so that they associate with negative charges (Hall et al 1996). Water becomes organised in multiple, interacting layers all over the electric charges. The number of layers depends on the strength of the charge. Additional water diffuses through the interstices of the extracellular matrix of the chondrocytes by an osmotic effect. This means that PG is associated with and organises large volumes of water many times its own weight. In both these ways PG controls water, and that water becomes the main component of cartilage.

The flexibility of cartilage arises directly from this water component. When pressure is put on cartilage, water is forced out from the charged domains of the sulphate and carboxyl groups. The negative charges of these groups come closer and the repulsive forces of their charges act against further compression. When the pressure is released water returns to the previous position.

The nutrients of cartilage in joints enter and leave in the fluid flows caused by the compression and relaxations arising from body movement and not via blood vessels. Therefore, a long period of immobility can damage joint cartilage, making it become thin and fragile (Caplan 1985).

For articular cartilage, the negative charge density changes according to the position and depth in the tissue (Malmonge and Arruda 2000). Therefore, the water content is not homogeneously distributed throughout the articular cartilage, decreasing in concentration from 80% at the surface to 65% in the deep zone (Torzilli et al 1987).

1.1.6. Chondrocytes

Chondrocytes are the only cellular component of adult hyaline cartilage. They have important properties and abilities which make them distinct from other types of mesenchymal cell. Mesenchymal cells differentiate into chondroblasts at the sites where cartilage will develop in the embryo. They become rounded and form a mass of chondroblasts with little intercellular material. They begin to secrete the macromolecular constituents of cartilage matrix. The chondroblasts continue to deposit the matrix. When chondroblasts are completely surrounded by matrix, they are described as chondrocytes (Sheldon 1983). Chondrocytes are fully differentiated cells that are the only secretors of cartilage which is the tissue that gives the permanent flexible part of the skeleton and is the temporary component for skeletal structure during development (Muir 1995). Typical chondrocytes are ovoid cells, although they can vary from a spheroidal to a flattered shape. Cell size also varies, with diameters ranging from 10 to 30 μm or more. These variations in shape and size are related to the type of cartilage, the position of cells in the cartilage, and the age and maturity of the tissue. Chondrocyte nuclei are round or ovoid, but become more irregular with age. Cell surfaces are irregular with projecting cell processes, which may continue far out into the extracellular matrix (Stockwell 1979, Gardner 1992b), but do not touch other cells (Poole 1997). These features can be explained by cell-matrix interactions without interaction with other cells.

Chondrocytes have some functions that are not shared by other types of cell. Their functions are very important to cartilage and their products are essential to function of the cartilage (Muir 1995). Chondrocytes produce and maintain the cartilage matrix under a normal condition of low turnover (Goldring 2000). The major components of the extracellular matrix synthesised by these specialised cells include type II collagen molecules, large aggregating proteoglycan, small proteoglycan, and other collagenous and non-collagenous matrix proteins (Hardingham et al 1991).

Chondrocytes are sensitive to mechanical stress, which plays an important role in matrix composition and the mechanical properties of the cartilage (Smith et al 2000). They are influenced not only by the patterns of load application, but also by matrix properties, which change load into signals. These signals can alter the intracellular ionic or osmotic environment of the chondrocyte, by influence on membrane transport, and cause rapid alterations in chondrocyte metabolism (Wilkins et al 2000). Chondrocytes undergo considerable changes in shape and volume under normal physiological conditions. Cellular deformation is one of many biophysical factors involved in the regulation of chondrocytes (Guilak 2000).

Chondrocytes can sense changes in the organisation of the extracellular matrix and react by running a balance between anabolism and catabolism and constant remodelling as the cells replace matrix macromolecules lost through degradation (Huber et al 2000).

The chondrocyte surface is limited by the cell membrane, and there are known cell surface markers such as syndecan. Each chondrocyte contains a nucleus and cytoplasm. The smooth endoplasmic reticulum produces glycosaminoglycans, which then shift to the Golgi complex. The Golgi apparatus is a packaging and shipping centre for substances intended for export to the matrix. The other role for the Golgi apparatus is secretion of collagenous components of matrix. The rough endoplasmic reticulum synthesises the collagen and the protein core of proteoglycans. All chondroblasts and chondrocytes have abundant rough endoplasmic reticulum (Sheldon 1983).

Finally, all products synthesised are transported in large vesicles to the surface of the chondrocytes and released into the moat or lacunar space (Gardner 1992b).

Chondrocytes live in small matrix spaces known as lacunae, which are surrounded immediately by fibrils. However, after histological fixation and processing they shrink and an artificial space is created between the cell and the lacunar boundary. The chondrocyte and its pericellular microenvironment together represent the chondron. A chondron is a complicated microanatomical unit, consisting of a single chondrocyte connected at its surface to a transparent pericellular matrix, which is limited and covered by a fibrillar pericellular capsule (Poole et al 1984). A high concentration of sulphated glycosaminoglycans was found around the chondrocytes of hyaline cartilage as shown by the binding of cationic dye (Stockwell and Scott 1967). Strong proteoglycan-collagen interactions are also recognised in the pericellular matrix (Kuhn and Mark 1978) and non-collagenous glycan and other small proteoglycans are in this area (Poole 1997).

1.1.7. Cell and matrix interaction

Cell and matrix interactions are essential for normal tissue development and function. Changes in these interactions can lead to alterations in the nature of the tissue. These interactions are based on collagen in combination either with PGs or with glycoprotein. Complex formations occur between collagen and CS and between collagen and HA (Rooney and Kumar 1993).

Certain interactions between collagen and PG are necessary for deposition as an insoluble complex within the extracellular matrix. However, the synthesis of collagen and PG are regulated independently. PGs interact with tropocollagen to control collagen fibrillogenesis (Vynios et al 2001). Collagen, in its triple helix structure, is stabilised by its interaction with chondroitin 6-sulphate (Gelman and Blackwell 1973). Collagen and

PG interaction is very important for maintaining the integrity of the extracellular matrix. The more GAGs that are bound within the extracellular matrix the more the cartilage is able to resist externally applied forces.

Collagens interact not only with PGs, but also with each other, to make cross-banded fibrils (Eyre et al 1991). Type IX collagen molecules bind covalently to the superficial layer of the cross-banded fibrils and project into the matrix, where they also can bind covalently to other type IX collagen molecules (Eyre et al 1987). Type XI collagen molecules bind covalently to type II collagen molecules and probably to part of the interior structure of the cross-banded fibrils. Type IX and XI collagens presumably help to form and stabilise the collagen fibrils assembled primarily from type II collagen (Mayne 1989). The projecting PGs, such as fibromodulin and decorin, of the type IX collagen molecules may also help to bind together the collagen fibril meshwork and to connect the meshwork with PGs. Type II collagen appears to form an important part of the matrix immediately surrounding the chondrocytes and to help chondrocytes attach to the matrix.

Cartilage contains two major classes of PGs. Large aggregating PG monomers, or aggrecan, and small PGs. The formation of aggregates helps to anchor PGs within the matrix, preventing their displacement during deformation of the tissue, and helping to organize and stabilise the interaction between PGs and the collagen meshwork. Biglycan (small PG) is concentrated in the pericellular matrix and may interact with type VI collagen. Noncollagenous proteins and glycoproteins may also have roles in matrix organisation and cell-matrix interaction (Buckwalter and Mankin 1998).

1.1.8. Cartilage classification

There are three broad classes of cartilaginous tissue present in the body: hyaline cartilage, elastic cartilage, and fibrocartilage. These tissues are distinguished by their biochemical composition, their molecular microstructure, and their biomechanical properties and functions.

1.1.8.1. Hyaline cartilage

The most common cartilage in the body is hyaline cartilage. The relative proportions of collagen and PG make the tissue glossy and translucent to the naked eye. The most common hyaline cartilage is articular cartilage. This tissue covers the articulating surface of long bones and sesamoid bones with synovial fluid. Another example of hyaline cartilage is the growth plate, which controls the growth of the long bones. Other tissues include the larynx, the support structures of the tracheal tube, ribs, costal cartilage, and nasal septum. In the light microscope hyaline cartilage matrix appears relatively

homogeneous and structureless and is characterised by its ability to stain metachromatically with toluidine blue or positively with alcian blue. These staining characteristics reflect the high concentration of polyanionic sulphated GAGs present in the matrix.

1.1.8.1.1. Articular cartilage

Under normal condition, the bone ends in synovial joints cannot contact one another, because the articulating surfaces are covered by cartilage. Articular cartilage resembles hyaline cartilage elsewhere in the body. However, articular cartilage has no perichondrium and the matrix contains more water than other types of cartilage. The surfaces of articular cartilage are slick and smooth. This feature alone can reduce friction during movement at the joint. However, even when pressure is applied across a joint, the smooth articular cartilages do not touch one another, because they are separated by a thin film of synovial material in the joint cavity, which acts as a lubricant, minimizing friction. Articular cartilage serves as a load-bearing elastic material, which is responsible for resistance to compression forces and distribution of load. The biological and mechanical properties of articular cartilage depend on the interaction between the chondrocytes and matrix (Kuettner et al 1991, Cohen et al 1998). Load-bearing areas of articular cartilage are thicker and mechanically stronger than non-load-bearing regions of the same joint (Hall et al 1996).

Articular cartilage can be considered as a series of four horizontal zones, superficial, transitional, deep, and calcified. The zonal organisation is defined according to the cells, the molecular composition and the macromolecular organisation of the matrix. The sizes of zones vary between joints and between species. Cells in the various layers differ in their metabolic activity (Clark 1990, Huber et al 2000).

Zone I:

The most superficial part of articular cartilage in the uncut joint merges with synovial fluid, so that the articular cartilage surface is an artefact (Gardner 1992a). It is the thinnest zone of articular cartilage, varying in thickness from 350 to 450 nm. It has the highest concentration of water, a dense packed layer of collagen, and a low concentration of PG (Buckwalter and Mankin 1998). The collagen fibres are thin in diameter and form a dense network with interlacing fibre bundles lying tangential to the articular surface. This zone consists of two collagen layers (Huber et al 2000). The first layer, known as the lamina obscurans, is composed of fine fibrils covering the articular surface (Clark 1990). They form thick packed bundles parallel to each other (Jeffery et al 1991). There are no cells in this part of the superficial zone. The deeper layer contains collagen fibres

arranged perpendicular to the articular surface. This area reacts with cationic dyes such as toluidine blue, and includes the free ends of the banded collagen fibres and chondroitin sulphate proteoglycan (Kumar et al 2001). The specific organisation of the superficial zone determines the mechanical properties of the tissue and also may act as a barrier to the passage of large molecules from synovial fluid to cartilage (Buckwalter and Mankin 1998). The chondrocytes in the superficial zone are flattened and lie 1 to 2 μm from the free disarticulated surface (Huber et al 2000).

The normal structure and properties of the superficial zone are very important for the weight-bearing function of cartilage. Removal of this zone is one of the first detectable changes in experimentally induced degeneration of articular cartilage (Guilak et al 1994). The intact superficial surface provides a very highly efficient lubrication mechanism with an extremely low coefficient of friction (Kumar et al 2001). Hence, any damage to the superficial cartilage zone may cause the cartilage to break down, with consequent osteoarthrosis (Orford and Gardner 1985).

Zone II:

The transitional zone, also termed the intermediate or middle zone, is composed of chondrocytes surrounded by matrix. The chondrocytes are ovoid or round and are usually single within lacunae, but they can be paired in the deeper part (Poole 1997). Their cytoplasm contains much rough endoplasmic reticulum, clusters of glycogen granules, lipid drops, and a few mitochondria (Buckwalter and Mankin 1998). The collagen fibres are arranged randomly, but are generally oriented toward the articular surface. The PG content is increased in this zone compared to the superficial zone (Huber et al 2000). Chondroitin sulphate concentration is maximal in the middle zone (Stockwell and Scott 1967).

Zone III:

The deep zone, otherwise called the radial or radiate zone, contains chondrocytes, which are single, wide, plump, ovoid, or round, in the upper part of the zone, but mainly forming columns in the deeper parts. The cell volume here is at its lowest, because in this zone, the greatest distance is seen between chondrocytes. The density of PGs is high and the concentration of water lowest. The collagen fibres have a generally radial orientation and they form large bundles, approximately 55 μm across. The concentration of PGs is high, but variable, and the aggrecan content is decreased (Bayliss et al 1983).

The principal GAG is chondroitin sulphate and it is more abundant than in zone II. Keratan sulphate is scanty (Gardner 1992a). However, towards the deeper parts, the proportion of chondroitin sulphate decreases, with a relative increase of keratan sulphate

(Bayliss et al 1983). The matrix of zone III dips into zone II as finger-like projections. A wavy irregular line, known as the tidemark, separates the deep zone from the calcified zone (Huber et al 2000).

Zone IV:

The calcified zone is the deepest part of the articular cartilage. It extends from the tidemark to the subchondral bone. It is characterised by round chondrocytes located in uncalcified lacunae and by the absence of PGs (Poole 1997). The collagen fibres are arranged perpendicular to the articular surface (Lane and Weiss 1975). They are anchored in a calcified matrix, and continue to the bone. The calcified zone contacts the underlying cortical bone, which is known as the articular end plate (Huber et al 2000). Articular cartilage appears normally to expand in thickness until about the age of 60 years, but the thickness of the calcified zone is decreased. However, other evidence suggests that the ratio of the thickness of the articular cartilage to the thickness of the calcified zone is fixed (Gardner 1992a).

The tidemark:

The junction of the mature non-calcified and calcified cartilage is separated by a band of fibrils called the tidemark (Poole 1997). The tidemark serves as the means of binding the collagen of the non-calcified zone to the calcified zone. Three distinct variations of the collagen were observed by histological studies. There is a band of randomly oriented fibrils continuous with those of the non-calcified and calcified zone, a band of flattened fibrils parallel to the surface of the calcified zone and a band of perpendicular fibrils which have a distinct, continuous transition between the non-calcified and calcified zones (Redler et al 1975).

The line of the tidemark is stained by a wide variety of dyes for protein and lipid, but not for PG, is still present after decalcification and is biochemically undefined. The tidemark normally forms a single line, but duplication of tidemark can be seen with age and in osteoarthritis (Hough et al 1974).

Small gaps in the tidemark may provide channels for the passage of nutrients from subchondral bone to the articular cartilage (Redler et al 1975, Malinin and Ouellette 2000). However, some investigators argue that there are no microanatomical connections between articular cartilage and subchondral bone and the main nutritional pathway is from the synovial fluid to the articular cartilage (Lee et al 1997) (Figure 1.3.).

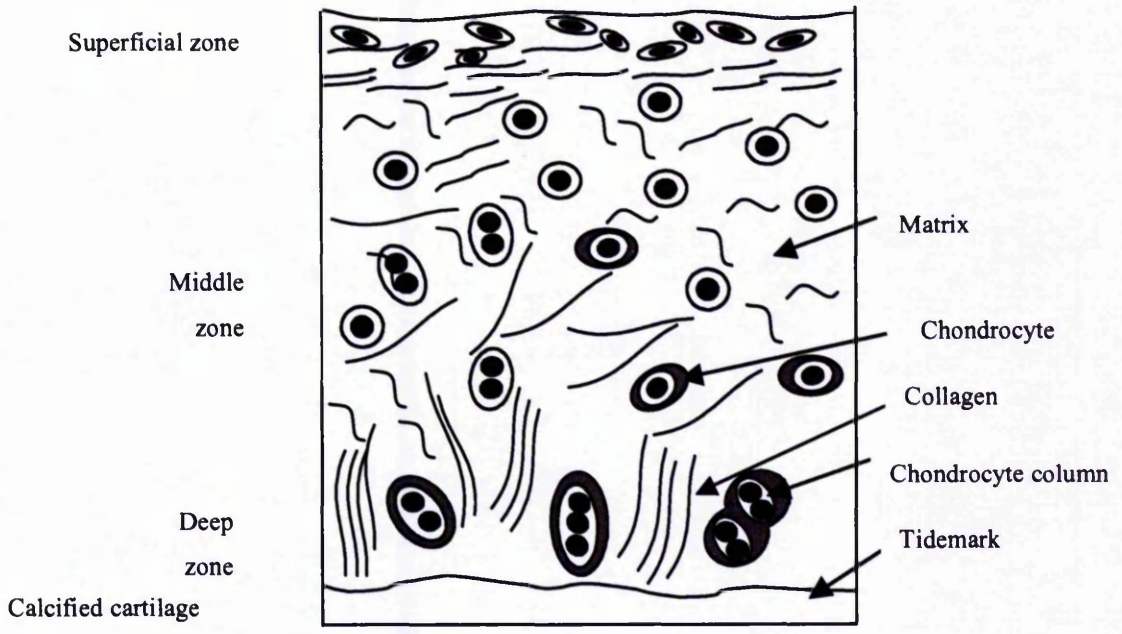


Figure 1.3. Diagram of the structure of articular cartilage

1.1.8.1.2. Extracellular matrix alteration in articular cartilage

The extracellular matrix surrounding each chondrocyte is subdivided into distinct zones. Chondrocytes in all the zones (as in 1.8.1.1 above) of articular cartilage are surrounded by pericellular matrix. Territorial matrix and interterritorial matrices frequently surround the pericellular matrix and enclose the chondrocytes. These subdivisions were made according to circumferential differentiation of the matrix component. In addition, the pericellular matrix in the medial and deep layers is often separated from the territorial matrix by a rim or boundary, which is termed the pericellular capsule. This is composed of fine fibrillar material, but it is not found in the superficial layer (Poole et al 1984).

The pericellular matrix contains a fine network of thin, branching, filamentous material, interspersed with matrix granules, which contain dense PGs. No collagen fibres were observed within the pericellular matrix (Buckwalter and Mankin 1998). Histochemical stains showed that the pericellular matrix contained a high concentration of

PG in the superficial layer. In the deeper layer the concentration of PG aggregates increases to the periphery of the pericellular matrix (Poole et al 1984). The pericellular matrix contains non-collagenous protein, glycoprotein including anchorin II, chondronectin, and non-fibrillar collagens such as type VI collagen. Cytoplasmic extensions from cells project into and through the pericellular matrix to the territorial matrix (Buckwalter and Mankin 1998).

The territorial matrix is an envelope, which surrounds the pericellular matrix of individual cells and, in some cases, pairs and clusters of cells and their pericellular matrix. In the radial zone the territorial matrix surrounds each chondrocyte column. The territorial matrix contains PG aggregates concentrated in the spaces between the collagen fibre bundles. Thin collagen fibrils in the territorial matrix adhere to the pericellular matrix and make fibrillar baskets around the cells to protect them when the cartilage is deformed (Buckwalter and Mankin 1998). Chondroitin sulphate is predominantly in the territorial matrix, while keratan sulphate appears more concentrated in the interterritorial matrix (Stockwell and Scott 1967).

Interterritorial matrix is the largest compartment of articular cartilage, responsible for the mechanical properties of the tissue. It contains the largest diameter collagen fibrils (Poole 1997). In the superficial zone the collagen fibrils in the interterritorial matrix are parallel to the joint surface, and in deeper areas are perpendicular to the joint surface (Buckwalter and Mankin 1998). The collagen fibres in the interterritorial matrix are more closely packed than in the territorial matrix and large amounts of electron dense material are associated with them (Poole et al 1984).

1.1.8.1.3. Perichondrium

The perichondrium is the only boundary of many types of cartilage. It is a dense membrane composed of fibrous tissue that closely wraps all cartilage except cartilage in the joints, which is covered by synovial membrane. It consists of two parts: an outer fibrous layer and an inner cellular chondrogenic layer. The outer layer contains many elastic as well as collagenous fibres, and blends with the surrounding connective tissue (Ortega et al 2001). The inner layer merges imperceptibly with the subperichondrial cartilage. It is associated with skeletoblasts, the pluripotential stem cells with a fibroblast-like form (Arai et al 2002). The perichondrium varies in thickness and in immature tissue the chondrogenic layer is the thicker (Horton 1993). The perichondrium contains some differentiated cells with the potential to make cartilage (Upton et al 1981). It contains glycoprotein, collagen and non-collagenous protein; of these, collagen is most

abundant in the perichondrium. The perichondrium also has higher water content than does the cartilage.

The chondrocytes of the subperichondrial zone become flattened near the perichondrium, lying tangential to the margin of the cartilage. In the perichondrium the cells are fibroblastic, lying within dense collagenous tissue. The outer layer of the perichondrium contains vascular tissue, which is found within the fibrous tissue, and water lies between the vessels and the cartilage, therefore nutrients can pass through the perichondrium to the subperichondrial cartilage (Stockwell 1979, Horton 1993).

1.1.8.1.4. Cartilage canals

Vascular canals known as cartilage canals pass through the hyaline cartilage of the epiphysis and small short bones before ossification. They are found in permanent cartilages, such as the costal and laryngeal cartilages, into adult life (Gruber et al 1990). Chondro-epiphyses can bleed from these canals (Gelberman and Mortensen 1983). The number of canals in the cartilage and small bones varies. There are species-specific patterns of canal entry and ramification within a single epiphysis. Canals do not penetrate the articular surface and they do not usually enter from areas of attachment of ligaments and tendons. The canals do not, at any time, enter the region of the presumptive articular cartilage. Each canal contains a few capillaries at its blind end. They are supplied and drained by an arteriole and a venule respectively, which run through the centre of the trunk of the canal from the perichondrium (Delgado-Baeze et al 1991). The connective tissue of the canals contains chondroclasts, chondroblasts or osteoprogenitor cells (Kugler et al 1979), polymorphic cells (Stockwell 1979), fibroblasts and macrophages (Wilsman and Van Sickle 1972), multivacuolated cells, and perivascular cells (Cole and Wezeman 1987). Occasional lymphatics and unmyelinated nerves are also present.

The main function of cartilage canals is to provide nutrition, by diffusion, to epiphyseal chondrocytes and, at a later time, they participate in forming the secondary ossification centres.

The most commonly cited hypotheses for the origins of the canals are:

1-Angiogenic differentiation (Roach et al 1998)

2-Chondrolytic activity of mesenchymal cells (Chappard et al 1986)

3-Extension of the perichondrium (Shapiro 1998).

1.1.8.1.5. Cartilage nutrition

The mechanism of transfer of nutrient fluid in cartilage is passive diffusion through the matrix, and active transport by the cells. Cartilage has a low permeability and diffusion rates vary with the molecular size of the solute. Negative ionic charges of the GAGs in

matrix means it is readily permeable to cationic dyes, but resists the passage of anionic dyes. The diffusion was found to be decreased with larger sized molecules (Torzilli et al 1987).

Cartilage matrix has maximum water content of about 80-85%. Since rates of diffusion depend on the water content of tissue, the diffusion of solutes in cartilage must always be less than those in pure water. Even with small molecules their diffusion in cartilage is less than in water. Large molecules have much slower diffusion in water than smaller molecules and in cartilage it is even further diminished as a result of resistance between them and the matrix macromolecules. Molecular size and shape of the solute, not molecular weight, are the important factors for diffusion. In addition, the ease of movement of solutes into cartilage is dependent on their interaction with the matrix macromolecules.

Variations in PG and collagen content of the matrix have little effect unless they materially alter the free water content of the tissue or the complex diffusion pathway. Another mechanism which cartilage might enhance nourishment has been thought to be by mass transfer of fluid through the matrix, resulting from mechanical compression on loading, followed by re-expansion at rest (Stockwell 1979).

1.1.8.1.6. Articular cartilage nutrition

The cartilage in the central part of the articular cartilage can effectively obtain nutrients from only two sources:

1-The synovial fluid via the articular surface.

2-The blood in the subchondral bone marrow.

There is a considerable nutritional supply to immature cartilage from the epiphyseal marrow spaces, and normal adult joint articular cartilage derives its nourishment mainly via the articular surface. However, in large joints with thick cartilage it is possible that the deepest tissue might obtain nutrition from the marrow spaces. It is likely, however, that diffusion from marrow spaces would be arrested at the deeper calcified layer of articular cartilage; therefore the synovial fluid route is probably the predominant mechanism of nutrition of articular cartilage. In avascular necrosis of the femoral head the cartilage survives, although the subjacent bone dies. This suggests that they are fed by different routes (Stockwell 1979). However, Malinin and Quellette (2000) claimed that interruption of contact between articular cartilage and vascularised subchondral bone resulted in degeneration of the cartilage.

Ysart and Mason (1994) stated that cartilage has a clear requirement for oxygen. However, in low oxygen tension, chondrocytes follow anaerobic glycolysis and increase

lactic production, while at physiological oxygen tension they follow a combination of aerobic and glycolytic metabolism (Lane et al 1977). However, Lee and Urban (1997) claimed that anoxia suppressed glycolysis.

1.1.8.2. Growth plate

1.1.8.2.1. Development of the growth plate

Mesenchymal cells differentiate during the sixth week of human embryonic development. Then they condense and differentiate into chondrocytes, which form a cartilaginous model of the future skeleton. Chondrocyte hypertrophy starts in the central portion of the cartilaginous anlage, where the matrix begins to calcify. The periosteal sleeve of bone is formed around the periphery of the anlage during the seventh embryonic week. The bone is formed directly in a collagenous matrix by osteoblasts and is termed intramembranous bone formation (Brighton 1987). The transition from embryonic to fetal stages, at the end of the eighth week, is signalled by the invasion of capillary buds into the central portion of the hypertrophied and calcified cartilaginous anlage. Mesenchymal cells are brought to the cartilaginous anlage by vascular invasion and differentiate into osteoblasts and osteoclasts. The osteoblasts form an osteoid matrix on cartilage cores producing the primary trabeculae, a process named endochondral bone formation. The osteoclasts remove bone to form a medullary canal. This process takes place at either end of the long bone. Thereafter longitudinal growth at the bone occurs by appositional growth of cells from the metaphyseal side of each growth plate. This process continues until closure of the growth plates at skeletal maturity (Iannotti 1990).

1.1.8.2.2. Structure of the growth plate

The growth plate, or physis, is a complex unit of cells that appears to vary from species to species and even among different bones within the same species. This variability becomes important in understanding specific diseases of bone growth. Variations are also present in physiological and biochemical activities. Being the basic system of endochondral ossification the physis is responsible for growth of all components of the axial and appendicular skeleton and has a characteristic and virtually unchanging cytoarchitectural structure from early fetal life until the slowdown phase of growth immediately preceding skeletal maturity (Morsher 1968). The major differences between the various growth plates may be found in the amount of cells in each zone, the overall heights of the physes and cellular modifications (Ogden and Rosenberg 1988).

The growth plate is an important structure that is divided anatomically into different components according to the three constituent tissues. These are the cartilaginous zone, the bony component or metaphysis, and the fibrous component.

The epiphyseal disc or cartilaginous growth plate lies between the epiphysis and the shaft of a long bone. The cartilaginous component is the most important part of the growth plate. It begins at the top of the reserve zone and ends with the last intact transverse septum at the bottom of each cell column in the hypertrophic zone. There are three different zones: the reserve zone, the proliferative zone and the hypertrophic zone (Brighton 1987).

The reserve zone

Several different names have been applied to this zone including the resting zone, the zone of small-sized cartilage cells and the germinal cells. However, these cells are not resting and are not small in comparison with the cells in the proliferative zone and their function is not clear (Hunziker 1994).

The cells in the zone, with a fibroblastic phenotype, are spherical in outline, exist singly or in pairs and have a low mitotic rate (Loveridge and Noble 1994). They are few in number, compared with the number of cells in other zones and are separated from each other by more extracellular matrix than cells in any other zone. The cell size is approximately the same as that of the cells in the proliferative zone. These chondrocytes contain abundant endoplasmic reticulum, and they actively synthesise protein.

The matrix in the reserve zone contains fewer lipids, GAGs, proteins, and polysaccharides than the matrix in any other zone. It contains the highest content of collagen of any zone in the plate (Irving and Wurthier 1968). Collagen fibrils in the matrix show random distribution and orientation. Matrix vesicles are also seen, but they are fewer in number than in other zones. The matrix shows a positive histochemical reaction for the presence of a neutral polysaccharide or an aggregated proteoglycan (Farnum and Wilsman 1986).

Oxygen tension measurement shows that pO_2 is low in the reserve zone. This must mean that the blood vessels pass through this zone without actually supplying it. The function of this zone is not clear. However, the high lipid and vacuole content may indicate the storage of these materials for later nutritional requirements, and, in this sense, the function of these materials is possibly one of storage (Brighton 1987). In addition, manipulation of the resting zone showed that it could regenerate the other zones (Abad et al 2002). However, it does not take part actively in longitudinal growth either through cell proliferation, matrix synthesis or calcification. Although most growth plate abnormalities affect the reserve zone, there is no known disease state that primarily affects this zone (Iannotti 1990).

Proliferative zone

The cells are flattened and aligned in longitudinal columns, in line with the long axis of the bone. The cells are filled with endoplasmic reticulum. The percentage of the cytoplasmic area occupied by endoplasmic reticulum increases from the top to the bottom of the zone.

The chondrocytes of this zone are, with few exceptions, the only cells in the cartilaginous portion of the growth plate that have divided. The top cell of each column is the true mother cartilage cell for each column, and that is the true germinal layer of the growth plate (Brighton 1987).

Longitudinal growth in the growth plate is equal to the rate of production of new chondrocytes at the top of the proliferative zone multiplied by the maximum size of the chondrocytes at the bottom of the hypertrophic zone (Hunziker et al 1987).

The matrix of the proliferative zone contains collagen fibrils, distributed at random, and matrix vesicles, limited mostly to the longitudinal septa. The matrix shows a positive histochemical reaction for a neutral polysaccharide or an aggregated PG (Iannotti 1990). Biochemical analysis shows that this zone contains the largest amount of hexosamine. Dermatan sulphate PG is synthesised and secreted into the extracellular matrix surrounding proliferative chondrocytes, but it is absent in other zones (Kielty et al 1985). The cells synthesise and secrete macromolecules such as types II and IX collagen. The highest content of PG in the matrix occurs in the proliferative zone. PGs combine with large amounts of calcium in the highly negatively charged matrix, therefore they protect the proliferative zone from undergoing calcification. Pyrophosphate, present in its highest amount in the proliferative zone, is another inhibitor of calcification (Brighton 1987).

Oxygen tension is higher in the proliferative zone than in any of the other zones of growth plates. This is due to a rich vascular supply present at the top of the zone.

The functions of this zone are matrix production and cellular proliferation. The combination of these two functions equals linear or longitudinal growth (Jaramillo and Shapiro 1998). These functions are responsible for the increase in linear growth of the long bone, but the cartilaginous portion of the growth plate does not itself increase in length (Brighton 1987).

It is obvious that any severe abnormality in chondrocyte proliferation in this zone will affect matrix production. These changes could be quantitative or qualitative (Ingalls 1933).

Hypertrophic zone

The flattened chondrocytes of the proliferative zone become spherical and greatly enlarged in the hypertrophic zone. The average chondrocyte has enlarged some five times compared to its size in the proliferative zone by the time it reaches the bottom of the hypertrophic zone. The hypertrophic cell achieves a similar cell volume in the proliferative phase in one tenth of the time needed for proliferation (Hunziker et al 1987). This zone contains three different portions: the zone of maturation, the zone of degeneration, and the zone of provisional calcification (Cowell et al 1987).

The cytoplasm of the chondrocytes in the top half of this zone (the zone of maturation) stains positively for glycogen, but near the middle of the zone (the zone of degeneration) the cytoplasm suddenly loses all staining for glycogen (Brighton 1987). The cells vacuolate and towards the bottom of the zone such vacuolation becomes conspicuous, then nuclear fragmentation occurs, and matrix vesicles are released to initiate the mineralisation process. The final destiny of the hypertrophic chondrocytes is considered by many to be death, as shown by the empty lacuna at the bottom of each column (Iannotti 1990).

The highest concentration of calcium and phosphorus is found in the chondrocytes of the hypertrophic zone. Histochemical localisation of calcium shows the mitochondria of chondrocytes in the top half of the hypertrophic zone to be filled with calcium. This mitochondrial calcium may be involved in cartilage calcification (Matthews et al 1970). The cells of this zone reduce their PG secretion, and the matrix also contains lesser amounts of PG link protein compared to the reserve zone. Collagen type I has been found in the cytoplasm of the cells in the hypertrophic zone (Kielty et al 1985) but not in the matrix. The matrix of this zone only contains type X collagen. This seems to play an important role in endochondral ossification. Collagen types II and I have not been found abundantly in the matrix of the hypertrophic zone supporting the view that collagen type X is the major collagen of the matrix in this zone (Horton and Machado 1988).

This zone is metabolically active (Iannotti 1990). It has the highest content of alkaline phosphatase, acid phosphatase and glucose-6-dehydrogenase (Hunziker 1988). It contains the lowest amount of hydroxyproline and hexosamine. During the proliferative phase, the volume of matrix production roughly equals that of the mean proliferative cell volume, but during hypertrophy, this matrix synthetic activity is about three times higher (Hunziker 1988). The degradation of the PG in the hypertrophic zone may allow the process of calcification since PGs will no longer trap calcium (Kawabe et al 1986). However, this view is contradicted by the study of Poole et al (1982), which suggests that PG degradation does not occur in the growth plate.

Oxygen tension in the hypertrophic zone is quite low, which is due to the avascularity of the zone.

The zone of provisional calcification is the transitional region between bone and cartilage. In this zone the osteoclasts digest away the cartilage and osteoblasts make new bone on the formed trabeculae. The adjacent cartilage matrix becomes progressively mineralised, forming the dense, but fragile, bone of the metaphysis spongiosa (Jaramillo and Shapiro 1998).

The functions of the hypertrophic zone seem clear - preparation for and calcification of the matrix (Ogden and Rosenberg 1988, Loveridge and Noble 1994). Abnormalities in this zone may be discussed in terms of the functions of that zone. The mass of cartilage in the metaphysis, the failure of the hypertrophic cells to degenerate, and the irregularity of calcification of the cartilage matrix, all disrupt the normal sequences of endochondral bone formation (Gram et al 1959).

1.1.8.2.3. Vascular supply of the cartilaginous part of growth plate

Small arterial branches pass through small cartilage canals in the reserve zone to terminate at the proliferative zone. The small arterial branches supply the top portion of the cell columns. The proliferative zone is well supplied with blood vessels. None of the arterial branches penetrates beyond the uppermost part of the proliferative zone, and no vessels pass through the proliferative zone to supply the hypertrophic zone. Low oxygen tension in the reserve zone and hypertrophic zone could be explained by their lack of vascular supply (Brighton 1987).

1.1.8.2.4. Regulation of growth in cartilaginous growth plate

A number of systemic and local factors may affect the rate of longitudinal growth. They exert their influence at various points in the developmental cascade from a resting, slowly proliferating phase, through rapid proliferation and finally through differentiation to the fully mature hypertrophic chondrocyte.

Growth hormone is considered the main regulator of post-natal endochondral bone growth. Growth hormone, insulin-like growth factor and fibroblast growth factor stimulate chondrocytes to proliferation and to matrix proteoglycan synthesis (Rosselot et al 1994). The concept of the direct action of growth hormone on the resting chondrocytes was established by *in vitro* studies (Abad et al 2002). However, other growth factors must be present for a direct effect of growth hormone on growth plate chondrocytes (Rosselot et al 1994). The proliferating chondrocytes have more receptors for IGF-1 than cells from other areas of the growth plate. Growth factors are abundant in the growth plate and they induce a differentiated phenotype of growth plate chondrocytes

(Richardson et al 2003). Other circulating factors such as oestradiol and thyroid hormones are known to affect longitudinal growth (Loveridge and Noble 1994).

1.1.8.2.5. Extracellular matrix alteration in cartilaginous growth plate

The growth plate chondrocytes change their surrounding extracellular matrix by starting to synthesise macromolecules needed for bone formation and not normally found in hyaline cartilage; they also show augmented or diminished synthesis, degradation and, possibly, modified processing of other elements of cartilage matrix (Hunziker et al 1987).

Histochemical studies have identified the extracellular matrix compartments (Poole et al 1984, Horton and Machado 1988, Buckwalter and Mankin 1998). In the pericellular matrix compartment, the matrix encloses chondrocytes closely, contains GAG as proteoglycan, but lacks collagen type II. Type II collagen was detected in the reserve zone, in its territorial matrix (which is the rim of matrix around the pericellular matrix). Various GAG proteoglycans have been found in the interterritorial matrix, which is that part of the matrix that fills the space between the territorial matrices. Territorial matrix and interterritorial matrix were spatially related to the chondrocyte clusters rather than individual cells. Cells within a cluster share a common territorial matrix and the interterritorial matrix corresponds to the septa separating the cluster.

The chondrocytes and extracellular matrix of growth plate cartilage express the GAG proteoglycan and proteoglycan link protein; however, they do not do so uniformly. In the reserve zone all the GAG proteoglycan tends to localise to the pericellular matrix more than in territorial and interterritorial matrices (Horton and Machado 1988). Chondroitin sulphate proteoglycan was more concentrated in the territorial matrix, as compared with the interterritorial matrix (Stockwell and Scott 1967). The proliferative zone also shows GAG proteoglycan, except that the pericellular and territorial matrices show a lesser amount of PG link protein, and territorial matrix often contains a lower concentration of keratan sulphate proteoglycan.

In the hypertrophic zone, the GAG proteoglycan was found uniformly, but PG link protein was localised primarily to the interterritorial matrix in the central regions of the septa.

In the hypertrophic zone, the concentration of the PG determinant was moderately diminished. Collagen type II was detected throughout the territorial matrix and interterritorial matrix of all areas. However, territorial and interterritorial matrices of the proliferative and the hypertrophic zones show lesser amounts of collagen type II than does the matrix of the reserve zone. Less type II collagen was found in the territorial matrix than in the interterritorial matrix (Irving and Wurthier 1968).

Collagen type I was detected only in the cytoplasm of mature and degenerative chondrocytes (Sasano et al 1992). PG was weakly detected in the cytoplasm of chondrocytes and the pericellular matrix of the reserve zone. However, it was detected more strongly in the cell cytoplasm and in the pericellular matrix of the proliferative and hypertrophic zones.

The variation in the arrangement and organisation of the extracellular matrix at different zones of the human growth plate did not correlate with age (Horton and Machado 1988).

1.1.8.3. Fibrocartilage

Fibrocartilage has been considered as a tissue intermediate between hyaline cartilage and dense fibrous tissue (Gardner 1992a). It is closer in structure to tendon and ligament than hyaline cartilage. However, it differs from the aponeurosis and joint capsules in composition and in its dependence on synovial tissue. It joins always with adjacent dense fibrous tissue or hyaline cartilage, so it is never found alone (Heinegard and Oldberg 1989). Fibrocartilage is exemplified by the intraarticular disc and the menisci (Junqueira and Carleiro 1980).

Fibrocartilage consists of dense, avascular fibrous connective tissue, which contains many collagen fibres, and a small number of fibroblasts and fibrocytes. It contains chondrocytes, which are round to ovoid in shape and located in rows between dense fibrous bundles (Wilson and Gardner 1984). The fine structure of the chondrocytes in fibrocartilage indicates that they have probably differentiated from fibroblasts into chondrocytes (Berkovitz and Pacy 2000). Fibrocartilage has a very high content of collagen, which is about 80% of dry weight. It may contain both types I and II collagen, but mainly type I (Boyde and Jones 1983). Collagen fibrils are organised into collagen fibres and fibre bundles. Thin, non-banded fibres surround the rows of cells forming a thick zone. Thick interwoven collagen fibres with a characteristic binding pattern occupy the greatest part of the extracellular space. The amount of matrix present between the collagen fibres is limited, so it does not stain metachromatically (Wilson and Gardner 1984). The surface of the fibrocartilage is irregular, and a rough meshwork of interlacing fibres is superimposed on this irregularity.

Fibrocartilage contains no definite perichondrium, blood vessels, lymphatics or nerve endings. Because of the low oxygen tension it can resist ischaemia (Horton 1993). In the human, any repair in cartilage that forms in osteoarthrosis is usually fibrocartilaginous rather than of truly hyaline cartilage (Stockwell 1979).

1.1.8.4. Elastic cartilage

Elastic cartilage has many of the characteristics of hyaline cartilage, but it differs from it by the presence of elastic fibres, which comprise about 20 percent of the dry weight of the tissue. These elastic fibres are extremely dense and the delicate fibrils seen in hyaline cartilage are rarely seen in elastic cartilage (Sheldon 1983).

In the matrix of the elastic cartilage a network of highly woven fibrils is found, which form a trabeculum or honeycomb-like net in the intercellular space (Kostovic-Knezevic et al 1981). Mature elastic fibres are composed of two morphologically distinct components, amorphous elastin and microfibrils. Elastic fibres are thinner than the collagen fibrils in hyaline cartilage and join end-to-end and, sometimes, side-to-side, to form a tightly woven mesh (Uitto et al 1991). The distribution of these thin fibres suggests that the matrix is composed of abundant proteoglycans. The major collagen type in elastic cartilage is type II collagen (Eyer and Muir 1975). In contrast to the uniform diameter exhibited by collagen fibres, elastin fibres appeared in irregular shapes, and are variable in diameter and length (Mecham and Heuser 1990).

The shape of the cells in elastic cartilage is round to ovoid and they may contain two nuclei (Gardner 1992a). They are similar in shape, size and frequency to the cells of hyaline cartilage, and lie singly or in pairs in chondrons. They are restricted by a permeable, basket-like microcapsule of collagen (Eyer and Muir 1975). They may contain fat droplets and glycogen aggregates. The ultrastructure of the cells of elastic cartilage is similar to that of other chondrocytes (Heinegard and Oldberg 1989).

Section 2

1.2. Classification of joints

1.2.1. Introduction

The functional classification of joints considers the amount of movement permitted by the joint, a property known as the range of motion. This functional category is further subdivided on the basis of the anatomical structure of the joint or the range of motion permitted (Martini 2001).

Joints are divided into three groups according to this classification. A synarthrosis is an immovable joint, which can be fibrous or cartilaginous. Over time, the two bones may fuse in this joint. An amphiarthrosis is a slightly movable joint, which is fibrous or cartilaginous. A diarthrosis is freely movable joint, which is a synovial joint.

1.2.1.1. Synarthrosis

Synarthrosis is a strong joint where the bony edges are kept close together and may even be linked, because movement between these bones must be prevented. There are four major types of synarthrotic joints. Suture, the first type of synarthrosis, has dense connective tissue interlocking the bony edges and holding them together. The joints located between the bones of the skull are suture joints. Gomphosis is a second type of synarthrosis, which binds the teeth to their bony sockets in the maxillary bone and mandible, having a periodontal ligament acting as fibrous tissue to connect them. Synchondrosis, the third type of synarthrosis, is a rigid, cartilaginous bridge between two articulating bones. The epiphyseal cartilage is a synchondrosis, even though the two bones are part of one skeletal element. The costochondral junction is another example of synchondrosis. A fourth and last type of synarthrosis is synostosis, which is a totally rigid, immovable joint created after two separate bones fuse and the edges between them disappear. The metopic suture of the frontal bone and the epiphyseal lines of mature bones are synostoses (Williams et al 1989).

1.2.1.2. Amphiarthrosis

Amphiarthrosis permits more movement than synarthrosis, but is not a freely movable joint. Collagen fibres or cartilage connect the articulating bones at the amphiarthrosis. There are two major types of amphiarthrotic joint. At a syndesmosis, the first type, bones are connected by a ligament. The distal articulation between the tibia and fibula is an example of syndesmosis. At a symphysis, the second type of amphiarthrosis, the articulating bones are separated by a block or pad of fibrocartilage. The articulation

between the bodies of vertebrae and the connection between the two pubic bones are examples of this type of joint (Martini 2001).

1.2.1.3. Diarthrosis

Diarthroses, or synovial joints, permit a wider range of motion than other types of joints. A synovial membrane lines the articular cavity, and synovial fluid fills it. An articular capsule surrounds the synovial joint. These joints are typically located at the ends of long bones (Stockwell 1981).

Synovial joints are divided into gliding, hinge, pivot, ellipsoidal, saddle, and ball and socket joints, on the basis of the shapes of their articulating surfaces. Gliding, or planar, joints have flattened or slightly curved faces. The amount of movement is very slight, but the articular surfaces slide across one another. Rotation is possible, but ligaments usually prevent or greatly limit such movement. Gliding joints are monoaxial or multiaxial. These joints are located at the ends of the clavicle, between the cartilages of the ribs and the sternum, and between the articular faces of adjacent vertebrae (Williams et al 1989).

Hinge joints permit angular motion in a single level. Hinge joints are monoaxial joints. The elbow, knee, ankle and interphalangeal joints of the appendicular skeleton are example of hinge joints (Martini 2001).

Pivot joints only permit rotation and they are also monoaxial. The articulations between the atlas and axis, and the head of the radius and shaft of the ulna are pivot joints (Williams et al 1989).

The ellipsoidal, or condyloid, joint has an oval articular face with a depression in the opposing surface, when it is at rest. In ellipsoidal joints, angular motion occurs in two planes, along or across the long axis of the oval, so making it a biaxial joint. Such a joint connects the radius with the proximal carpal bones, the phalanges of the fingers with the metacarpal bones, and the phalanges of the toes with the metatarsal bones (Williams et al 1989).

Saddle, or sellaris, joints have saddle-shaped articular faces, that is, the faces are curved to be concave in two opposing directions. Thus each face is concave on one axis and convex on the other, and the opposing faces nest together. These joints permit angular motion, including circumduction, but prevent rotation. They are usually considered to be biaxial. The carpometacarpal joint at the base of the thumb is the best example of a saddle joint (Stockwell 1981).

In a ball and socket joint, the round head of one bone rests within a cup-shaped depression in another. All combinations of angular and rotational movement can be

performed at this joint. This is a triaxial joint. Examples include the shoulder and hip joints (Williams et al 1989).

1.2.2. Synovial membrane

The synovial membrane, covering the internal surfaces of the synovial joint, has an intimal lining of synovial cells resting on a subsynovial layer of less cellular connective tissue. Between the intimal cells are a moderate dense amorphous material and a few collagen fibres. The synovial membrane is highly vascular and its capillary network lies in contact with the basal (outer) surface. The blood plasma is separated from the joint cavity only by endothelium and a variable thickness of extracellular matrix (Stockwell 1979).

The synovial membrane secretes synovial fluid, which is a thick, viscous solution. The synovial fluid within a joint has several important functions (Ettlinger and Hunder 1979). These consist of lubrication, distribution of nutrients and shock absorption. Lubrication reduces friction between moving surfaces. For distribution of nutrients, the fluid may flow into and out of the cartilage matrix and will also remove waste material. With shock absorption, it reduces the shock by distributing it evenly across the articular surface (Martini 2001).

1.2.3. Sternoclavicular joint

1.2.3.1. Anatomy

1.2.3.1.1. Sternum

The sternum, or breast bone, is subcutaneous and palpable. It forms the anterior aspect of the rib cage in the midline of the chest. The sternum has articulations with the upper seven ribs and the clavicle each side. The human sternum consists of a manubrium, a body and xiphoid process. The manubrium, the most superior part of the sternum, is also called the prosternum. It is the thickest and strongest part of the sternum. It forms the only connection between the shoulder girdle and the axial skeleton (Williams et al 1989). The large notch in its superior border is known as the jugular or suprasternal notch: to both sides of this notch are other notches, called "clavicular facets", for articulation with the clavicles. These facets are concave mediolaterally and slightly convex from anterior to posterior. This shape, with the help of a fibrocartilaginous disc, allows the clavicle to rotate when the arm is abducted, adducted, flexed or extended. The first rib joins with the manubrium below the clavicular facets, on the upper part of its lateral margins. The costal cartilage of the second rib joins the manubrium at its junction with the body of the sternum and articulates with both parts. The articulation between the manubrium and the

body make the sternal angle, which is a very important surface landmark for identifying the level of the second rib.

The body of the sternum, the intermediate part, is called the mesosternum or gladiolus. It is made up of four separate parts, or sternebrae. These are completely fused in adult humans. The third, fourth, fifth and sixth pairs of ribs articulate directly with the sternum. The seventh pair articulates at the junction between the body and the xiphoid process. The sternum at the fourth month of intra-uterine life is formed of hyaline cartilage. Ossification centres appear in the manubrium and the body and the manubriosternal joint is, in first instance, a synchondrosis. The hyaline cartilage becomes fibrocartilage at a later date and, therefore, the definitive form of the joint between them is a symphysis (Stockwell 1981).

A xiphoid process, the most inferior part of the sternum, is also called the metasternum. The xiphoid is highly variable in both length and shape. Its lower end is normally divided in two parts. It is set on a deeper level than the sternal body, and its lower tip frequently projects forward. It fuses with the sternal body in middle life (Williams et al 1989).

The total length of the sternum in males is greater than in females, and its sits lower in females than it does in males (Hatfield et al 1984).

1.2.3.1.2. Clavicle

The clavicles, or collar bones, extend laterally and almost horizontally across the neck from the manubrium of the sternum to the acromion process of the scapula. They are wholly subcutaneous and can be palpable beneath the skin. They support the shoulders and enable the limbs to swing clear of the trunk, transmitting part of the weight of the limbs to the axial skeleton (Stockwell 1981). The acromial end, which is the flat lateral part of each clavicle, articulates with the medial aspect of the acromion. The sternal end, the enlarged medial part of each clavicle, articulates with the clavicular facets of the manubrium, and the first costal cartilage. Just lateral to each of these articular surfaces is a roughened area that marks the attachment of the costoclavicular ligament. This ligament binds the clavicle to the underlying costal cartilage and therefore, acts to stabilise the sternoclavicular joints.

The shaft of each clavicle is twisted, being convex forward in its medial two-thirds, and concave lateral to this (Moseley 1968). The clavicles are sites for muscle attachments. The forward convexity of each medial part leaves room for the large vessels and nerves that cross the upper surfaces of the first ribs to pass behind and below the

clavicles on their way to the limbs (Flatow 1993). The feature may be changed according to muscular development or position of the body.

1.2.3.1.3. Sternoclavicular joint

The sternoclavicular joint (SCJ) is a true diarthrodial joint, which is a synovial articulation (Stockwell 1981). It is formed by an inferior portion of the medial end of the clavicle, which articulates with the clavicular facet of the manubrium of the sternum and the cartilage of the first rib (Yood and Goldenberg 1980). The enlarged medial end of the clavicle is concave from front to back and it is much larger than the clavicular facet of the sternum. Because of the small area of contact between the two articulating surfaces and their congruity, the joint is potentially unstable (Cope et al 1991). However, an intraarticular disc partly compensates for this instability (De Palma 1959). In addition, a joint capsule encloses the entire joint and it is reinforced by ligaments on the anterior, posterior, superior, and inferior sides (Pratt 1994). These ligamental attachments stabilise the joint, and resist the tendency for medial displacement of the clavicle (Peat 1986).

The anterior and posterior sternoclavicular ligaments reinforce the articular capsule anteriorly and posteriorly. Both ligaments pass downward and medially from the sternal end of the clavicle to the anterior and posterior surfaces of the manubrium of the sternum (Williams et al 1989). The posterior sternoclavicular ligament is stronger and functionally more important (Dennis et al 2000). The posterior sternoclavicular ligament becomes taut during protraction, and the anterior sternoclavicular ligament is lax. During retraction the anterior part becomes taut and the posterior part is lax. These ligaments are the major resistance to upward displacement of the medial end of the clavicle and are the most important for preventing inferior displacement of the lateral end of the clavicle (Spencer et al 2002).

The interclavicular ligament runs across the superior aspect of the SCJ, joining the medial ends of the clavicles (Williams et al 1989). Therefore, it may help to prevent lateral displacement of each clavicle. It becomes taut when the arm is elevated or shoulder protracted. It probably has only a minor effect on SCJ stability (Peat 1986).

The costoclavicular ligament extends from the cartilage of the first rib to the clavicle. It is a short, flat, strong, rhomboid-shaped ligament, which has anterior and posterior sheets separated by a bursa. The costoclavicular ligament is a major stabilising structure for the SCJ and strongly binds the clavicle to the first rib (De Palma 1959). This ligament becomes taut when the arm is elevated or the shoulder protracted. This ligament is very important for stability of the SCJ (Yood and Goldenberg 1980) (Figure 1.4.).

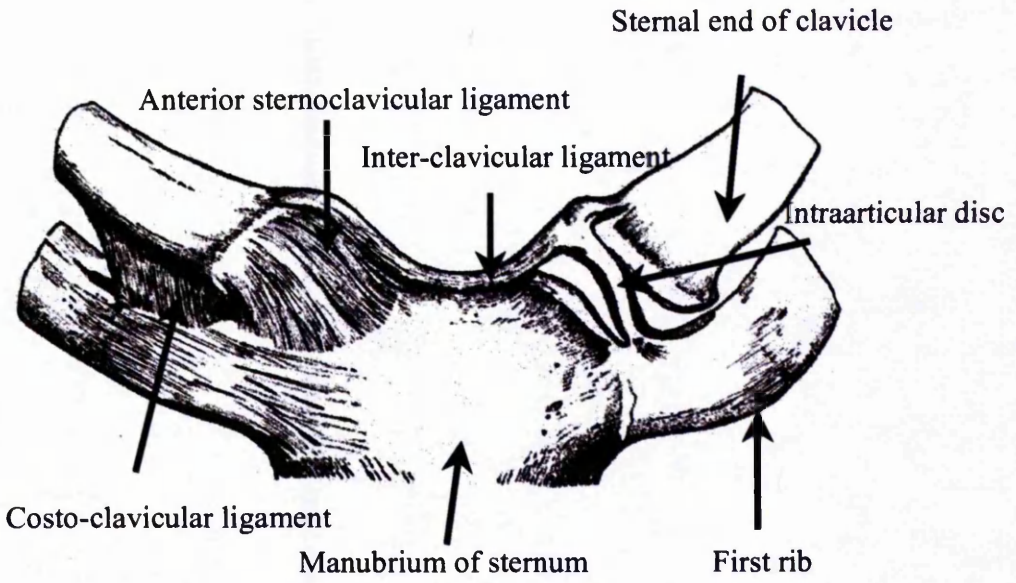


Figure 1.4. Sternoclavicular joint viewed from the anterior
(Origin of Figure: Gray's Anatomy).

1.2.3.1.4. Intraarticular disc

The intraarticular disc typically is thick, strong and nearly circular. It usually divides the joint cavity into two separate joint spaces (De Palma 1959). It attaches superiorly to the upper medial end of the clavicle and passes downward between the articular surfaces, and joins to the first costal cartilage and the manubrium of the sternum. This configuration permits the disc and its attachment to act as a hinge, which supplies the total range of joint movement and enables the bones to move freely (Pratt 1994). This method of disc attachment also stabilises the joint against forces from the shoulder, transmitted through the clavicle to the axial skeleton. Without this attachment these forces would tend to cause medial dislocation of the clavicle. This ability to resist forces is augmented by its thickness, which also contributes to its function as a shock absorber (Peat 1986, Pratt 1994).

1.2.3.2. Function of the SCJ

The SCJ is the only synovial articulation between the upper extremity and the trunk. The movement occurring at the SCJ somewhat resembles that of a ball-and-socket joint (Peat 1986). However, it is a gliding diarthrodial joint (Martini 2001). The amounts of motion are relatively small, and the movement is magnified at the lateral end of each clavicle. This is essential for normal mobility of the shoulder complex (Pratt 1994).

The area of compression between the articular surface and the intraarticular disc varies with movement of the clavicle (De Palma 1959). When the clavicle moves in one direction during elevation, depression, protraction, or retraction the ligaments on the side of the motion become lax. Those on the opposite side of the joint become taut and limit the movement. This causes the compression of the clavicular and sternal articular cartilages and the intraarticular disc.

During elevation and depression of the clavicle, most motion occurs between the clavicle and the disc. During protraction and retraction, the greatest movement occurs between the disc and sternal articular surface. The combination of taut ligaments and pressure on the disc and articular surfaces are important in maintaining stability in the plane of motion. Forces acting on the clavicle from the upper limb rarely cause dislocation of the SCJ. Excessive forces applied to the clavicle are most likely to cause a fracture of the clavicle, near to the attachment of the coracoclavicular ligament.

The clavicle can be elevated, depressed, moved anteriorly (protracted), moved posteriorly (retracted) and rotated (Peat 1986, Pratt 1994), and motion at the SCJ accompanies every movement of the arm. Therefore this joint is one of the most frequently used joints and can be involved in localised or systemic disease (Yood and Goldenberg 1980).

Section 3

1.3. Skeletal dysplasias

1.3.1. Introduction

The human skeletal dysplasias, also known as "bone dysplasias", "osteochondrodysplasias" or "chondrodysplasias", are a heterogeneous group of heritable connective tissue disorders, which are associated with abnormalities in the size and shape of the limbs, trunk and/or skull, that frequently cause disproportionate short stature (Sillence et al 1979).

Individual skeletal dysplasias are rare, but as a group they are common and have a significant effect on morbidity and mortality at all ages (Azouz et al 1998). The skeletal dysplasias are caused by defects in endochondral ossification, which is normally responsible for the embryonic development and subsequent linear growth of most of the skeleton (Horton 1982).

Skeletal dysplasias occur in all vertebrates, and well over 150 different forms have been described in man (Horton and Hecht 1993). They are characterised clinically by skeletal deformities, which result from abnormalities at the growth plate or in resting cartilage (Byers 1989). The clinical consequences may range from mild shortness, without other complications, to severely reduced stature and the most severe chondrodysplasias are lethal in the uterus or immediately after birth (Sillence et al 1979). The short stature may be the most obvious, but other organs or tissues can be affected in individuals with skeletal dysplasias (Horton and Hecht 1993).

1.3.1.1. Classification of chondrodysplasias

The international classification of heritable osteochondrodysplasias defines them as developmental disorders of chondro-osseous tissue, while dysostoses are disorders of single bones.

The international classification of osteochondrodysplasias was worked out by the International Working Group on Constitutional Disease of Bone in 1991. In spite of the rapid progress of biochemistry, molecular biology and information on gene localisation and defective proteins, the working group still felt that current aetiopathological knowledge was too fragmentary to allow for a causal classification. The classification is thus based on radiodiagnostic criteria grouping morphologically similar disorders.

Radiological classification is based on the different parts of the long bones that are abnormal. Thus there are epiphyseal dysplasias and metaphyseal dysplasias, which can be divided depending on whether or not the spine is also involved (Rimoin and Lachman

1997). The list of osteochondrodysplasias has been expanded considerably, but is not complete yet.

The international classification divides the constitutional disorders of the skeleton into five major groups:

1-Osteochondrodysplasias

2-Dysostosis

3-Idiopathic osteolyses

4-Skeletal disorders associated with chromosomal aberrations

5-Primary metabolic disorders

The osteochondrodysplasias are further divided into:

1-Defects of growth tubular bones and/or spine, which are frequently referred to as chondrodysplasias

2-Disorganised development of cartilage and fibrous components of the skeleton

3-Abnormalities of density or cortical diaphyseal structure and/or metaphyseal modelling.

1.3.1.2. Pathogenesis

The chondrodysplasias are caused by gene mutations that disrupt growth or development of cartilage and/or bone, and endochondral ossification. The mutations seriously influence chondrogenesis and/or cartilage hypertrophy qualitatively or quantitatively or both (Balint and Szebenyi 2000). Many candidate genes can be identified, and they can be categorised. For instance, one group contains those genes which encode cartilage collagen chains synthesised by differentiated chondrocytes. Another contains the genes coding non-collagenous components of cartilage matrix. Cases show a quantitative reduction of proteoglycan in the matrix and qualitative changes in sulphated acid polysaccharide protein complex (Scheck et al 1978). Other groups might include genes that code for collagenous and non-collagenous matrix proteins expressed by hypertrophic chondrocytes. Other genes manage the expression of genes within a particular chondrocytic phenotype or regulate the proliferation of chondrocytes within the growth plate or their response to local growth factors (Horton 1982).

Candidate genes need not be expressed only in connection with bone development or growth. Many chondrodysplasias exhibit signs in non-skeletal tissue that cannot be explained as secondary phenomena. This suggests that the mutant genes are also expressed in other tissues (Silengo et al 1980).

1.3.1.3. Pathology of cartilage in chondrodysplasias

The explanation of the fine structural changes in chondrodysplasias is dependent on the understanding of hyaline cartilage and growth plate in normal humans. A light microscopical study showed that radiographically abnormal skeletons had identical pathological abnormalities (Rimoin 1974, Lee et al 2002)).

Pathological studies of chondro-osseous tissue have shown specific abnormalities in many of the skeletal dysplasias (Rimoin 1974, Rimoin et al 1976, Hwang et al 1979, Sillence et al 1979). Histological examination of chondro-osseous tissue may be useful in making an accurate diagnosis of the specific skeletal disorder. Because chondrocytes and matrix of different stages of maturity are found spatially separated in the growth plate, it is sometimes possible to deduce the general nature of abnormalities by histological examination of growth plate cartilage and, from this, to postulate possible affected genes (Byers 1989).

In some disorders, histopathological alterations are not present, or they are non-specific, and, in these cases, pathological examination is useful only in ruling out a diagnosis (Rimoin et al 1976).

Using histological examination the chondrodysplasias can be divided into those disorders which show no qualitative abnormalities in endochondral ossification, those with abnormalities in cellular morphology, those that have abnormalities in matrix morphology and those with abnormalities localised to the area of chondro-osseous transformation. In certain disorders, abnormalities in two or more of these areas can be seen (Rimoin and Lachman 1993).

For instance, achondroplasia, the most common type of chondrodysplasia, is characterised by well-organised, regular, endochondral ossification. Other than an increased number of dead cells, no characteristic abnormalities were found in typical achondroplasia (Rimoin 1974, Rimoin and Lachman 1997). However, a histopathological study of several cases showed abnormal areas of endochondral ossification that were identified by irregular column formation, with a short growth zone and a wide zone of randomly disposed hypertrophic chondrocytes (Sillence et al 1979).

In thanatophoric dysplasia, the most lethal type of chondrodysplasia, generalised disorganisation of endochondral ossification was seen in every growth plate. There were columns of chondrocytes only in particular areas and these were very unproductive (Hwang et al 1979, Lemyre et al 1999). In some areas there was a cartilaginous matrix of fibrous appearance directly adjoining bony spicules. Resting cartilage appeared to be normal by light microscopy (Rimoin 1974). Short-limb dwarfism, one type of autosomal

recessive disorder, is characterised by the bilateral absence of fibulae and severe abnormalities of all digits. Shortness of the limbs is much less than in other disorders and there is also hypoplasia of the distal ulna leading to bowing of the radius (Kohn et al 1989).

Achondrogenesis refers to a lethal, chondrodysplastic phenotype with a short trunk and very short extremities (Horton and Hecht 1993). The growth plate showed a severe disturbance in endochondral ossification. All zones of the growth plate were densely hypercellular. Chondrocytes of the reserve zone were slightly enlarged and vacuolated. The normal progression of chondrocytes was lost, although some increased hypertrophy appeared in cells adjacent to the metaphysis. Calcification of cartilage and trabecular formation were very disorderly, with irregular capillary penetration and little column formation (Sillence et al 1979).

Campomelic syndrome is an autosomal dominant skeletal malformation characterised by shortness and bowing of long bones, especially of the lower limbs. It is a severe skeletal dysmorphology syndrome, which is due to premature mineralisation. Despite the severe hypoplasia of cartilage, the overall organisation and cellular composition of the growth plate are otherwise normal. Additional findings are 11 pairs of ribs and bell-shaped thorax, hypoplastic scapulae, narrow iliac wings, non-mineralised thoracic pedicles, clubbed feet, typical facial anomalies and tracheomalacia. The disorder is frequently lethal due to respiratory distress. Campomelia (bowed limbs) is seen in most but not all patients, defining also-called acampomelic campomelic syndrome (Lee et al 2002).

1.3.1.4. Biochemical and molecular abnormalities

In recent years, great progress has been made in knowledge about the basic biology of collagens and PGs and the advances in analysis of the human genome have begun to inform knowledge of the underlying biochemical and molecular defects in the skeletal dysplasias (Byres 1989). Using the "candidate gene" approach and positional cloning, some specific gene defects that produce the skeletal dysplasias have been identified and may be classified into several categories. One category consists of qualitative or quantitative abnormalities in the structural proteins of cartilage. For instance, over 30 mutations have been found at the type II collagen genes (Spranger et al 1994). The general clinical phenotype is spondyloepiphyseal dysplasia. Specific phenotypes have ranged from achondrogenesis type II and hypochondrogenesis, at the severe end of the spectrum, to very mild spondyloepiphyseal dysplasia with clear osteoarthritis (Horton

1996). The majority of these patients also have electrophoretically detectable abnormalities in collagen type II in cartilage (Rimoin and Lachman 1997).

Another category consists of inborn errors of cartilage metabolism. For instance, in diastrophic dysplasia diminished sulphate transport appears to have a dramatic effect on sulphation of chondroitin sulphate-containing PGs (Superti-Furga et al 1996).

Defects in local regulators of cartilage growth form another category of biochemical and molecular abnormalities. For instance, mutations in fibroblast growth factor receptor 3 (FGFR3) are responsible for achondroplasia. Other identified abnormalities include systemic defects that influence cartilage development (Arikawa-Hirasawa et al 2001). Other disorders exist with an identified gene but an unknown pathogenesis and vice versa (Rimoin and Lachman 1993).

1.3.1.5. Radiological evaluation

After clinical evaluation of the patient, a full set of skeletal radiographs should be obtained. A full series of skeletal views, with separate views of the hands and feet is usually required. Skeletal radiographs alone will often be sufficient to make an accurate diagnosis, since the classification of skeletal dysplasias has been based primarily on radiographical criteria. The skeletal radiographical features of many of these diseases change with age, and it is usually beneficial to review radiographs taken at different ages when possible. In some disorders, the radiographical abnormalities after epiphyseal fusion are non-specific; therefore the accurate radiographical diagnosis of an adult disproportionate dwarf may be impossible unless prepubertal films are available.

Some radiographical features characterise certain disorders. For example, in achondroplasia, the acetabula of the pelvis are flat with tiny sacrosciatic notches and rather square iliac wings with round corners. Oval translucent areas in the proximal femora and humeri may be seen in infants.

In many instances an accurate diagnosis can be made by skeletal radiographs, but in other disorders further information may be required to diagnose its exact form (Rimoin and Lachman 1997).

Section 4

1.4. Osteoarthritis

1.4.1. Introduction

Osteoarthritis (OA) is a common disorder of synovial joints, affecting about 15% of the adult population in the world and causing pain and disability (Cole and Kuettner 2002). OA is the fourth most important painful condition in women, and the eighth most important in men in the developed world. It is a slowly developing joint disease that is obviously important because it is the main reason for joint replacement surgery (Dieppe 1999).

OA is not a single disease and represents a group of diseases with rather different underlying pathophysiological mechanisms. The most common complication of OA in patients is pain. However, only about half of patients with radiographical OA have symptoms. It is usual for joint pain to progress to reduction of function and to disability with the presence of deformity, instability and/or periarticular muscle weakness (Altman 1997).

1.4.1.1. Aetiology

In the past, OA was thought to be an inevitable disease of ageing as a result of the wear and tear of joints over a lifetime. This hypothesis now seems to be incorrect, as recent research into the aetiopathogenesis of this disease suggests that an autodestructive process affects cartilage and that this process is initiated by an aetiological agent. The aetiology of OA is unknown in the majority of patients (Fiske et al 1995). However, there are a few conditions that appear to be aetiologically related to OA (Altman 1997). Generally OA is classified as primary or idiopathic when there has not been any known condition identifiably associated with it. OA is defined as secondary when there are any noticeable related factors (Loughlin 2002).

OA is the most common form of arthritis, which is an important cause of disability in the functioning of the lower extremities in the elderly (Martin 1994).

Age is one of the most important risk factors for OA (Ettinger 1984, Hamerman 1993, Lohmander 1994, Petersson 1996, March 1997, Kerin et al 2002). OA is variously explained as part of a process of age-related changes or disorders (Passiu and Carcassi 1989, Poole 1999).

"Age and OA are interrelated, as people live longer, OA incidence increases, and as OA is a non-fatal condition, it is additive to the other disabilities of older people" (Hamerman 1993).

The prevalence of OA increases with age, increasing dramatically between ages 40 and 50 years (Altman 1997). However, during normal ageing without osteoarthritic changes, some changes have been found in cartilage and these will be discussed later.

Other risk factors include being overweight or obese (Bullough 1987, Altman 1997, March 1997, Kerin et al 2002). Overweight people are at a higher risk of OA of the knee than people of normal weight. A cross relationship has been showed between obesity and OA; after developing OA, the level of activity in overweight persons is decreased due to painful joints. This reduces the amount of exercise taken and so leads to a further gain in weight. Being overweight at an average age of 37 years, when OA of the knee is very uncommon, increases the risk of developing knee OA in patients when in their eighth decade (Felson 1995). However, there is no evidence of excess weight having an association with progression of hip OA (Hochberg 1996).

There is strong evidence that occupational factors predispose to OA (Lohmander 1994). Mechanical forces may be the start of the disease process, even if it is mediated by biochemical processes. Weakness of the quadriceps muscle might lead to the development of knee OA (Vertullo 2001). This suggests that any definitive answer to OA will require understanding of the biomechanics of joints as much as the biochemistry (Dieppe 1999).

Low impact exercise increases the risk factor for developing OA in abnormal or injured joints, but it does not affect normal joints (Lane 1995).

The prevalence of OA is higher in women than in men (Lohmander 1994, Petersson 1996, Altman 1997, Poole 1999). Female gender has been found to be a major factor associated with progression of hip OA, but there was no influence on the prevalence of hand OA (Hochberg 1996). Reproduction and female hormonal variability have been considered as important factors in OA (March 1997).

Another risk factor for OA is race (Lohmander 1994, Petersson 1996). There are considerable racial differences in prevalence of OA, and the distribution of the joints involved (March 1997). It may be because of congenital differences, or due to differences in risk factors. Genetic susceptibility (see section 4.1.8) (Lohmander 1994, Machtey 1997, March 1997, Fukui 2001), nutritional habits (Machtey 1997), trauma and overuse (Bullough 1987, Lohmander 1994, March 1997, Fukui 2001) are other effective risk factors in OA.

OA is known as a degenerative disease, but inflammation is involved at the initiation and during the progression of lesions of articular cartilage. OA begins, usually, with inflammatory signs which are prominent in most patients (Passiu and Carcassi 1989,

March 1997). Initial damage to the cartilage is chiefly enzymatic, which is the breakdown of the matrix by degradative enzymes.

The prevalence of OA is much higher in some joints than in others. For example, in the knee joint, OA is common - especially in obese women, but in the ankle it is rare, despite the fact that these two joints bear similar loads (Huch et al 1997). This may be caused by metabolic, biochemical or biomechanical differences between different joints (Petersson 1996, March 1997, Cole and Kuettner 2002). Chondrocytes from different articular cartilages showed different metabolic reactions. For example, chondrocytes from the ankle are less sensitive to degradative enzymes than chondrocytes from the articular cartilage of the knee. In addition, chondrocytes from knee articular cartilage express matrix metalloproteinase, which may be involved in proteoglycan degradation (Huch et al 1997).

Deposition of various crystals (Katazenstein et al 1990) in articular cartilage and deposition of amyloid (Egan et al 1982) may also be factors related to OA.

1.4.1.2. Definition

OA is defined by two major abnormal processes that lead to joint failure. The first is the structural breakdown of the hyaline cartilage and the second is the proliferation of new bone and fibrous cartilage within the joint (Ettinger 1984). In addition, OA is a polymorphic disease defined as a heterogeneous group of conditions that leads to joint symptoms and signs, which are associated with degeneration of articular cartilage and related lesions in the underlying bone and at the joint margins (Menkes 1991).

OA cartilage is a rich source of inflammatory mediators, which assist tissue injury (Fosange et al 1993). The inflammatory process increases vascular permeability and leakage, and the classical signs of inflammation, redness, swelling and heat, appear in most joint tissues. However, in OA these changes are not seen. Granulation tissue is not formed in OA, because there is no extravasation of body fluids and inflammatory cells. There are no nerves in cartilage, therefore there is no painful sensation in OA cartilage during the early stages of the disease (Attur et al 2002).

OA disease is clinically evidenced by joint pain, tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of inflammation (Mollenhauer and Erdmann 2002).

1.4.1.3. Pathogenesis

OA can be considered as the result of an imbalance between the load to which the articular cartilage is submitted and its ability to withstand mechanical stress. The balance can be altered by an increased load in the presence of a normal cartilage and/or by

structural alteration of the cartilage, which is not able to bear a normal load. Osteoarthritic disease is a result of both mechanical and biological events that uncouple the normal balance between degradation and synthesis of extracellular matrix by articular cartilage chondrocytes (Bullough 1984). Ultimately OA disease is manifested by morphological, biochemical and biomechanical changes in whole joints (Mollenhauer and Erdmann 2002). It is generally accepted that OA may result as a consequence of multiple causes. The joint has only a limited capacity to react to various insults and the osteoarthritic lesion may reflect a common endpoint.

Chondrocytes play an important role in the pathogenesis of OA (Gibson et al 2001). The biochemical events, which start the OA process, are as yet undetermined. The first known changes in the articular cartilage are synthesis of matrix and increase in its content by chondrocytes. They reinitiate synthesis of cartilage macromolecules and initiate synthesis of type IIA and III procollagens and proteolytic enzymes (Sandell and Aigner 2001). Chondrocytes in OA produce interleukin-1 (IL-1), which releases a cascade of cytokines including tumour necrosis factor-α (TNF-α), TGF-β and various prostaglandin derivatives (Goldering 2000). The cytokines stimulate chondrocytes to release lytic enzymes, including metalloproteinases (Arner and Dipasquale 1993). PGs are the first to break down, and various proteases can digest the protein core of PGs. These enzymes are located in the lysosomes of chondrocytes, or close to the lysosomes. They act at low pH in lysosomes, therefore they are probably active only inside the chondrocytes or in the pericellular area, where the pH is acid, whereas in the matrix the pH is neutral. Metalloproteinases are active at neutral pH in matrix, and they have been found in whole cartilage, matrix and chondrocytes. They are capable of disrupting the collagen network (Wu et al 2002). They are a family of molecules that includes collagenase, stromelysin and gelatinase (Dourado et al 1996). Collagen as well as PGs can be degraded enzymatically. Collagenase activity in OA is higher than in normal cartilage (Ettinger 1984, Passiu and Carcassi 1989).

At the same time, chondrocytes stop normal synthesis of matrix. At the molecular level, these events result in a diminished amount of GAG in matrix and decreased binding between GAGs and collagen type II (Hamerman 1989). These changes cause an obvious net loss of matrix proteoglycan, which leads to an initial decrease in water content. These changes result in a loss of compressibility and shock absorption. Fibrillation then starts and breakdown of the matrix occurs (March 1997). Damage to - and loss of - the collagen network finally occurs. This is part of the hypertrophic phase, which clearly causes

failure of cartilage. In this phase there are changes in the expression of the small PGs (Dourado et al 1996).

These general ideas of pathogenesis in OA emphasise the direct activation of cartilage and bone with minor involvement of the synovium. The reaction of the chondrocytes alters matrix stress, resulting in the generation of mediators by chondrocytes (Sardle and Aigner 2001). They may diffuse to synovium and stimulate synovial cells, which cause a broad range of inflammatory processes. Inflammatory factors released by the inflammatory cells can vary at different stages of the inflammatory process. Monocyte infiltration is commonly found in the synovial membrane, while immunoglobulin deposits and complement fractions can be found in the articular cartilage.

In addition, some cartilage components can behave like antigens. The most important antigens recognised are components of PGs, which are located in the central part or hyaluronan-binding portion. Three major antigenic sites have been identified in collagen (Ettinger 1984). Chondrocyte membranes also have antigenic components, which are species-specific as well as specific for cartilaginous tissue. In normal conditions, the cartilage matrix prevents the stimulation of inflammatory cells by these antigens, but following osteoarthritic cartilage destruction these antigens are released and activate both cellular and humoral immune responses. The articular cartilage then degenerates (Passiu and Carcassi 1989).

All these biochemical changes in the cartilage matrix decrease its tensile strength and resilience and impair its normal function in transmitting forces, supporting chondrocytes and protecting subchondral bone.

1.4.1.4. Pathology

OA is characterised by alteration in the structure and function of the articulation or joint. These changes occur in the joint as a whole, including the articular cartilage, underlying bone and soft tissue (Altman 1997, Dieppe 1999, Poole 1999).

Early morphological changes of osteoarthritic cartilage are represented by tiny superficial irregularities. As the disease progress, the superficial irregularities become deeper and form fissures, involving also the deeper layers of cartilage. This process, known as fibrillation, develops in relation to progressive wear and damage of articular cartilage (Gardner 1994). Tangential clefts at the cartilaginous surface and vertical clefts, which continue to the calcified zone, are seen in the advanced stages (Kerin et al 2002). The cartilage becomes thinner and more fragile at an early stage of the disease. In the more advanced stages, the cartilage can be completely destroyed and the exposed subchondral bone becomes thicker, worn and polished (Poole 1999).

In the early stages of OA, structural changes in the extracellular matrix induce chondrocyte proliferation, also called 'clonal growth'. This chondrocyte expansion is one of the changes associated with cartilage catabolism. The clonal chondrocytes 'try' to increase matrix synthesis to compensate for the losses of PG and collagen and, partially, repair the matrix by 'intrinsic repair' (Aigner and McKenna 2002, Hedbom and Hauselmann 2002). However, degradative enzymes strongly affect this synthetic capability and the repair fails. Clones of chondrocytes are seen frequently in degenerated cartilage. These clones are usually limited to areas of fibrillation or loosely textured matrix. After cell division and proliferation, the chondrocytes stay in clumps, bound together by sheets of type VI collagen. The pericellular distribution of type VI collagen increases during OA, in contrast to the loss of type IX collagen (Huber et al 2000).

The stiffness and viscosity of the chondrocytes increase in OA, but it is not clear whether these changes are part of the degenerative process or potentially an active cellular response to an altered mechanical environment (Trickey et al 2000).

One of the biochemical disorders of osteoarthritic cartilage which occurs, together with these anatomopathological changes, is an increase in the water content of the matrix. This has been shown also in cartilaginous fragments of normal thickness without fibrillation and is thus specific to an early stage of OA (Mankin 1993). The decreased amounts of PG, increase in the water content and the collagen fibre network pressure affect the normal function of the cartilage. The loss of matrix PG leads to a loss of compressive stiffness and elasticity and affects self-lubrication of cartilage. Furthermore, decreases in chondroitin sulphate:keratan sulphate ratio and increase in chondroitin 4-5sulphate:chondroitin 6-5sulphate ratios occur; this is probably due to increased GAG degradation rather than to diminished synthesis (Passiu and Carcassi 1989). PG aggregation is lessened, so smaller aggregates are found, rather than those of normal size, together with increased concentrations of non-aggregating PGs (Adam and Musilova 1979, Vasan 1980). The lack of two populations of PG, one with its hyaluronan-binding-protein region of core protein intact and the other possessing an inactive binding region, is evident in osteoarthritic cartilage (Vasan 1980). However, PG synthesis is increased until the lesions reach an advanced stage. Thus the diminution in PG content is due to their increased catabolism. The loss of PGs from articular cartilage is a hallmark of the osteoarthrotic process but it is not possible to categorically assert that the start of the pathogenetic processes of OA accompanies the alterations in cartilage PGs (Goldring 2000). In cartilage, most of the matrix fails to stain with metachromatic dyes in the later stages of OA which indicates the loss of PG (Mankin 1993). However, at this time, the

pericellular matrix shows intense metachromatic staining (Malemud 1991). Initial PG loss occurs around the chondrocytes. This may be caused by proteinases that degrade not only PGs, but other proteins also (Adam and Musilova 1979).

Collagen fibres do not show quantitative changes; at least in the early stages of OA, but qualitative alterations with increased fibre diameter and disruption of the collagen network in the superficial layers are observed by electron microscopy. The disorganisation of the superficial collagen network seems to be one of the first structural changes, before the appearance of overt osteoarthritic lesions (Panula et al 1998). In the advanced stage, turnover of collagen is faster than normal until the chondrocytes' capacity for synthesis diminishes. Chondrocytes in OA were shown to express and synthesise types I and III collagens, but these syntheses were independent of each other and were in part simultaneous with the synthesis of type II collagen (Aigner et al 1993, Lohmander 1994).

Changes in the tidemark interface occur early in the osteoarthritic cartilage. Two changes can be seen, duplication of the tidemark and irregularity in its appearance. When the disease progresses with overt fibrillation there is increasing duplication of the tidemark and as many as eight separate well-defined lines may be seen (Schunke et al 1988). In advancing OA, there is increasing disruption of this region of the joint with merging of subchondral bone and new cartilage formation. Invasion of the vascular tissue extends across the tidemark into uncalcified cartilage (Ball 1980). The end-stage of cartilaginous degeneration is characterised by damage to the whole of the tissue of the joint with uncovered bone-plate regions, microfractures in the subchondral plate and formation of subchondral bone cysts as a result of focal resorption (Ball 1986). The vascular supply of the bone marrow allows for extrinsic repair. The newly formed tissue, covering the bone-plate, is mechanically suboptimal fibrocartilage. These changes are accompanied by sclerosis of subchondral bone and development of osteophytes at the joint margins, as a response to new stress patterns. However, there is evidence that changes in the metabolism of subchondral bone may precede and determine the loss of cartilage. Cartilage and subchondral bone are regarded as a functional unit, therefore, changes in the dynamic interplay between them may determine the development of OA (Huber et al 2000).

The synovium and joint capsule show different pathological changes. Fibrosis may entrap bony or cartilaginous debris, which often induces the formation of multinucleate giant cells. Inflammatory changes such as proliferation of synovial lining cells and mild to moderate infiltration by mononuclear cells are often seen (Ettinger 1984). In the synovial membrane, monocyte infiltration is usually found (Passiu and Carcassi 1989). In

addition, the accumulation of cartilage debris in the synovium may start inflammatory reactions by synovial fibroblasts and macrophages, which maintain the destructive process. Nakashima et al (1998) found the cartilage fragments in synovium by immunohistochemical staining.

1.4.1.5. Degradation enzyme in osteoarthritis

Degradation and loss of articular cartilage are characteristic features of OA. The appearance of fibrillation, matrix depletion, cell clusters and changes in matrix composition are caused by chondrocytes. Although biomechanical factors are strongly concerned, at present it is not clear what initiates the hyperactive phenotype of osteoarthritic chondrocytes, including their ability to express degradation proteinases.

The matrix metalloproteinase (MMPs) are considered important for the chondrolytic processes that contribute to the degenerative changes in osteoarthritic cartilage. MMP1,13 (Wu et al 2002) and MMP8 (Tetlow et al 2001), as collagenase, are believed to be involved in collagen type II degradation in osteoarthritic cartilage. MMP2 (Tetlow et al 2001), MMP3 (Ishiguro et al 1999), MMP7 (Fosang et al 1993) and MMP9 (Fosang et al 1992) are probably involved in proteoglycans degradation. Many factors are implicated in the complex regulation of MMP production by chondrocytes, including interleukin1 (IL-1), transforming growth factor-beta (TGF-beta) (Pujol et al 1991, Lafeber et al 1997) and tumour necrosis factor (TNF) (Tetlow et al 2001). IL-1 and TNF decrease matrix component synthesis and increase specific degradation enzyme synthesis and activity (Tetlow et al 2001, Morris and Treadwell 2003). However, TGF-beta stimulates synthesis of collagen type II and XI (Pujol et al 1991) as well as proteoglycans (Lafeber et al 1997). Serum amyloid A-activating factor1 (SAF-1) is involved in the regulation of MMP1 in osteoarthritic cartilage (Ray et al 2003). In contrast, OA induces increased activities of mannosyl, glycosyl, N-acetylglucosaminyl-galactosyl, sialyl and fucosyl transferases (Richard et al 1990).

1.4.1.6. Radiology

The radiological examination of OA remains the most practical method of assessing osteoarthritic structural changes (Doherty and Lanyon 1996). However, radiological diagnosis is not possible until the disease is well advanced (Wotton and Duance 1991).

Radiographical findings may have little relationship to symptoms. Joint space narrowing, osteophyte formation, and subchondral bone changes may all occur independently of the clinical syndrome that is called OA. It is not known whether radiographical progression will stop if symptomatic OA is controlled or if successful repair or protecting mechanisms are effectively activated. In spite of these unknowns,

when considering the techniques, the radiograph is probably the best tool for the measurement of progression in OA (Altman et al 1987).

The methods for grading radiographical changes in OA were developed by Kellgren and Lawrence (1957). The basis of their scale is the presence of osteophytes, which is considered the pathognomonic feature of OA. Joint space narrowing, subchondral bone changes and joint deformity are considered to be insufficiently specific changes, unless seen in conjunction with osteophytes (Kellgren and Lawrence 1957). Experience determines that, in OA, joints may in fact be narrowed, sclerotic and deformed, without the presence of osteophytes, but according to the Kellgren and Lawrence scale, the joint must be graded as negative for OA if osteophytes are not present (Kallman et al 1989).

Radiographical examination can give a diagnosis of OA if there is a finding of narrowing of the joint space as cartilage is destroyed (Senior 2000). Osteophytes and subchondral sclerosis and/or cysts, of other grades of OA, can be seen also when the osteoarthritic changes in articular cartilage continue (Menkes 1991). Joint space narrowing has a greater value than the presence of osteophytes and subchondral bone changes in the assessment of the progression of OA (Altman et al 1987). However, the most useful radiographical variables differ according to anatomic site, and the evaluation of OA at specific joint regions is different (Lohmander 1994). Serial radiographs are very helpful in estimating the progression of the OA, because OA generally tends to progress very slowly (Kallman et al 1989).

The radiographical findings come from the pathological changes. The radiographical-pathological correlation in OA is summarised in Table 1.1.

Table 1.1. Radiographic-pathological correlation in OA
(Adapted from paper published by Hamerman 1989)

Pathological change	Radiographical abnormality
Cartilage fibrillation/erosion	Decrease in interosseous distance
Subchondral new bone formation	Sclerosis
New cartilage formation and endochondral ossification	Osteophyte
Fibrous-walled pseudocysts resulting from fluid intrusion or myxoid degeneration	Subchondral cyst
Trabecular compression	Bone collapse/attrition
Fragmentation of osteochondral surface, cartilage and bone metaplasia in synovium	Osseous (loose) bodies

1.4.1.7. Diagnosis

Unfortunately, there is no accurate diagnostic test for OA. Diagnosis of OA involves a combination of clinical findings with radiographical and/or laboratory evidence (Altman 1997). Therefore, the principle upon which the condition is diagnosed will vary between, for example, cartilage biochemist, general practitioner, orthopaedic surgeon, radiologist and epidemiologist, depending on their different tools, requests and reasons (Felson 1995).

For clinical diagnosis, a combination of symptoms, clinical signs and radiographical finding are used (Cole and Kuettner 2002). These criteria can help in the advanced stage of OA, but other criteria are needed to diagnose OA in the early stages. Biochemical assays have been developed as prognostic markers to evaluate OA and have been tested to see whether they predict the later occurrence or worsening of OA (Fukui et al 2001). Biochemical markers can show the early stages of cartilage degradation. Increased PG concentration in the synovial fluid has been related to disease severity, but this can happen under a variety of conditions and it is also reversible (Lohmander 1997). The first irreversible change in cartilage degradation in OA is the breakdown of collagen. Collagen-related markers might be potentially more reliable signs of degradation than PG markers. Type IX collagen is an especially strong candidate (Brierley et al 1991). This collagen includes non-collagenous domains, which are sensitive to enzymatic attack releasing fragments of the molecules, and it is known to be located on the surface of type II collagen fibrils (Wotton and Duance 1991).

Promising techniques, currently being evaluated, are magnetic resonance imaging, bone scintigraphy, arthroplasty and body fluid markers of joint cartilage turnover (Lohmander 1997).

1.4.1.8. Genetic susceptibility

The available evidence suggests that genetic factors have a major role in OA. It has been believed for over 50 years that a strong genetic susceptibility for OA is present (Cicuttini and Spector 1996). This genetic influence has now been shown by a recent study of twins (Spector et al 1996), and it has been demonstrated that the search for specific genes for OA in the population is possible (MacGregor and Spector 1999). Separation analysis of population data suggests that OA is a polygenic disorder, but the identity of the genes involved remains unknown (Senior 2000).

OA is placed in the "complex multifactorial" class of genetic disorders (Loughlin 2002), which means that it would be expressed as a consequence of the interaction between genetic and environmental factors (Hamerman 1993). The nature of the genetic influence in OA is suggested to involve either structural defects, such as collagen, or alternatively a genetic influence on a known risk factor for OA such as obesity (Cicuttini and Spector 1996). The genetic effects may be change by sex or by environmental factors distributed differently between men and women (Kaprio et al 1996). For example, in women a clear genetic effect was found in OA of the hand and the knee (Spector et al 1996).

Epidemiological studies have demonstrated that primary OA has a major genetic susceptibility (Loughlin 2002). It is occasionally expressed as an autosomal dominant trait, clearly demonstrating that DNA mutations can cause the disease. Primary OA is a complex trait, in which genes play a major role in susceptibility, and environmental factors can modify the phenotype (Meulenbelt et al 1997). A genetic predisposition for primary OA has been suggested by association with HLA-A1B8 and HLA-B8 (Cicuttini and Spector 1996). The possibility of abnormalities of type II collagen caused, by a mutation in the type II procollagen gene, should be considered in the differential diagnosis of childhood arthropathy, osteochondrosis, or primary OA with mild chondrodysplasia, in the context of a positive family history (Mier et al 2001).

1.4.1.9. Ageing cartilage and osteoarthritis

The rate of chondrocyte maturation is slow during normal ageing (Corvol 2000). It compensates for the absence of the recruitment of new chondrocytes. The turnover of matrix protein, particularly PG, the half-life of which is shorter than collagen, decreases with age. In addition, degradation of the matrix protein is not activated, so the balance

supports the maintenance of these proteins (Kempson 1991). Ageing chondrocytes remain capable of secreting a number of growth factors and of reacting to them. However, there is a decrease in the ability of the chondrocytes to respond to a variety of stimuli (Huber et al 2000). They are less firmly anchored in the matrix because the GAG chains are altered both quantitatively and qualitatively. These phenomena act together to decrease the number of chondrocytes in the cartilage and to weaken the cartilage matrix. However, the structure and arrangement of the cells are preserved and a balance is maintained between the reduced population of cells and the small amount of matrix (Corvol 2000).

The size of aggrecan is progressively reduced in ageing cartilage (Bayliss et al 2000). Type II collagen content decreases, because undoing of the triple helix occurs and type II collagen is damaged. The damaged collagen network cannot withstand the osmotic swelling pressure of the hydrophilic proteoglycans, and localised swelling occurs (Huber et al 2000). However, the newly synthesised PGs have slightly different properties from those of younger cartilage and the length, number and nature of GAG chains are smaller and, as a result, the water content is lower (Corvol 2000). The softening of tissue is caused by localised swelling and, because of chronic mechanical forces, the degenerative process accelerates and cartilage destruction occurs (Kerin et al 2002). Surface fibrillation, accompanied by further loss of PG, can be shown as an early change in OA (Huber et al 2000).

Also, in ageing cartilage PGs, there is an increase in 6-sulphation relative to 4-sulphation in chondroitin sulphate and an increase in the size and number of keratan sulphate chains (Elliott and Gardner 1979). Aggrecan shows a progressive decrease in the extent of the chondroitin sulphate-rich region and a reduction in the molecular weight of the core protein. Multiple-sized fragments of core protein may be produced with attached GAGs (Watanabe et al 1997). The larger fragments may be retained in the matrix, and the smaller fragments may diffuse into the joint fluid (Hamerman 1993). This debris of hyaline cartilage and/or fibrocartilage causes the inflammatory response of the synovial membrane (Nakashima et al 1998). The result is production of cytokines and metalloproteinase by the chondrocytes or by the synovial cells. Whether these changes suggest age-related degenerative joint disease or the reactive response of early OA remains unclear (Corvol 2000).

However, "*OA at least in the early stages was thought to be the result of progressive aging of articular structures and especially of cartilage*" (Passiu and Carcassi 1989).

In spite of these biochemical changes tensile strength and stiffness of ageing cartilage is only moderately reduced (Hamerman 1993).

Section 5

1.5. Amyloidosis

1.5.1. Introduction

Amyloidosis is the term for a heterogeneous group of diseases defined by the deposition of extracellular proteinaceous material, amyloid, in various tissues and organs (Skinner and Cohen 1983). The term amyloid was introduced by Virchow in 1854, when he noticed a macroscopic tissue abnormality that showed a positive iodine-staining reaction. Now the term amyloid is used, as a generic term, to describe proteinaceous tissue deposits characterised by specific structural and histochemical properties. By light microscopy amyloid is as an amorphous pink material, using haematoxylin and eosin, and it stains with Congo red to give an apple green birefringence under polarised light. Ultrastructurally, amyloid appears as non-branching fibrils, usually 7-10 nm in diameter. Using X-ray diffraction techniques, polypeptide chains were found to be arranged in an anti-parallel β-pleated sheet, which consists of sequences of β sheet formation interspersed between lengths of more usual protein structure (Kisilevsky and Young 1994). Amyloid is, thus, an extracellular deposit of insoluble protein fibrils composed of a disease-specific component and proteoglycan (Kisilevsky 2000). Amyloid fibrils have never been found in normal tissue and they all have the same ultrastructure by electron microscopy. In addition, the ultrastructure of the fibrils in human amyloidosis is similar to that in amyloid experimentally induced in animals (Husby and Sletten1986). Amyloid can deposit in all organs, but the various amyloid deposits have different localised and systemic patterns (Rumpelt et al 1996).

1.5.1.1. Amyloid structure

An amyloid deposit is a mixture of three components: amyloid fibril, P component and glycosaminoglycan. The amyloid fibril protein is seen as a mass of twisted unbranched fibrils, usually with random orientation, or, occasionally, with parallel orientation and the amyloid proteins vary in different amyloid deposits (Francis 1996). The amyloid P component is believed to be part of all the amyloid deposits (Pepys 1992), but it is not a part of the 'amyloid' deposits in Alzheimer's disease (Husby and Sletten 1986). It is not known how the amyloid fibrils and amyloid P component join together. Amyloid P component is a non-fibrillar glycoprotein, which forms 15% (w/w) of any amyloid deposit. Serum amyloid P component is a normal plasma protein (Husby and Sletten 1986), and is a precursor of AP component, which is like the C-reactive protein family (Pepys 1992). AP component binds to fibrillar amyloid in a union dependent on calcium.

It is not known whether AP component plays a role in amyloid deposit formation (Pepys 1992). Glycosaminoglycans are the third part of amyloid deposits. All glycosaminoglycans have been found to be present in amyloid deposits, except hyaluronan (Kisilevsky 1992). Heparan sulphate seems to be the common GAG in all amyloid deposits. It has been suggested as an important factor in amyloidogenesis facilitating the assembly amyloid fibrils (Kisilevsky and Fraser 1996).

1.5.1.2. Classification of amyloid and amyloidosis

According to chemical and clinical findings, various types of amyloid protein have been found in two major groups of amyloidosis. In systemic and localised human amyloidosis, 17 forms of amyloid protein have been recognised, 10 types of which were found in systemic amyloidosis and 7 types in localised amyloidosis (Westermark 1997). Some of these deposits are rare and some have a main role in the pathogenesis of disease affecting millions of people (Kisilevsky 2000). In the classification of amyloids, the capital A is always the first letter, followed without a space, by an abbreviation for the first letter of the amyloid protein in the tissue deposits (Husby 1992). Some of these amyloid proteins are described below.

1.5.1.2.1. AL amyloid

AL amyloid is seen in primary or idiopathic amyloidosis and amyloidosis associated with monoclonal gammapathies. The nature of AL amyloid is an immunoglobulin, which is most frequently κ or λ light chains (Husby and Sletten 1986). The incidence of AL amyloid in the human population is 8.9 per million per year, and the median age of diagnosis is 62 years. Involved organs are kidney, liver, heart, joints or sural nerve (Kyle 1992). The numbers of plasma cells in the bone marrow are increased in patients with idiopathic amyloidosis. It is suggested that this disorder is caused by plasma cell dyscrasias and it has the same basic pathogenetic mechanism as myelomatosis. In many cases, clinical distinction between idiopathic amyloidosis and myeloma amyloidosis is difficult, because many patients with myeloma will subsequently develop AL amyloidosis (Husby and Sletten 1986). The major difference between these disorders is the destructive bone lesions of multiple myeloma, which are not present in primary amyloidosis (Sundar et al 1989).

1.5.1.2.2. AA amyloid

AA amyloid is the major ingredient of amyloid fibrils of human secondary amyloidosis (Skinner and Cohen 1983). Secondary amyloidosis still is a major problem in underdeveloped countries, because it is a serious complication of many chronic

inflammatory diseases of either infectious or non-infectious origin, such as tuberculosis, bronchiectasis, osteomyelitis and rheumatoid arthritis (Gertz 1992).

The precursor of tissue AA protein is serum amyloid A (SAA) protein, which is synthesised by the liver as an apoprotein (Shirahama et al 1984). However, in rheumatoid arthritis, SAA is produced by inflamed synovial tissue (O'Hara et al 2000). SAA must be in a sufficient amount in a suitable anatomic location for amyloid fibrils to assemble (Kisilevsky and Young 1994). SAA is synthesised in enormous quantities during an inflammatory reaction as a positive acute-phase protein (Buxbaum and Tagoe 2000). If large quantities of SAA were the only necessity for AA amyloidosis, it would be almost certainly a very common consequence of inflammatory disease. As this is obviously not the case, other factors must be necessary for AA amyloidosis (Skinner 1992). AA amyloid deposition is found mostly in kidney, gastrointestinal tract and skin (Gertz 1992). The amyloid fibril in familial Mediterranean fever, called recurrent polyserositis, is AA amyloid (Gardner 1992c).

1.5.1.2.3. AF amyloid

The fibril protein in heredity and familial (heredofamilial) amyloidoses, AF protein, is a variant of prealbumin with only a slight difference in the structure (Husby and Sletten 1986). The genetic mutation can be recognised in early life, before evidence of the disease, and is usually associated with a polyneuropathy. Amyloidosis of the heart may also be another form of the "heredofamilial" amyloidoses (Gardner 1992c).

It is interesting that the protein of senile amyloidosis, the most common form of systemic amyloidosis, also shows partial sequence homology with normal prealbumin, as in heredofamilial amyloidosis, so it may be part of the ageing process. It is an autosomal dominant form of amyloidosis (Cornwell et al 1995).

1.5.1.2.4. Aβ_2-M amyloid

β_2-Microglobulin (β_2-M) proteins are deposited in Aβ_2-M amyloidosis, which was found first in carpal tissue and joints of chronic haemodialysis patients (Ogawa et al 1995, Danesh and Ho 2001). It was not reported in any other circumstances. β_2-M deposits may be found in the joints early after the beginning of haemodialysis, but the duration of haemodialysis (Tran et al 2001) and age at onset of haemodialysis (Jadoul et al 1997) affect the distribution of β_2-M depositions. Amyloid deposition in vertebral discs was not seen to progress with longer duration of dialysis (Ohashi et al 1992).

β_2-M is a normal serum protein, which is a minor component of the surfaces of many cells, including lymphocytes. β_2-M is present in serum, CSF and urine of normal people (Gejyo and Arakawa 1992). Lymphocytes are the main source of β_2-M and it is

catabolised in renal tubules. In renal failure, it accumulates in the serum, and it is not removed by haemodialysis, thus the serum concentration of β_2-M becomes elevated (Maury 1990). This could result in the deposition of β_2-M in various tissues, such as joints, heart and gastrointestinal tissues, in haemodialysis patients (Saito 1994). However, β_2-M concentrations in sera from patients with carpal tunnel syndrome or cystic radiolucencies do not differ from those in sera of normal people (Gejyo and Arakawa 1992). Therefore, other factors may be involved in β_2-M deposition (Danesh and Ho 2001), and this argues against its arising by simple precipitation (Tran et al 2001).

β_2-M has a binding affinity for soluble collagen in a dose dependent way, therefore early β_2-M deposits were found preclinically and preferentially in joints (Maury 1990). It deposits first on the surface of the cartilage in the absence of macrophages (Jadoul et al 2001). The factors causing deposition of β_2–M proteins into amyloid fibrils still remain unclear. Macrophages infiltrate, in the later stages of amyloid deposition, after the death of synovial cells probably because of amyloid fibril toxicity. Therefore, macrophages are not the origin of amyloid fibrils (Garbar et al 1999) and infiltration by macrophages should be a phenomenon secondary to β_2-M deposition (Argiles and Mourad 2000). In addition, multinucleated cells and granulation tissue are often observed around amyloid deposits (Ohashi 2001). The observation of macrophages in the late stage of β_2-M deposition, suggests a possible role for these cells in transformation of clinically silent deposits into symptomatic osteoarticular destruction (Hou et al 2001). β_2-M deposits in synovial joint tissue show a close association with subchondral bone cysts (Zingraff et al 1990). When macrophages infiltrate after β_2-M deposition in synovial tissue, their secretions, such as cytokines and metalloproteinase, might contribute to the formation of cystic bone lesions (Ogawa et al 1995). β_2-M deposits were found commonly in SCJs after long-term haemodialysis (Zingraff et al 1989, Maury 1990, Cameron et al 1997). Hip, knee and shoulder joints are other common sites for β_2-M deposits.

1.5.1.2.5. ATTR amyloid

Transthyretin (TTR) is a normal serum protein that carries one quarter of serum thyroxin and all of retinal-binding protein (Buxbaum and Tagoe 2000). It is a 127 amino acid residue protein, synthesised mainly by the liver and in several minor sites including the choroid plexus and the eye. A single gene on human chromosome 18 encodes TTR. Mutation in this gene causes a familial polyneuropathy or cardiomyopathy, associated with amyloidosis, and this has been found in various countries (Buxbaum and Tagoe 2000). It is caused by the deposition of wild type TTR in tissue and organs. TTR is also

the precursor protein in apparently sporadic senile systemic cardiac amyloidosis (Westermark 1997).

1.5.1.2.6. AE amyloid

AE amyloid is found in localised endocrine-associated amyloidosis. This has been recognised as arising locally in association with a variety of endocrine tumours and forms intratumour deposits. Most commonly it occurs with medullary carcinoma of the thyroid (Looi 1986). Local deposits of amyloid AE are also associated with tumours producing insulin and growth hormone. The chemical nature of amyloid AE in humans is not certain. However, prohormones or peptides are believed to be the source for local amyloid AE deposits (Francis 1996). AE deposition is thought to represent a form of calcitonin (Husby and Sletten 1986) or precalcitonin (Gardner 1992c). Amyloid AE can deposit at the site of insulin injection, when injections are repeated.

1.5.1.2.7. AS amyloid

AS amyloid deposits in organs of elderly people as a localised amyloidosis. Cardiac amyloidosis is one form of senile, organ–limited amyloidosis, which involves the left side of the heart more than the right and, especially, the ventricle (Klien et al 1989). However, another type of senile amyloidosis is limited to the atria and occurs at an earlier age. This is referred to as atrial amyloidosis and is an age and sex-independent predictor for the presence of atrial fibrillation, therefore it affects atrial function and increases the risk of onset of atrial fibrillation at an earlier age (Rocken et al 2002). Both types may be observed in the same heart tissue, but they are pathologically unrelated (Cornwell and Westermark 1980). ASc amyloidosis shows diffuse amyloid deposits in the ventricles; it is characterised by the subunit protein, which is thyroxin-binding prealbumin (Hesse et al 1993). Aortic valve amyloidosis appears to be the most common form of localised amyloidosis, occurring in nearly all people over the age of 50 years. It was found by echocardiography of aorta and heart (Cornwell et al 1995).

Extracellular depositions of amyloid in islets of Langerhans are very common in old people. The degree of deposits in extracellular part of the islets may correlate with the severity of diabetes mellitus, but is unlikely to be a cause of clinical diabetes (Clark et al 1996). It often involves islet cells in a patchy distribution, more commonly in the head than the tail of the pancreas. The islet cell amyloid may be composed of prohormones of insulin or glucagon (Gorevic et al 1985). In old age, asymptomatic amyloid deposits are found as localised amyloidosis (see below) in various parts of synovial joints, such as cartilage, synovial membrane and tendon.

The senescence-accelerated mouse (SAM) is a model for showing normal ageing characteristics (Takeda et al 1997). SAM shows a high frequency of senile amyloidosis in synovial joints and intervertebral discs: these lesions are histologically quite similar to those of humans (Takeshita et al 1982). The biochemistry of these deposits shows an additional type of amyloid, murine systemic amyloid (Shimizu et al 1992). AA amyloid was found as well, and the incidence of AA was correlated with systemic signs of inflammation at autopsy.

1.5.1.2.8. Aβ amyloid

The Aβ amyloid fibril protein, called β protein, is a proteolytic product of amyloid precursor protein, which is encoded by a gene located on the long arm of chromosome 21 (Buxbaum and Tagoe 2000). Aβ protein can be produced as a soluble peptide by metabolically normal cells (Takahashi et al 2002). Cleavage in the middle of Aβ amyloid precursor protein stops deposition of amyloid, but cleavage close to the N-terminus or C-terminus leads to production and deposition of Aβ amyloid. During normal ageing, production and destruction of Aβ protein is balanced (Hyman et al 1993). In old age, this balance may be disrupted, and deposition of Aβ amyloid plays a key role in development of Alzheimer's disease, which is characterised by the deposition of amyloid and neurofibrillary tangles in the ageing brain (Cornwell et al 1995). Alzheimer's disease is one of the most common forms of amyloidosis and death from Alzheimer's disease is more common than that from strokes (Husby and Sletten 1986).

Another form of Alzheimer's disease is autosomal dominant familial Alzheimer's disease, which occurs in mid adulthood, rather than late (Buxbaum and Tagoe 2000). Familial Alzheimer's disease represents about 5-10% of all cases of Alzheimer's disease. A mutation of the gene encoding the amyloid β protein precursor causes the clinical syndrome, with complete penetration, although a few cases with the appropriate genotype have no disease phenotype (Buxbaum and Tagoe 2000). The amyloid deposits found in Down's syndrome are very similar to Aβ amyloid (Husby et al 1993).

1.5.1.2.9. AD amyloid

Primary amyloid (AL) and secondary amyloid (AA) can be found in skin, but in distinct cutaneous amyloidoses, such as lichen amyloidosis, AA and AL amyloid were not observed, and the amyloid deposits are designated as amyloid (AD). Amyloid of localised cutaneous amyloidosis was positively stained for the antiserum, which contains components derived from epidermal fibrous protein (Kobayashi and Hashimoto 1983).

1.5.1.3. Pathogenesis

Amyloid is a large biochemically and clinically heterogeneous, group of folded proteinaceous materials. Various peptides and proteins have been recognised from several types of amyloid deposits (Sipe and Cohen 2000). The mechanisms for converting these proteins to amyloid fibrils are unknown (Husby and Sletten 1986, Zingraff and Drueke 1998). In some familial amyloidoses a gene mutation is responsible for these depositions, but more often they are wild type amyloidoses (Buxbaum and Tagoe 2000). Many of the proteins, such as immunoglobulin light chains, have a high degree of normal β pleated-sheet structure (Kisilevsky and Young 1994). Other components of amyloid deposits, such as glycosaminoglycan or AP component, may play an important role in pathogenesis of amyloidosis (Kisilevsky 1992). Amyloid P component may increase the resistance of the AA protein to proteolysis, and they may be important in pathogenesis of AA amyloid. Snow and Kisilevsky (1985) showed by experimental amyloidogenesis that AA amyloid is not deposited without amyloid P component. GAGs, such as heparan sulphate, are usual components of the amyloid structure. Heparan sulphate is a basement membrane proteoglycan, which can support protein aggregation into amyloid fibrils (Kisilevsky 1992).

Many of the amyloid protein precursors are shortened at the N-terminal or C-terminal, and then changed to amyloid deposits. Therefore the proteolytic step probably is important in many of the amyloidoses (Westermark 1998). Immunoglobulins are components of some amyloid deposits, therefore alteration and abnormality of immunoglobulin products may be a reason for amyloid deposition (Gardner 1992c).

1.5.1.4. Diagnosis of amyloidosis

Diagnosis of amyloidosis is still often best achieved by biopsy and microscopic examination of the deposits. The tissue for examination can be taken from any organ with clinical evidence of involvement. However, biopsy is taken commonly from an easily available tissue in systemic amyloidosis, which may be, for example, skin or gastrointestinal tract (Westermark 1995). After preparing the tissue, staining with Congo red is used. Congo red has a selective affinity for amyloid deposits and it is a diagnostic test for amyloid (Francis 1996). It is not completely specific, because it stains elastic tissue as well, but careful decolourisation minimises this problem (Westermark 1995). Amyloid deposits are seen as congophilic areas with green birefringence with polarising light. This is the single most useful procedure for establishing the presence of amyloid (Skinner and Cohen 1983). It is not only important to find the deposition of amyloid in tissue, but also to characterise the type of amyloid deposit, as this is central to prognosis

and treatment (Fujihara et al 1980). The exact chemical form of amyloid can be found using immunohistological techniques (Linke and Nathrath 1982). Such antibodies as are now used for the accurate diagnosis and classification of amyloidosis, work on formalin-fixed and paraffin–processed tissue (Levo et al 1982). Some examples of these antibodies are anti amyloid P component, anti AA amyloid, anti κ and λ light chains, anti β_2-M and anti β protein.

The evaluation of the distribution and extent of amyloidosis by magnetic resonance (MR) is useful in the hip joints of patients undergoing long-term haemodialysis. MR examination may improve the ability to predict the risk of pathological fractures in patients with a long history of haemodialysis (Otake et al 1998).

1.5.1.5. Amyloid of articular tissue

Amyloid has been recognised in synovial joint tissue with four different types of amyloidosis. In primary amyloidosis, AL amyloid is deposited in joint tissue (Fautrel et al 2002). Miyata et al (2000) noted two types of amyloid lesions in amyloid arthropathy. The most common form involved both synovia and periarticular tissue with AL amyloid deposits. Palpable subcutaneous nodules, joint swelling, limited movement and synovial hypertrophy were described. In the second type of AL amyloid, subchondral bone was replaced by amyloid, causing swelling and pathological fracture. Both types of AL amyloidosis can be recognised in the amyloid deposits of myelomatosis. The frequency of AL amyloid arthropathy is approximately 5% in myeloma cases (Gardner 1992c).

AA amyloid is also deposited in synovial joint tissue in association with immunological, systemic, connective tissue disorders, such as rheumatoid arthritis. In rheumatoid arthritis, AA amyloid is both produced and deposited in cartilage, which may lead to progressive cartilage degeneration (Vallon et al 2001)

In old age, senile amyloidosis widely involves articular structures. Small deposits of amyloid can often be recognised in the articular cartilage of normal elderly people (Goffin et al 1981, Ladefoged 1986), and comparable deposits are found in senescence mice (Shimizu et al 1992). There is a correlation between the amount of amyloid deposited and age (Ladefoged 1982). These deposits are usually symptomless (Cary 1985) and fail to react with anti AA and AL amyloid (Goffin et al 1985). The most frequent sites of articular amyloid deposition were found to be first, the sternoclavicular joints and, second, the hip joints (Goffin et al 1981).

The dialysis-associated amyloid deposit, β_2-M, is found in articular cartilage in patients on long-term haemodialysis. The prevalence of amyloid deposits is clearly related to the length of dialysis therapy (Zingraff et al 1990). The joint most commonly affected by

dialysis-related amyloidosis arthropathy is the shoulder, in which it causes pain, stiffness and restricted movement (McDonald et al 1998, Danesh and Ho 2001).

The way amyloid comes to form in articular tissues and to deposit there is uncertain (Rumpelt et al 1996). One hypothesis proposes that soluble amyloid precursors can diffuse from the synovial fluid into the articular cartilage (Bywaters and Dorling 1970). Another hypothesis argues that amyloid fibrils are found within, or adjacent to, the chondrocytes in all cases, and that they are secreted by the chondrocytes (Cornwell and Westermark 1980).

1.5.1.6. Amyloid and osteoarthritis

Amyloid deposits in articular tissue are found in primary amyloidosis (Gardner 1992c), secondary amyloidosis (Vallon et al 2001), senile amyloidosis (Ladefoged 1986) and dialysis-related amyloidosis (Zingraff et al 1990). Localised amyloid deposits can occur in primary joint disease, such as osteoarthritis (Goffin et al 1981). However, the precise frequency, nature, tissue distribution and pathogenesis of amyloid deposition in OA are uncertain (Ladefoged 1986). Similar microdeposits of amyloid are seen in the fibrillated and unfibrillated articular cartilage of elderly people, with no clinical or pathological evidence of OA (Ladefoged 1982, Goldman and Bansal 1996). In addition, in OA, fibrillated and fissured as well as smooth cartilage contained amyloid microdeposits (Athanasou and Sallie 1992). However, amyloid is often found in the surface of osteoarthritic articular cartilage, and there is a correlation between the presence of amyloid deposits in cartilage with the age of the patient and the duration of articular symptoms (Egan et al 1982). The surface location of amyloid in the articular cartilage may be a sign of increased mechanical trauma at this site. Since OA occurs more frequently in elderly patients the presence of amyloid deposits in articular cartilage may just be an accompanying feature of aged joints (Goffin et al 1981).

The type of amyloid deposited in OA is not certain (Goffin et al 1981), but amyloid A is not found in localised amyloid deposits in OA, and this is confirmed by the absence of immunohistochemical staining for amyloid A (Athanasou and Sallie 1992). A predominance of β_2-M amyloid in articular tissue was found with minor deposits of other types of amyloid and, therefore, joints may have specific features that lead to the development of β_2-M deposition (Moe and Chen 2001). β_2-M deposits may stimulate release of metalloproteinases and cytokines, mediators of tissue degradation and inflammation, leading to an articular degradation, without infiltration by inflammatory cells (Moe and Chen 2001).

1.6. Aims and Objectives

A better understanding of the aetiopathogenesis and pathology of osteoarthritis will depend on the continuing elucidation of the molecular structural and metabolic events of normal cartilage compared to early disease events and established changes.

Whilst conventional histological and histochemical studies have advanced knowledge and much better understanding has been achieved by the immunohistological identification of specific molecules, there is a paucity of investigational data concerning one of the major chemical constituents of cartilage – carbohydrates. Antibodies against specific cartilage carbohydrate epitopes are few and the use of lectin histochemistry to explore differences in glycan composition and distribution in health and disease has been under exploited.

The larger human articulations are complicated and difficult to study overall. In addition, there are few unifying theories to explain and predict all the changes of osteoarthritis. Most studies have concentrated on the changes in articular cartilage with the changes in subchondral bone being considered to be secondary to the primary events in cartilage. Alternatively, in repetitive impulsive loading of joints the subchondral bone responds by becoming sclerotic and stiffer and less effective as a shock absorber. Changes in the tidemark and subchondral bone may, therefore, be regarded as early events (Radin et al 1972). Either way, an attractive unifying view is that the changes in the relationship between articular cartilage and subchondral bone represent a process of endochondral ossification and a recapitulation of the process normally seen in the growth plates of the growing skeleton.

Therefore by choosing to study a small, readily accessible, human adult articulation and the human foetal and neonatal costochondral junction, it would be possible to test **the hypothesis that a comparative histological, histochemical and lectin histochemical study of joint and junction will demonstrate molecular changes (especially in glycans) which are characteristic and predictive of osteoarthritic change and which support the view that such change is similar to an endochondral ossification.**

The studies reported in this thesis were therefore performed to:

I. Define the structure of the human sternoclavicular joint in health and disease;
II. Define the structure of the human foetal and neonatal costochondral junction in normal samples and in those showing certain congenital and known structural abnormalities;

III. To compare and contrast adult articulations with the foetal/neonatal growth plate to determine the presence or absence of shared microanatomical and molecular features.

The work focussed on the following objectives:

a. The definition of the structure of normal and osteoarthritic human sternoclavicular joint by histological, histochemical, immuno- and lectin histochemical techniques;
b. The definition and chemical specification of articular amyloid depositions in aged and osteoarthritic sternoclavicular joints;
c. The definition of the histological, histochemical and lectin histochemical features of normal and abnormal foetal/neonatal growth plates;
d. The demonstration of the similarities and differences between articular and growth plate cartilages and an evaluation of the significance of these observations.

Chapter 2

Materials and Methods

2.1.Introduction

Two groups of tissues were used in the present study: sternoclavicular joint (SCJ) and costochondral junction (CCJ). The SCJs were obtained from 31 subjects (29 right and left SCJs and 2 only left SCJs), aged from 23 to 98 years (mean 71), 15 of them male (age 35-83) and 16 of them female (age 23-98). The SCJs studied contained the fibrocartilage and articular cartilages attached to the subchondral bone and the first rib. The retrieved neonatal CCJs were received already fixed in formalin and processed to wax blocks. They were 75 subjects, 45 male and 30 female, stillbirths and infants.

For SCJ sections a number of staining procedures were used to demonstrate the structure and components of the joint. The stains applied were haematoxylin and eosin, toluidine blue, picro sirius red, alcian blue (in critical electrolyte concentrations) and alkaline Congo red. Macroformat photomicrographs were prepared for all samples and used for the formal assessment of the accepted features of disorders including osteoarthritis and amyloidosis.

Three histological staining procedures were applied on CCJ, which were haematoxylin and eosin, toluidine blue and picro sirius red.

The microanatomy of tissues can be recognised by haematoxylin and eosin (H&E) staining. In this study, using H&E helped to identify morphological differences between the various types of cartilage. It helped in understanding the position of cells and the disposition of matrices in each cartilage zone. A standard procedure was used in the present study (see page 104).

Toluidine blue (TB) stains glycosaminoglycans (GAGs) in connective tissues. The demonstration of changed TB staining of GAGs in cartilage is an established method, which may be used as one criterion for evaluating the severity of disease. This dye is able to differentiate fairly minor differences in metachromasia between pericellular, territorial matrix and interterritorial matrix and in the various zones of cartilage. There is a relationship between the hexuronic acid content of cartilage and the intensity of TB staining. If normal hexuronic acid is diminished, the consistency of TB staining is lost; therefore it is sufficiently sensitive to show loss of proteoglycan (PG) in early osteoarthritis (OA) without any superficial cartilage pathology (Sachs et al 1982). In the present study a standard procedure was used for every sample of cartilage (see page104).

Collagen molecules are rich in basic amino acids, which react strongly with acidic dyes. Picro sirius red (PSR) is one of the strongly acidic dyes, which show the presence of collagen. It has been used, for many years, to stain connective tissues rich in collagens (Junquiera et al 1979). PSR is most useful when combined with polarising microscopy

(Junqueira et al 1979). Collagen staining using PSR is a qualitative test, but in combination with polarising techniques a quantitative approach can be taken. A standard procedure was used in the present study (see page 104).

Alcian blue (AB) is a metachromatic stain that differentiates cartilage components when used with critical electrolyte concentrations. Critical electrolyte concentrations can be used to show the different components of the tissue, such as keratan sulphate and chondroitin sulphate, by preventing combination of the dye with some of the components (Scott and Dorling 1965). For example, carboxylate and phosphate do not bind to the dye in high concentrations of magnesium chloride (more than 0.2M). Using this technique makes it possible to demonstrate altered concentrations of CS and KS within both various zones and regions of normal and osteoarthritic cartilage. In the present study a standard procedure with three concentrations of magnesium chloride (0.05, 0.5 and 0.9M) was used (see page 104).

Alkaline Congo red (ACR) staining has selective affinity for amyloid deposits (Khurana et al 2001). It may be applied to tissue sections and used *in vivo*. This staining works through hydrophobic and electrostatic interaction between dye and tissue. Using an alcoholic solvent and salt solution has the effect of suppressing background staining leaving only the amyloid stained. It is important to use control sections, both positive and negative. Positive controls confirm the reactivity of the staining solutions and negative controls exclude autofluorescent tissue elements, such as collagen, in polarising light. The bright apple green birefringence of amyloid following Congo red staining is easily visualised, highly selective and thought by many to be the most reliable diagnostic test in current use. In this study a standard procedure was used (see page 105), and polarising light was used to confirm the existence of amyloid deposits in sections. Immunohistochemical staining was used to find the type of amyloid deposited in the joint tissues (see page 105 for details).

Lectin histochemical staining tested for the presence and distribution of specific glycans as detailed on page 107. The SCJs assessed in this study were sub-divided into three groups: normal, mild to moderate osteoarthrosis, and severe osteoarthrosis. A panel of 14 biotinylated lectins was applied to these three different groups under the same conditions: HHA, PSA, LCA, e-PHA, l-PHA, UEA-I, MAA, ECA, AHA, DBA, VVA, MPA, HPA and WFA (see Table 2.2 for specifications).

The growth plates from the CCJs assessed in this study were sub-divided into two groups: normal (and with no report of skeletal abnormalities in the autopsy report) and pathological (reported congenital disorder). A panel of 15 biotinylated lectins was

applied to these two groups under the same conditions: HHA, PSA, LCA, e-PHA, l-PHA, UEA-I, AAA, ECA, CTA, AHA, DBA, VVA, HPA, WFA and DSA.

The following materials were used for the present study. Details of the chemicals and other materials used in this study are given in Appendix A.

Mouse anti-human AA amyloid, mouse anti-human kappa light chain, mouse anti-human lambda light chain, swine anti-rabbit, goat anti-mouse, streptavidin peroxidase and anti-CD68 were purchased from **DAKO**. Haematoxylin, eosin, calcium chloride, DPX, celestine blue, picric acid, toluidine blue, Congo red, sodium hydroxide, sodium chloride, methanol and acetone were from **BDH**. Hydrogen peroxidase, avidin-peroxidase conjugate, methyl green, crude trypsin, DAB, EDTA, 3-aminopropyl-triethoxysilane (APES), human β2 microglobulin antigen, λ light chains (free) human myeloma plasma, κ light chains (free) human myeloma plasma, bovine serum albumin and hyaluronidase were from **SIGMA**. Normal swine serum and normal goat serum came from **TCS**. Rabbit anti human P component came from **Novocastra Laboratories**. Sirius red came from **Raymond A Lamb**, and TRIS buffered saline (TBS) from **Boehringer Mannheim**. Mouse anti-human β2 microglobulin from **Serotec**.

2.2. Tissue preparation

The right and left sternoclavicular joints were removed at post-mortem examination. When histological sections are prepared, there should be no recognisable loss of cartilage components or alteration in staining characteristics. For this fixation and decalcification are two critical procedures, therefore choosing suitable solutions and methods is important. Formaldehyde is recommended for the fixation of proteoglycan and glycoprotein. It combines with proteins forming bridges between adjacent molecules and causes only a slight loss of staining for glycoprotein, but performs better fixation than any other fixing solution employed (Allison 1973). Formaldehyde (10% v/v) was therefore used for fixation in this study. An anteroposterior X-ray of all the samples was taken to help in the understanding of subsequent microscopical and pathological changes. The samples were cut into two parts from the middle part of the sternum to the right and left SCJ. Two sagittal cuts were made in each portion and the middle section was used, this made thinner blocks for more complete decalcification. The blocks were about 2cm by 2cm in length and width and 1cm in depth. Every sample consisted of parts of two bones (clavicle and sternum), two articular surfaces (clavicular and sternal), the intraarticular disc and the first rib. For decalcification, use of EDTA preserves the integrity of the intracellular structures and the stainability of the extracellular deposits despite the long

demineralisation time required (Eggert 1981). Cationic dyes, such as toluidine blue and alcian blue, that bind to negative charges of the glycosaminoglycans, are commonly used for the histological demonstration of these macromolecules after fixation or decalcification. Decalcification was performed in 0.19M EDTA (pH 7.4-7.6) at room temperature for 40-70 days. The EDTA was changed every 7 days and at that time new X-rays were taken. The X-ray determined the time of termination of decalcification. A variety of combinations of techniques of fixation and demineralisation are available. For this study the favoured technique was 24-48 hours of 10% (v/v) formaldehyde fixation and, if demineralisation was required, EDTA was used.

The preparations were completed by processing to paraffin wax. The sections were processed automatically by a Shandon Citadal 2000 with:

1-50% (v/v) industrial methylated spirit (IMS)

2-70% (v/v) IMS

3-100% (v/v) IMS (4 times by separate container)

4-Xylene (mixture of Isomers, 3 times by separate container)

5-Wax (Ralwax, melting point 58-60°C, 2 changes - the second one under vacuum)

6-In vacuum oven (LTD Qualivac).

After processing they were embedded using Ralwax since is harder than other types of wax and, therefore, better for cartilage sections. Then they were put on a cold plate for 1-2 hours. A number of sections, 7μm thick, were cut from each block using a Leitz 1512 model base sledge microtome. They were mounted on glass slides using albumin and dried on a hot plate. Slides were coated by 3-aminopropyl-triethoxysilane (APES) for the extra adhesion needed in the relatively long immunohistochemistry and lectin histochemistry procedures. This adhesive is excellent for the adherence of sections but does not react with the reagents used in immuno- or lectin histochemistry. The slides were APES coated by the standard procedures before using them in immunohistochemistry and lectin histochemistry (see page103)

2.2.1. Trypsin digestion

In the present study, sections were subjected to proteolytic digestion with type II (crude) trypsin. Fixed material was treated with trypsin, which improves intensity of the stain and also the number of stained sites (Huang et al 1976). Trypsin is an endopeptidase that breaks the cross-links of the precipitated protein, which form during fixation masking some antigens. It breaks peptide bonds connecting the carboxyl group of the basic amino acids arginine and lysine, which allows better penetration of reagents to show antigen/carbohydrate in formalin-fixed paraffin-embedded sections (Jeffrey et al 1987).

However, in fixation a number of chemical groups, such as amine, amide, imine, peptide, guanidine, hydroxyl, disulfide, carboxyl and so on, react with formaldehyde, so they are unavailable to the trypsin. Chymotrypsin cleaves peptide bonds of aromatic amino acids, which do not react with formaldehyde. Chymotrypsin thus provides a wider spectrum of proteolytic activity in processed material. So the value of the trypsin digestion is dependent on the reagent used for fixation and how it affects the antigen/carbohydrate binding sites.

2.2.2. Disclosing methods

Specific antigen-antibody and carbohydrate-lectin reactions in immunohistochemistry and lectin histochemistry must be identified by microscopically visible labels. This identification can be made by binding visible markers to the lectins or antibodies. Binding sites are disclosed by either direct or indirect methods.

In direct methods a label is linked directly to the antibody or lectin. The labels used for this method consist of fluorochrome, enzyme or gold. The fluorochrome conjugates directly to antibody or lectin and is then applied to the tissue. This method needs expensive equipment (a fluorescence microscope) and also needs a high concentration of antibody or lectin.

Enzymes, such as horseradish peroxidase, are the most commonly used for direct disclosing. The peroxidase reaction is a very simple and stable reaction, but requires blocking of endogenous peroxidase by 0.3% (v/v) hydrogen peroxide in water or methanol prior to staining. It also requires a high concentration of lectin.

The use of gold particles is another direct disclosing method and is a very sensitive technique. It is suitable for electron microscopy at medium and high power but it is expensive and gold-protein linkage is unstable causing failure of disclosure or producing high background staining.

Indirect disclosing methods include one-step antibody, two-step antibody, sugar-antibody/lectin-sugar sandwich and avidin-biotin systems. In the one-step antibody technique, an antibody is raised against the antigen/lectin and labelled with a marker such as fluorochrome, enzyme or gold. It is more expensive but more sensitive than direct methods. However it is not completely specific and needs additional controls.

The two-step antibody technique uses a second antibody against the primary anti-antibody/lectin, for which PAP (peroxidase-anti-peroxidase) or APAAP (alkaline phosphatase-anti-alkaline phosphatase) enzyme visualising systems are required. It is more sensitive than the direct method, but it is not completely specific. In addition, it is expensive where multiple lectins are used as each lectin needs its own antibody.

In the sandwich technique, lectin is disclosed by conjugated peroxidase. Peroxidase has been conjugated to a specific sugar for the lectin. It is not necessary for a few lectins, such as LCA and PSA, because peroxidase contains the glycans that can bind to those lectins.

In the avidin-biotin system, antibodies or lectins bound to biotin are used. The biotin is disclosed by peroxidase attached to avidin or streptavidin. Avidin is a glycoprotein, which consists of four subunits. Each subunit represents a single polypeptide chain and includes an oligosaccharide containing mannose and glycosamine. Four hydrophobic pockets are present on the surface of the avidin molecules, which are able to bind to biotin residues (Green 1975). The structure of streptavidin is similar to avidin, but it does not contain oligosaccharide residues and has a neutral isoelectric point, which prevents it from non-specific binding (Chaiet and Wolf 1964). Avidin can be used as long as the molarity of the diluting buffer is increased which prevents any non-specific binding (Jones et al 1987).

Biotin is a water-soluble vitamin, which enables it to bind to one of the four binding sites of the avidin or streptavidin molecule (Bayer and Wilchek 1980). The reaction is via non-covalent bonds and is extremely rapid and strong. Biotinylation is a conjugation of biotin with a variety of molecules, such as enzymes or lectins. Because of the small size of biotin molecules more than 100 molecules can be bound with each antibody or lectin molecule for biotinylation but additional spacer arms maintain a distance between the biotin molecules.

2.3. Immunohistochemistry

Immunohistochemical staining methods are specialised staining techniques using specific antibodies. The antibodies used in immunohistochemical staining belong to the IgG group of immunoglobulins. Polyclonal antibodies are mostly produced by lymphocytes and react with various antigens. The most frequently used animal for the production of polyclonal antibodies is the rabbit, followed by goat, pig, sheep, horse, guinea pig and others (Boenisch 1989).

Clones of plasma cells produce monoclonal antibodies. They are immunohistochemically identical and react with a specific antigen. Mice are used as the source of cells for the production of monoclonal antibodies (Boenisch 1989).

In the present study immunohistochemical staining was used to find out which type of amyloid was deposited in the joint tissues (articular cartilage, fibrocartilage, synovial tissue and tendons). The major fibril protein component of amyloid has been regarded as non-immunogenic because of the lack of tissue response to its presence, and also the

inability to raise a specific antibody to intact amyloid fibril protein in experimental animals (Francis 1996). The marked resistance of amyloid fibrils to enzymatic degradation, and the relatively poor antigenicity of the variable region of immunoglobulin short chains, have been suggested to account for this lack of immune response. This has now been overcome by partial chemical degradation of purified amyloid fibril protein to produce low molecular weight fragments or monomers, which are antigenic. Antibodies have now been produced and used experimentally to detect amyloid with a greater sensitivity and selectivity than standard histological methods. They frequently detect even intracellular amyloid fibrils, which showed no congophilia (Zucker-Frankin and Frankin 1970). These antibodies work in formalin-fixed paraffin-embedded tissue sections. The monoclonal antibodies, which were used in this study, consisted of anti-AA amyloid (diluted in dilution buffer 1/100 v/v), anti-β2 microglobulin (diluted 1/100 v/v), anti-κ light chain (diluted 1/800 v/v), anti-λ light chain (diluted 1/1000 v/v) and anti-P component (dilutes 1/300 v/v), to find the types of amyloid which exist in the sections. The data sheets for these antibodies are shown in Appendix B. Standard procedures were used to find types of amyloid deposit in targeted sections (see page 105).

One other immunohistochemical staining reaction was used in the present study with anti-CD68 antibody. Anti-CD68 antibody is a mouse monoclonal anti-human antibody. It is suitable for immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections only for *in vitro* study. This antibody reacts with CD68 antigen, which is a highly glycosylated transmembrane protein, mainly located in the cytoplasm of cells. This antibody detects macrophages in a wide variety of human tissues (Audran et al 1995).

2.3.1. Controls for immunohistochemical and lectin histochemical staining

Any immunohistochemistry and lectin histochemistry staining must simultaneously satisfy two independent requirements, method specificity and antibody or lectin specificity. Method specificity was tested by simply omitting the primary antibody or the lectin from one slide in every regular staining run (negative control). For lectins this control slide was kept in TBS (TRIS-buffered saline), 0.05M, pH 7.6, with calcium chloride (0.1M), when lectins were applied on the sections and for antibody the control slide was kept in dilution buffer (0.05M TBS pH 7.4-100ml, bovine serum albumin-0.5g and sodium azide-0.1g). The highest dilution of primary antibody is used in the positive control, which still stains the antigen in sections with target antigen (detectable antigen).

As positive controls in immunohistochemistry, the tissues used were bone marrow for β2-M, kidney for λ light chain, liver for κ light chain and tongue for AA amyloid. Separate positive controls were not needed for lectin histochemistry as other tissues in the sections acted as controls.

Antibody specificity was confirmed by the technique of absorption (or saturation) for anti β2-M (using human β2 microglobulin), and for anti κ and λ light chains (using κ and λ light chains human myeloma plasma). The specific antigen was added to a solution of the antibody in five different dilutions. One dilution was the same as the working dilution of the antibody, others were x ½ dilution, x ¼ dilution, x 2 dilution and x 4 dilution. These mixtures were added to sections of the same test tissues and left overnight at 4°C. The staining was blocked in three dilutions of antigen, the same dilution with antibody, x 2, and x 4 dilution of antibody.

Control of lectin specificity had been tested by competitive inhibition with free sugars. For each lectin, the biotinylated lectin was applied to one section with its specific inhibitory sugar (see Table 2.1). The results were negative or decreased staining for lectin, which showed the lectin binds via its specific sugar-binding site.

Table 2.1. Specific inhibitory sugars for lectin staining

Lectin	Specific inhibitory sugar	Concentration of inhibitory sugar
HHA, LCA, PSA	α-methylmannopyranoside	0.2M
UEA-I and AAA	L-fucose	0.2M
DBA, VVA, MPA, SBA, HPA, WFA	α-N-acetylgalactosamine	0.2M
ECA, CTA and PNA	D-Galactose	0.2M

2.4. Lectins

Lectins are cell-agglutinating proteins or glycoproteins, particularly haemagglutinin, which were found first in plants (Lis and Sharon 1998). Various characteristics have been found for lectins, which demonstrate that lectins have chemical properties and biological functions previously overlooked. First they were used as a toxic material that would be a useful model antigen for the solution of immunological problems caused by toxins of bacteria. Lectins injected into animals stimulated the production of antibodies and they inhibited both their toxic and agglutinin activities. They were used to show how immunity to the toxins transfers from a mother to her child in pregnancy and by milk (Kocourek 1986). The sugar specificity of lectins was discovered as their next important characteristic and then a few years later, lectins were discovered to be agglutinins specific for human blood groups (Sharon and Lis 1989). A later discovery was that the

lymphocytes were stimulated to undergo mitosis by some lectins and then it was reported that lectins agglutinated malignant cells but not normal cells. The mitogenic and agglutination characteristics were then attributed to new and previously undiscovered lectins (Kocourek 1986). Other lectins have been extracted from mammalian tissues, which probably work as clearance agents in the circulatory system. These characteristics of lectins showed their usefulness in detecting and studying carbohydrates in matrix and on cell surfaces. In addition, investigations of the interaction of lectins with carbohydrates are providing information on the precise molecular details of the macromolecules in general, and in relation to cells. Therefore they serve as invaluable tools in biological and medical research.

2.4.1. Lectin specificity

Lectins are highly specific carbohydrate-binding proteins or glycoproteins of non-immune origin and have been used to study carbohydrate components in tissue at the light microscopical level (Goldstein and Poretz 1986). Lectins can be used as histochemical markers to demonstrate glycoconjugate distribution in tissue to obtain more specific information on the localization of carbohydrate moieties of macromolecules (Strosberg et al 1983). A number of different lectins have been used to study the complex carbohydrates of cartilage, cells and matrix, taking account of their ability to agglutinate cells or precipitate glycoconjugates. Lectins are sensitive and specific tools for the study of degenerative joint disease (Lyons 2000).

The high specificity of lectins to certain glycoproteins has been well investigated and this is the main advantage of the lectin as a histological probe in comparison to the other staining methods for carbohydrates. Binding may involve several forces, mostly hydrophobic and hydrogen bonds. Rarely electrostatic forces are involved, because most carbohydrates are devoid of electrical charges. Lectins have a similar degree of specificity as monoclonal antibodies. They are usually smaller molecules than antibodies, but their binding sites are larger than that of antibodies (Mirelman and Ofek 1986).

According to specificity, lectins can be classified into the following groups (Lis and Sharon 1998):

1. Mannose binding lectins
2. L-Fucose binding lectins
3. N-Acetylneuraminic acid binding lectins
4. Galactose binding lectins
5. N-Acetylgalactosamine binding lectins
6. N-Acetyllactosamine binding lectins.

2.4.2. Lectins used in this study

The lectins used in this study, shown in Table 2.2, were chosen from different groups as defined by their sugar-binding affinities. Most of the lectins were obtained from **Sigma**, and a few of them from **Boehringer Mannheim** and **Vector Laboratories** (details in Appendix A).

2.4.2.1. Group 1: Mannose-binding lectins to subsets of N-glycans

Hippeastrum hybrid agglutinin (HHA):

This lectin is extracted from amaryllis bulbs (*Hippeastrum hybrid*). It is a mannose-binding lectin, of which the carbohydrate specificity is for non-reducing terminal α-D-mannose probably α1,2, α1,3, and α1,6 linked (Kuku et al 1990).

Pisum sativum agglutinin (PSA):

It is derived from garden peas (*Pisum sativum*). It is composed of two subunits and total molecular weight of 49 kD. Like HHA, it is a mannose-binding lectin. The carbohydrate specificity for this lectin is for bi/tri-antennary non-bisected complex N-glycans with core fucosylation. It 'prefers' one or two terminal mannose residues, but it can bind with terminal substitution of the outer chain with either galactosamine or N-acetylgalactosamine (Kornfeld et al 1981).

Lens culinaris agglutinin (LCA):

LCA is obtained from lentils (*Lens culinaris*). The total molecular weight of the LCA is 49 kD and it contains two subunits. This N-glycan-binding lectin prefers to bind to complex N-linked sequences rather than to simple high mannose residues. LCA binds to bi/tri antennary non-bisected sequences especially those with terminal GlcNAc linked with fucose (Kornfeld et al 1981).

Phaseolus vulgaris haemagglutinin (e-PHA):

This mannose-binding lectin is derived from kidney beans (*Phaseolus vulgaris*). It is composed of four subunits with a total molecular weight of about 128 kD. E-PHA (the erythroagglutinin) binds to bi/tri antennary bisected complex N-glycans. E-PHA binds preferably to glycans with bisection at the β-mannose with GlcNAc (Yamashita et al 1983).

Phaseolus vulgaris leukagglutinin (l-PHA):

L-PHA binds to GlcNAcβ1,2Man in tri/tetra antennary non-bisected complex N-glycans, unless there is a nearby terminal sialic acid residue. Its structure and molecular weight is similar to e-PHA. L-PHA is more likely to bind to tetra antennary structures (Yamashita et al 1983).

2.4.2.2. Group 2: Fucose-binding lectins

Ulex europaeus (UEA-I):

This lectin is extracted from the seeds of gorse (*Ulex europaeus*). The molecular weight of this glycoprotein is 46 kD; it contains 7.2% (w/w) carbohydrate. The carbohydrate-binding specificity of this lectin is for fucosyl residues attached to β-galactose terminals of outer chains and especially for L-fucosyl termini linked to α1,2 to Galβ1,4GlcNAc. It has less affinity to the β-GlcNAc residue N-glycosidically linked to asparagine in the protein core. It also shows the ability to agglutinate red cells of people with A and AB blood groups, and has high affinity for blood group O structures, especially type 2 antigen (Pereira et al 1978).

Anguilla anguilla agglutinin (AAA):

This is obtained from the freshwater eel (*Anguilla anguilla*). It is a dimer with identical subunits. The molecular weight of AAA is about 140 kD, and each subunit is approximately 40 kD. AAA has higher affinity for fucosylated type I chains in blood group H structures but also binds to blood group B type I and type II chains (Kelly 1984).

2.4.2.3. Group 3: α-N-Acetylneuraminyl-binding lectins

Maackia amurensis agglutinin (MAA):

Two lectins have been extracted from the seeds of the leguminous plant (*Maackia amurensis*). Both are composed of four subunits about 33-37 kD. One is a strongly haemagglutinating lectin and the other is mitogenic. The carbohydrate specificity is NeuNAcα2,3Galβ1- having affinity for tetrasaccharides and trisaccharides. The haemagglutinating lectin is one of the few plant lectins to bind sialic acid (Sata et al 1989).

2.4.2.4. Group 4: β-Galactosyl-binding lectins

Erythrina cristagalli agglutinin (ECA):

This lectin is obtained from the seeds of the coral tree (*Erythrina cristagalli*). The molecular weight of this glycoprotein is about 58 kD and it is composed of two subunits. It has no true monosaccharide inhibitor. It binds specifically to the disaccharide Galβ1,4GlcNAcβ1-. Binding is higher with two or more disaccharides on separate outer chains of the oligosaccharides (Iglesias et al 1982).

Erythrina corallodendron agglutinin (CTA):

This lectin is extracted from seeds of the West Indian coral tree (*Erythrina corallodendron*). It contains two subunits with molecular weights of 30 kD, which are composed of one carbohydrate binding site as well as Mn^{2+} and Ca^{2+} binding sites that are

essential for the lectin activity. Terminal Galβ1,4GlcNAc, especially in multiple branches, is the specific carbohydrate binding site of CTA (Gilboa-Garbar and Mizrahi 1981).

Arachis hypogaea agglutinin (AHA):

It is extracted from peanuts (*Arachis hypogaea*) and it is also referred to as peanut agglutinin (PNA). The molecular weight of this glycoprotein is 110 kD with four subunits. It binds specifically to terminal Galβ1,3GalNAcα1, but a recognition site is often masked by terminal sialic acid residues thus requiring pre-treatment with neuraminidase for its identification. It has a much lower affinity for Galβ1,4GlcNAcβ1 (Lotan et al 1975).

2.4.2.5. Group 5: α-2-Deoxy,2-acetamido-galactose-binding lectins

Dolichos biflorus agglutinin (DBA):

This lectin is extracted from the seeds of the horse gram plant (*Dolichos biflorus*). This glycoprotein contains four subunits with 2% (w/w) carbohydrate and its molecular weight is 140 kD. Specific carbohydrate binding of this lectin is α-2-deoxy,2-acetamido-galactose (N-acetylgalactosamine), and it is greatly increased with fucosyl substitution of the subterminal galactosyl residue linked to GalNAc. This lectin is capable of agglutinating red blood cells of the blood group A. It recognises both α and β anomers. DBA shares staining affinities with fucose-binding lectins, because its binding site has sites for GalNAc and fucosyl residues (Hammerstrom et al 1977).

Vicia villosa agglutinin (VVA):

It is obtained from the hairy vetch plant (*Vicia villosa*). Like DBA, specific binding is to N-acetylgalactosamine with high specificity for α anomers (Tollefsen and Kornfeld 1983). VVA binds preferentially to the disaccharide of N-acetylgalactosamine α1,3 linked to terminal galactose and terminal N-acetylgalactosamine. Hydrophobic interaction may also be important for binding.

Maclura pomifera agglutinin (MPA):

This lectin is extracted from seeds of the osage orange tree (*Maclura pomifera*). The total molecular weight of this lectin is 40 kD and it contains two subunits. It binds to N-acetylgalactosamine with various linkages both terminal and internal. It can also bind with low affinity to terminal galactose (Young et al 1989).

Glycine max agglutinin (SBA):

It is obtained from soybeans (*Glycine max*). The molecular weight of this glycoprotein is 110 kD and it consists of four subunits. It binds to terminal N-acetylgalactosamine

residues, but it may also weakly bind to N-acetyllactosamine and terminal α or β linked galactosyl residues (Hammerstrom et al 1977).

Helix pomatia agglutinin (HPA):

It is obtained from the edible snail (*Helix pomatia*), and has a molecular weight of 79 kD. It contains six polypeptide chains with molecular weight of 13 kD, which contains one intra-chain disulphide bond and a single carbohydrate binding site. It has carbohydrate specificity for terminal N-acetylgalactosamine. It has been considered as a useful marker for breast cancer (Baker et al 1983).

Wisteria floribunda agglutinin (WFA):

This lectin is extracted from seeds of Japanese wisteria plant (*Wisteria floribunda*). The molecular weight of this glycoprotein is about 70 kD and contains two subunits with molecular weights of 32 kD. It binds to N-acetylgalactosamine-containing glycoproteins, preferring terminal N-acetylgalactosamine in the α1,6 linkage more than the α1,3 linkage. WFA is a haemagglutinin, which agglutinates human A, B and O erythrocytes non-specifically, but has no mitogenic activity (Baker et al 1983).

2.4.2.6. Group 6: N-acetyllactosamine-binding lectins

Datura stramonium agglutinin (DSA):

This lectin is derived from seeds of the Jimson weed (*Datura stramonium*). It binds to N-acetyllactosamine. This lectin has affinity for highly branched saccharides with at least one Galβ1,4GlcNAc repeating unit (Yamashita et al 1987).

Table 2.2. Lectins used in this study and their specificities

Abbreviation	Source	Carbohydrate Specificity
HHA	*Hippeastrum* hybrid /amaryllis	Non-reducing terminal αDMan in α1,2, α1,3 and α1,6 linkages
PSA	*Pisum sativum* /garden pea	αDMan in non-bisected bi/tri antennary, complex N-glycans with core fucosylation
LCA	*Lens culinaris* /lentil	Similar to PSA, 'prefers' terminal GlcNAc
e-PHA	*Phaseolus vulgaris* haemagglutinin /kidney bean	Bi/tri antennary, bisected complex N-glycans
l-PHA	*Phaseolus vulgaris* leukagglutinin /kidney bean	GlcNAcβ1,2Man in tri/tetra antennary, non-bisected complex N-glycans, unless there is a proximate sialyl residue
UEA-I	*Ulex europaeus* /gorse	α-L-Fucosyl termini especially L-Fucα1,2Galβ1, 4GlcNAcβ1
AAA	*Anguilla anguilla* /freshwater eel	α-L-Fucosyl termini (especially where clustered)
MAA	*Maackia amurensis*	NeuNAcα2,3Galβ1-
ECA	*Erythrina cristagalli* /cocks comb coral tree	Galβ1,4GlcNAcβ1- (and Galα1,3Galβ1,4GlcNAcβ1-)
CTA	*Erythrina corallodendron* /West Indian coral tree	Galβ1,4GlcNAc- especially in multiple branches
AHA (PNA)	*Arachis hypogaea* /peanut	Galβ1,3GalNAcα1- > Galβ1,4GlcNAcβ1-
DBA	*Dolichos biflorus* /horse gram	GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1
VVA	*Vicia villosa* /hairy vetch	GalNAcα1,3Galβ1- > GalNAcα1,6Gal-
MPA	*Maclura pomifera* /osage orange	Galβ1,3GalNAcα1- and GalNAcα1 > Galα1
SBA	*Glycine max* /soybean	Terminal GalNAcα1- > Galα1- or GalNAcβ1- in longer chains
HPA	*Helix pomatia* /edible snail	GalNAcα1-
WFA	*Wisteria floribunda* /Japanese wisteria	GalNAcα1,6Galβ1- > GalNAcα1,3Galβ1-
DSA	*Datura stramonium* /jimson weed	$(-4GlcNAc\beta1-)_{2-4}$ also to NAcLac Galβ1,4GlcNAcβ1-

2.4.3. Specific pre-treatments

Adjacent sugars in a saccharide chain may have a noticeable effect on the binding of a lectin to a specific sugar sequence, either enhancing or diminishing it. In order to determine further staining, tests were carried out on samples after pre-treatment of the sections with β-elimination or hyaluronidase followed by 12 different lectins.

Using alkaline treatment (β-elimination reaction) makes it possible to identify whether glycans are O or N-linked to the backbone protein, because the O-linked glycans linked to core protein through serine and threonine are alkali-labile, while N-linked glycans, linked through asparagines, are not.

Hyaluronidase was used for enzyme pre-treatment. This enzyme catalyses the hydrolysis of endo-N-acetylhexosaminic bonds of hyaluronan and depolymerises hyaluronan monomers. The results of these pre-treatments could be a decrease or an increase in staining or no change in the level of staining. A decrease in staining is due to removal of some glycans, an increase in the staining may be due to exposure of new glycans, formerly blocked by others, and no change in the level of staining may be due to glycan removal equal to new exposure.

The standard procedures were used for these pre-treatments (see pages 108 and 109).

2.4.5. Consistency of observations and sources of error

The author and a qualified pathologist examined the histological and immunohistochemical staining reactions by light microscopy for diagnosis and grading sections of osteoarthritis tissues and the determination and typing of amyloid depositions. The lectin staining reactions were examined by the author and a second pathologist. The author diagnosed and graded sections of osteoarthritic tissues, determined the types of the amyloid deposition and ranked lectin intensity on several occasions for the purpose of measuring the reliability and the validity of her own observations. The sections were re-examined with observers, if disagreement was found in any aspect. These re-examinations improved the accuracy, consistency and reliability of the results.

2.5. Methodology

Sources of all chemicals, dyes and other materials used in the following procedures are given in Appendix A.

2.5.1. APES-coated slides for paraffin section adhesion

1-The slides were cleaned and degreased by treating them with 1% (v/v) acid alcohol (1% hydrochloric acid in 70% (v/v) industrial methylated spirit - IMS) for 2 minutes.

2-The slides were rinsed in 3 changes of tap water 1 minute each and once in distilled water.

3-They were rinsed in 99% (v/v) IMS for a quick dry.

4-They were dried in a 37°C oven.

5-The dry slides were placed in a freshly made solution of 3% (v/v) 3-aminopropyl-triethoxysilane (APES) in acetone for 2 minutes (9ml APES in 300ml acetone) in a fume cupboard.

6-The slides were rinsed in acetone quickly to remove excess APES.

7-They were washed in distilled water.

8-The slides were dried in the 50°C oven overnight

9-They were protected from dust by storing in the original slides boxes until required.

For immunohistochemistry and lectin histochemistry, the sections were floated on to APES-coated slides by using a water bath and they were dried in a 50°C oven at least overnight.

2.5.2. Haematoxylin and eosin

1-The sections were dewaxed in four changes of xylene 15 minutes each or overnight.

2-The slides were rehydrated in two changes of IMS, one change in 95% (v/v) IMS, followed by a rinse in running tap water.

3-The slides were stained in freshly filtered Mayer's haematoxylin for 5 minutes.

4-They were rinsed in hot running tap water until clear and blued.

5-The slides were stained in slightly acidified eosin for 30 seconds.

6-The slides were dehydrated in one change of 95% (v/v) IMS, two changes of IMS, the cleared in four changes of xylene using a fume cupboard.

7-They were mounted in DPX.

2.5.3. Toluidine blue (alcoholic)

1-The sections were dewaxed in four changes of xylene 15 minutes each or overnight and rehydrated in two changes of IMS and one change of 95% (v/v) IMS.

2-The slides were rinsed in tap water.

3-They were stained in toluidine blue solution (0.5% w/v in 0.5% v/v ethanol in distilled water) for 10 minutes.

4-The excess stain was removed by rinsing in several changes of distilled water.

5-The slides were dehydrated in 95% (v/v) IMS and two changes of IMS then cleared in four changes of xylene and mounted in DPX.

2.5.4. Alcian blue

1-The sections were dewaxed in 4 changes of xylene 15 minutes each or overnight and rehydrated in two changes of IMS and one change of 95% (v/v) IMS.

2-The slides were rinsed in tap water.

3-They were stained overnight in the three alcian blue solutions (0.05% w/v alcian blue 8GX in 0.05M, 0.5M or 0.9M $MgCl_2$ in 0.025M acetate buffer pH5.8).

4-The sections were rinsed in buffers of the same concentration for five minutes.

5-They were then rinsed in several changes of distilled water.

5-The slides were dehydrated in 95% (v/v) IMS and two changes of IMS then cleared in four changes of xylene and mounted in DPX.

2.5.5. Picro sirius red

1-The sections were dewaxed in four changes of xylene 15 minutes each or overnight and rehydrated in two changes of IMS and one change of 95% (v/v) IMS.

2-The slides were rinsed in tap water.

3-The nuclei were stained in celestine blue for 5 minutes.

4-The slides were rinsed in tap water.

5-They were stained in Mayer's haematoxylin for 5 minutes.

6-They were washed in running tap water for 30 minutes.

7-The slides were stained in picro sirius red solution (0.1% (w/v) sirius red in saturated aqueous picric acid) for 30 minutes.

8-They were dehydrated rapidly in three changes of absolute alcohol, cleared in 4 changes of xylene and mounted in DPX.

2.5.6. Alkaline Congo red

1-The sections were dewaxed in four changes of xylene 15 minutes each or overnight.

2-The slides were rehydrated in two changes of IMS, one change in 95% (v/v) IMS, followed by a rinse in running tap water.

3-The nuclei were stained in haematoxylin for 5 minutes, rinsed and blued in hot tap water.

4-The slides were incubated in salt-saturated, alkaline 80% (v/v) alcohol for 20 minutes.

5-They were transferred to 0.5% (w/v) Congo red in salt-saturated, alkaline 80% (v/v) alcohol and incubated for 20 minutes.

6-The slides were rinsed briefly in 80% (v/v), 95% (v/v), and two changes of undiluted IMS, cleared in xylene and mounted in DPX.

2.5.7. Immunohistochemical staining for amyloid

1-Paraffin sections were cut and mounted onto APES-coated slides. They were dried overnight at 50°C.

2-The sections were dewaxed in four changes of xylene 15 minutes each or overnight.

3-The slides were rehydrated in two changes of IMS, one change in 95% (v/v) IMS, followed by a rinse in running tap water.

4-Endogenous peroxidase activity was blocked by incubating sections in 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes.

5-The slides were rinsed in 95% (v/v) alcohol followed by tap water.

6-They were placed in 0.05M TBS pH 7.6 to equilibrate to the optimum pH for immunohistochemistry.

7-For pre-treatment, humidity chambers were prepared by wetting the tissue paper between the racks. The back of each slide and around each section was dried. A waxy line was drawn around the sections and type II (crude) trypsin (2 tablets in 2ml distilled water) was put on them and left at 37°C for 12 minutes.

8-The slides were transferred to 0.05M TBS for 5 minutes.

9-To block non-specific staining the slides were put back in the humidity chambers and a drop of normal swine serum (NSS, if primary antibody was polyclonal) or normal goat serum (NGS, if primary antibody was monoclonal) was placed on each section and left for 20 minutes.

10-Excess NSS or NGS was tipped off and treated with polyclonal or monoclonal primary antibody diluted in dilution buffer (0.05M TBS pH 7.4-7.6 - 100ml, bovine serum albumin - 0.5g and sodium azide - 0.1g) and incubated overnight at 4°C. One slide was included with diluent only – negative control.

11-Excess antibody was washed off by dipping each slide into a Coplin jar full of TBS. The slides were placed into a rack and washed x 2 in 0.05M TBS using the magnetic stirrer (3 minutes each).

12-The slides were placed in the humidity chambers again and 1/300 swine anti-rabbit (SAR, if primary antibody is polyclonal) or 1/300 goat anti-mouse (GAM, if primary antibody is monoclonal), diluted in dilution buffer, was applied for 40 minutes at room temperature.

13-They were washed x 2 in 0.05M TBS as before.

14-The slides were placed in the humidity chambers again and 1/500 streptavidin peroxidase, diluted in 0.05M TBS, was applied for 30 minutes.

15-They were washed x 2 in 0.05M TBS as before.

16-The slides were treated with DAB/hydrogen peroxide solution. A DAB tablet (5mg) and an H_2O_2/urea tablet were dissolved in 5ml distilled water and used to treat each section for 5 minutes. DAB is a suspect carcinogen: gloves were worn, and all DAB containers and any spillages were cleaned by soaking in bleach and rinsing in running tap water.

17-The slides were rinsed in running tap water for 2-3 minutes.

18-They were counterstained in Mayer's haematoxylin for 30 seconds, rinsed and blued in hot water and transfer to TBS for 30 seconds.

19-They were washed by tap water.

20-The slides were dehydrated, cleared and mounted in DPX.

2.5.8. Immunohistochemical staining for CD68

1-Paraffin sections were cut and mounted onto APES-coated slides. They were dried overnight at 50°C.

2-The sections were dewaxed in four changes of xylene 15 minutes each or overnight and rehydrated in two changes of IMS and one change of 95% (v/v) IMS.

3-The slides were rinsed in tap water.

4-Endogenous peroxidase activity was blocked by incubation in 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes.

5-The slides were rinsed in distilled water and placed in 0.05M TBS for 5 minutes.

6-The area of the slides around the sections was wiped, a waxy ring was drawn around the section, the slides were put in the humidity chambers and the sections covered with type II (crude) trypsin and incubated at 37°C for 12 minutes.

7-Each slide was dipped into a Coplin jar containing cold tap water, placed into a rack and washed in 0.05M TBS for 5 minutes.

8-The slides were wiped, put in the humidity chambers, the sections were covered with drops of CD68 antibody and incubated at room temperature for 60 minutes. A negative control section was covered by EPOS.

9-The slides were dipped into TBS in a Coplin jar to wash off the antibody, then rinsed in two changes of 0.05M TBS, each of 5 minutes.

10-The section were covered with DAB/hydrogen peroxide and incubated for 10 minutes at room temperature (5mg DAB tablet and H_2O_2/urea tablet in 5ml distilled water).

11-The slides were rinsed in distilled water.

12-The sections were counterstained with Mayer's haematoxylin for 1 minute.

13-They were rinsed in tap water and blued by TBS for 30 seconds.

14-The slides were dehydrated in 95% (v/v) IMS and then in IMS twice.

15-They were cleared in xylene and mounted in DPX.

2.5.9. Lectin histochemical staining

1-Paraffin sections were cut and mounted onto APES-coated slides. They were dried overnight at 50°C.

2-Sections were dewaxed in four changes of xylene for 15 minutes each or overnight.

3-Sections were rehydrated in two changes of IMS only.

4-Endogenous peroxidase activity was blocked by leaving slides in 300ml methanol plus 1.2ml 1N HCl, to which 3ml 30% (v/v) H_2O_2 was added just before use, for 30 minutes.

5-They were rinsed in tap water.

6-Type II (crude) trypsin was used for pre-treatment in a water bath. The water bath was switched on and two glass dishes of 300ml 0.05M TBS pH7.6 were put in. $CaCl_2$ (300mg) was added to one of them and the water bath and dishes were allowed to warm up to 37°C (about 30 minutes). After warming up, the slides were put into the TBS dish and 300mg trypsin was added to dish containing TBS and $CaCl_2$. After 5 minutes the slides were transferred to the TBS dish with $CaCl_2$ and trypsin and they were left for 15 minutes.

7-The slides were washed immediately in cold water to stop the reaction. Then they were washed in three changes of TBS, 5 minutes each.

8-A humidity chamber was prepared and around the sections was wiped, and then they were covered by biotinylated lectin (10µg/ml in 0.05M TBS with 1mM $CaCl_2$ pH7.6) for 30 minutes at room temperature.

9-The slides were washed gently with TBS and $CaCl_2$ and placed in a rack and washed in three changes of TBS + $CaCl_2$, 5 minutes each.

10-Sections were placed in the humidity chambers and wiped around, then they were covered by avidin peroxidase (5µg/ml) in 0.125M TBS (with 0.374 NaCl) pH7.6 for 60 minutes at room temperature.

11-Slides were washed with TBS from a wash bottle and placed in a rack and washed in three changes of TBS, 5 minutes each.

12-Slides were covered by DAB (280ml TBS + 150mg DAB in 20ml TBS pH 7.6 + 45 µl 30% (v/v) H_2O_2 added just before use) for 5 minutes.

13-They were washed in tap water and counterstained in 0.25% (w/v) methyl green for 30 sec.

14-Slides were washed in water and dehydrated in 70% (v/v) IMS and two changes of IMS.

15-They were cleared in xylene and mounted in XAM.

2.5.10. β-Elimination

This was modified from the standard method in stage 8 because the cartilage tended to float off when put in a 60°C oven.

1-Paraffin sections were cut and mounted onto APES-coated slides. They were dried overnight at 50°C.

2-The sections were dewaxed in four changes of xylene, 15 minutes each, or overnight and rehydrated in two changes of IMS and one change of 95% (v/v) IMS.

3-The slides were rinsed in tap water.

4-Endogenous peroxidase activity was blocked by incubation in 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes.

5-The slides were rinsed in distilled water and placed in two changes of 0.05M TBS for 3 minutes each.

6-The slides were rinsed briefly in distilled water.

7-The following mixture was made up and used to cover the slides:

50ml	DMSO (dimethylsulphoxide)
40ml	distilled water
10ml	IMS
0.954g	potassium hydroxide (final concentration is 0.17M).

(DSMO is a carcinogen and may have mutagenic or teratogenic effects. Always wear gloves and use in a fume cupboard.)

8-The sections were incubated in the above solution overnight at room temperature (modified by the author instead of leaving for 1 hour in 60°C oven).

9-The slides were washed very carefully with 10mM HCl (X 2) to neutralise.

10-The slides were rinsed in 0.1M sodium phosphate buffer with pH 7 (or PBS).

11-The slides were rinsed in distilled water, followed by TBS and they were then ready for normal lectin staining procedures as in Section 2.5.9 from step 6.

2.5.11. Hyaluronidase digestion of paraffin sections

1-Paraffin sections were cut and mounted onto APES-coated slides. They were dried overnight at 50°C.

2-The sections were dewaxed in four changes of xylene, 15 minutes each or overnight and rehydrated in two changes of IMS.

3-Endogenous peroxidase activity was blocked by incubation in 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes.

4-The slides were rinsed in distilled water and placed in 0.05M TBS for 3 min (X2).

5-The slides were rinsed briefly in distilled water.

6-The slides were covered by testicular hyaluronidase, 2000 units/ml in 0.05M sodium acetate buffer pH7.0, with proteinase inhibitors, at room temperature for 120 minutes.

7-They were rinsed in tap water and normal lectin staining procedures as in Section 2.5.9 from step 6.

Chapter 3

Results

Section 1

3.1. Sternoclavicular joint

In this study 60 sternoclavicular joints (from adults aged between 23-97, median 71) were investigated by a variety of techniques.

SCJs were X-rayed for initial assessment. Prepared sections were stained by haematoxylin and eosin (H&E), toluidine blue, picro sirius red, alcian blue (with three concentrations of magnesium chloride) and alkaline Congo red. Further investigations were by immunohistochemistry using anti-amyloid antibodies and by lectin histochemistry, using 14 different lectins.

3.1.1. Radiology of SCJ

X-rays of whole bilateral SC joints were made as initial assessments. This was done to obtain initial information about the degree and extent of articular damage and to provide a base line for the decalcification process. Typical feature are illustrated (Fig 3.1)

3.1.2. Microanatomy

The tissue sections of the SC joints stained by H&E were examined microscopically and divided into normal, mild to moderate and severe OA. Each joint contained two articular cartilage surfaces and fibrocartilage interposed between them. Overall grading was given on the basis of the grading of osteoarthritis proposed by Mankin et al (1971) (Table 3.1). It was very difficult to differentiate between the mild and moderate osteoarthritic changes therefore only three groups were considered after grading, normal articular cartilage, mild and moderate osteoarthritic changes and severe changes. See Table 3.3 for the grading assigned to each block.

In normal or nearly normal articular cartilage the distribution of chondrocytes in a full depth section differed from the superficial to the deep zone. Near the articular surface, chondrocytes were more numerous, had a discoid shape and were placed parallel to the surface. The number of chondrocytes decreased from the surface to the deep zone, and the deep zone matrix showed areas completely lacking in chondrocytes. The deep zone chondrocytes were arranged in units and circular clusters. These normal features were seen in 18 cases. Surfaces irregularities were seen in eight cases. The tidemark was intact in 52 cases.

Table 3.1. Histological-histochemical grading system for osteoarthritis

Category	Subcategory	Score
Structure	Normal	0
	Surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to transitional zone	3
	Clefts to radial zone	4
	Clefts to calcified zone	5
	Complete disorganisation	5
Cells	Normal	0
	Diffuse hypercellularity	1
	Cloning	2
	Hypocellularity	3
Safranin O staining	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No dye noted	4
Tidemark integrity	Intact	0
	Crossed by blood vessels	1
Total		**0-14**

Grade of osteoarthritis: 0-1 – normal, 2-5 – mild, 6-9 – moderate and 10-14 – severe.

In the present study, structure, cells and tidemark integrity in histological histochemical grading system (Mankin grading) were discovered using H&E staining. Toluidine blue staining was used instead of safranin O staining (section 3.1.3.1).

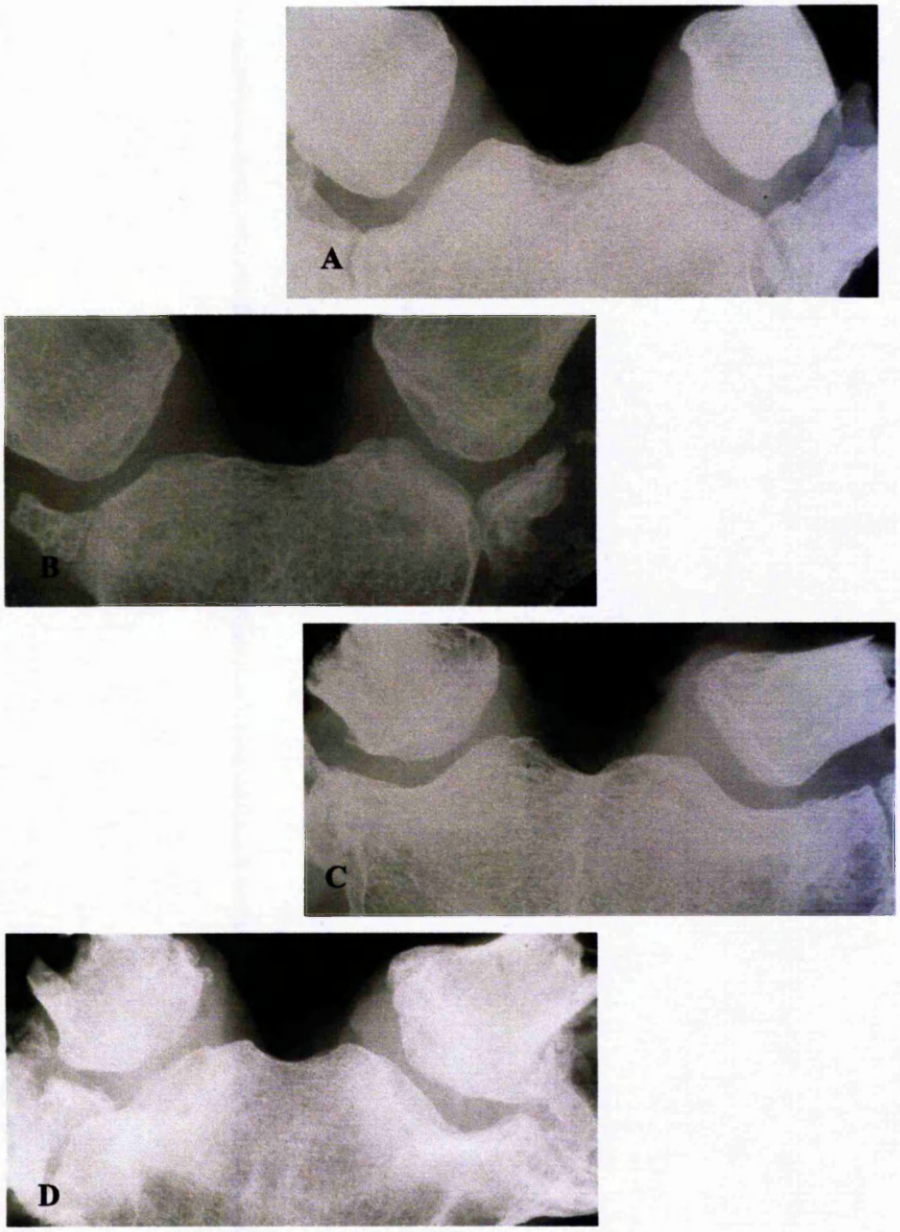


Figure 3.1 X-rays of the sternoclavicular joints.
A: Normal sternoclavicular joint. B,C: Mild to moderate osteoarthritic sternoclavicular joints. D: Severe osteoarthritic sternoclavicular joint.

H&E staining showed the microanatomical changes in SC joints affected by osteoarthritis. In OA, the chondrocytes in the surface were ellipsoid or round in shape, which was found along with hypercellularity. The normal arrangement of chondrocytes was disrupted in severe OA. Clones of chondrocytes were found in osteoarthritic cartilage, their frequency and size depending on the severity of the OA. Clones of chondrocytes were found predominantly nearer to the margins of clefts and fissures. These clones were seen in 78 cases. Hypocellularity was seen in 11 cases, but diffuse hypercellularity was seen in 15 cases. Surface irregularity and clefts were found in osteoarthritic cartilage and grading of OA was dependent on the irregularity and depth of the clefts in the zones. Clefts to the transitional zone were seen in 21 cases, clefts to the radial zone were seen in 14 cases and clefts to the calcified zone in 12 cases. Twenty-nine cases showed complete disorganisation in articular cartilage. Superficial and intramatrical fibrosis was found close to the fibrillated cartilage in severe OA.

The tidemark in osteoarthritic cartilage was duplicated or occasionally triplicated, and was crossed by blood vessels. These changes were seen in 68 cases. Subchondral cysts and osteosclerosis were seen in subchondral bone of osteoarthritic cartilage. Osteophytosis was found at the joint margins of severe osteoarthritic disease.

There is no standard technique for measuring OA changes in fibrocartilage so the changes described here were used. In normal fibrocartilage, the distribution of the chondrocytes was completely different from that of articular cartilage. The chondrocytes were usually single and dispersed randomly. Irregularity of the surface, clefts in fibrocartilage and clones of chondrocytes were taken to represent osteoarthritic changes in fibrocartilage. Surfaces irregularities were seen in 86 cases, clefts to fibrocartilage in 53 cases and clones of the chondrocytes in 69 cases.

In the first rib of normal cases, chondrocytes and territorial matrix were stained purple. Interterritorial matrix was stained homogeneously eosinophilic in all areas. The chondrocytes were dispersed randomly and were present in larger numbers than in articular cartilage and fibrocartilage.

Examples of these features are seen in Figures 3.2. to 3.22.

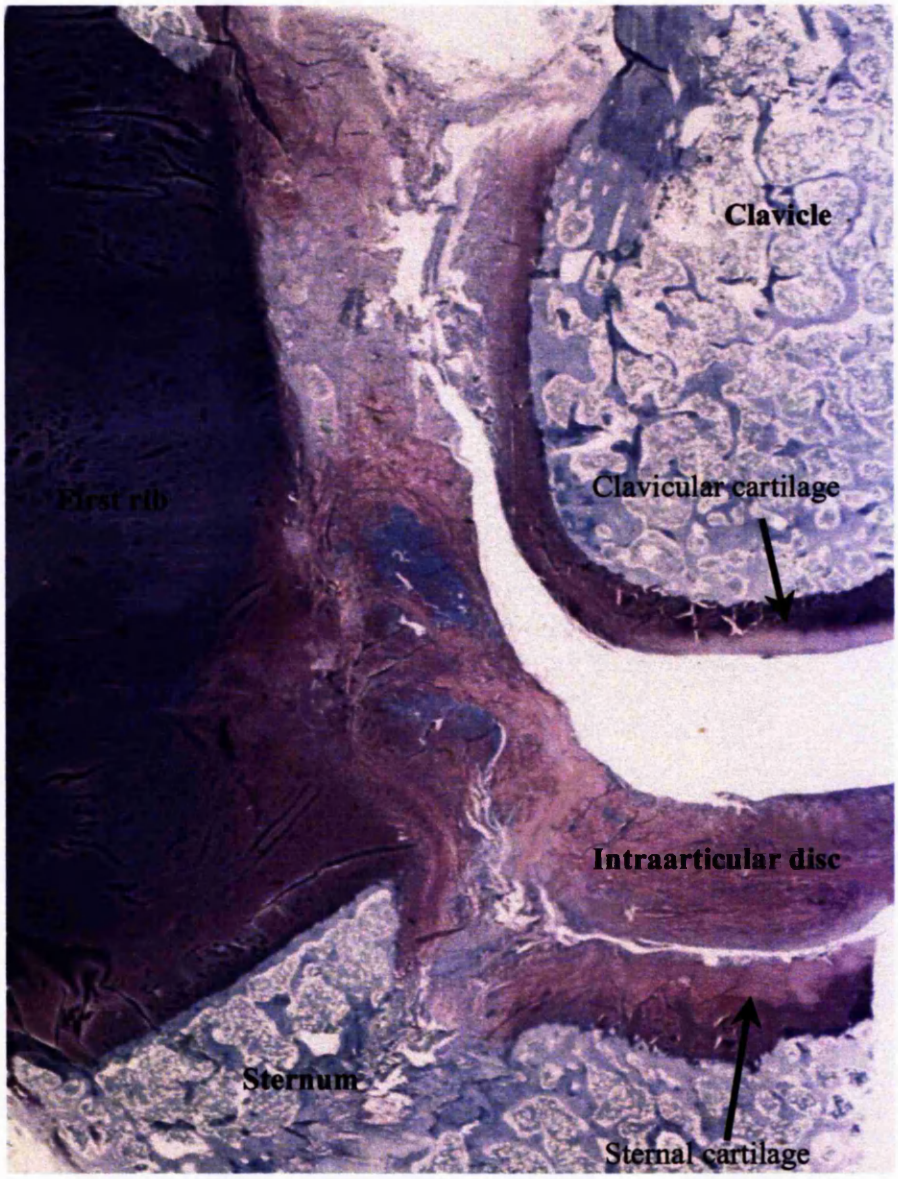


Figure 3.2. Inferior part of the right sternoclavicular joint (H&E X1). The normal structures of the sternoclavicular joint.

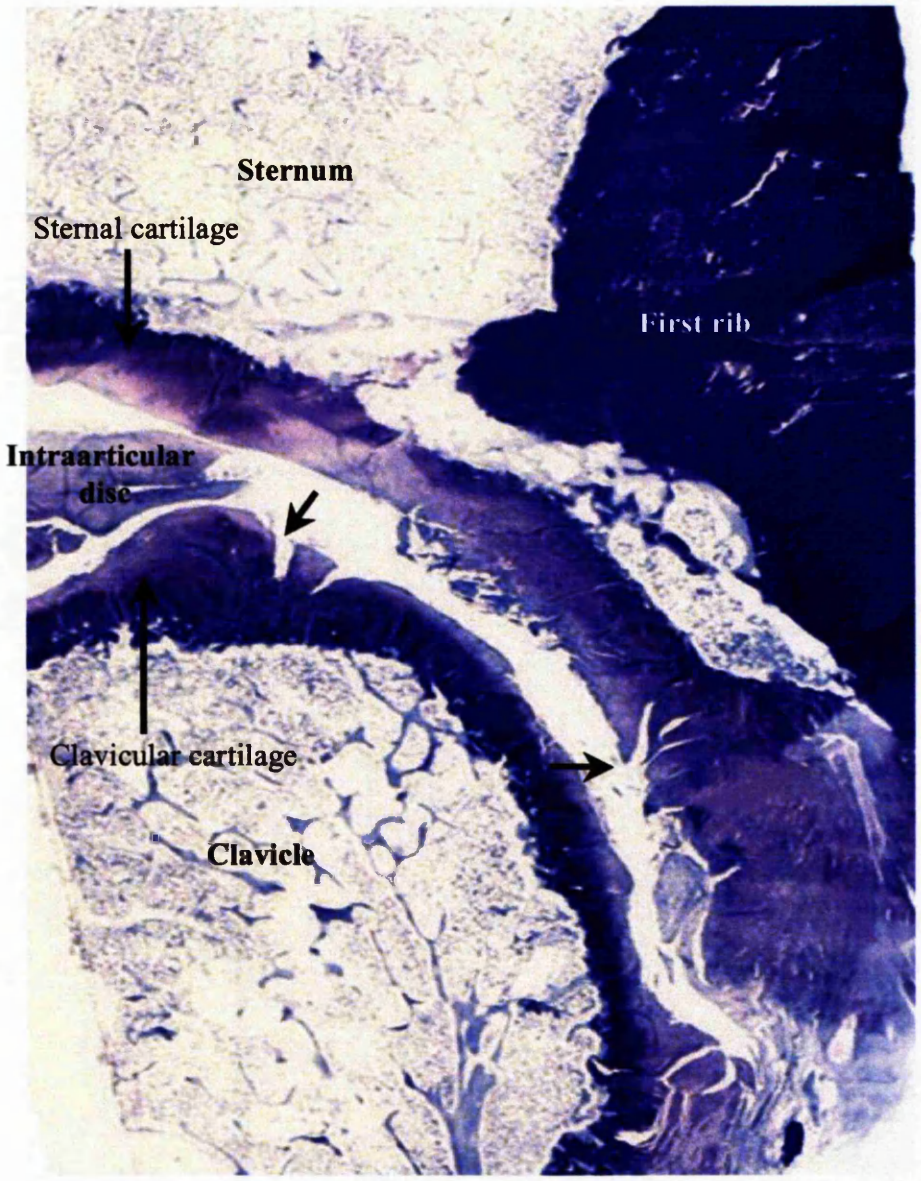


Figure 3.3. Inferior part of the right sternoclavicular joint (Toluidine blue X1). There are mild to moderate osteoarthritic changes in the articular cartilage (arrows) and splits in the intraarticular disc.

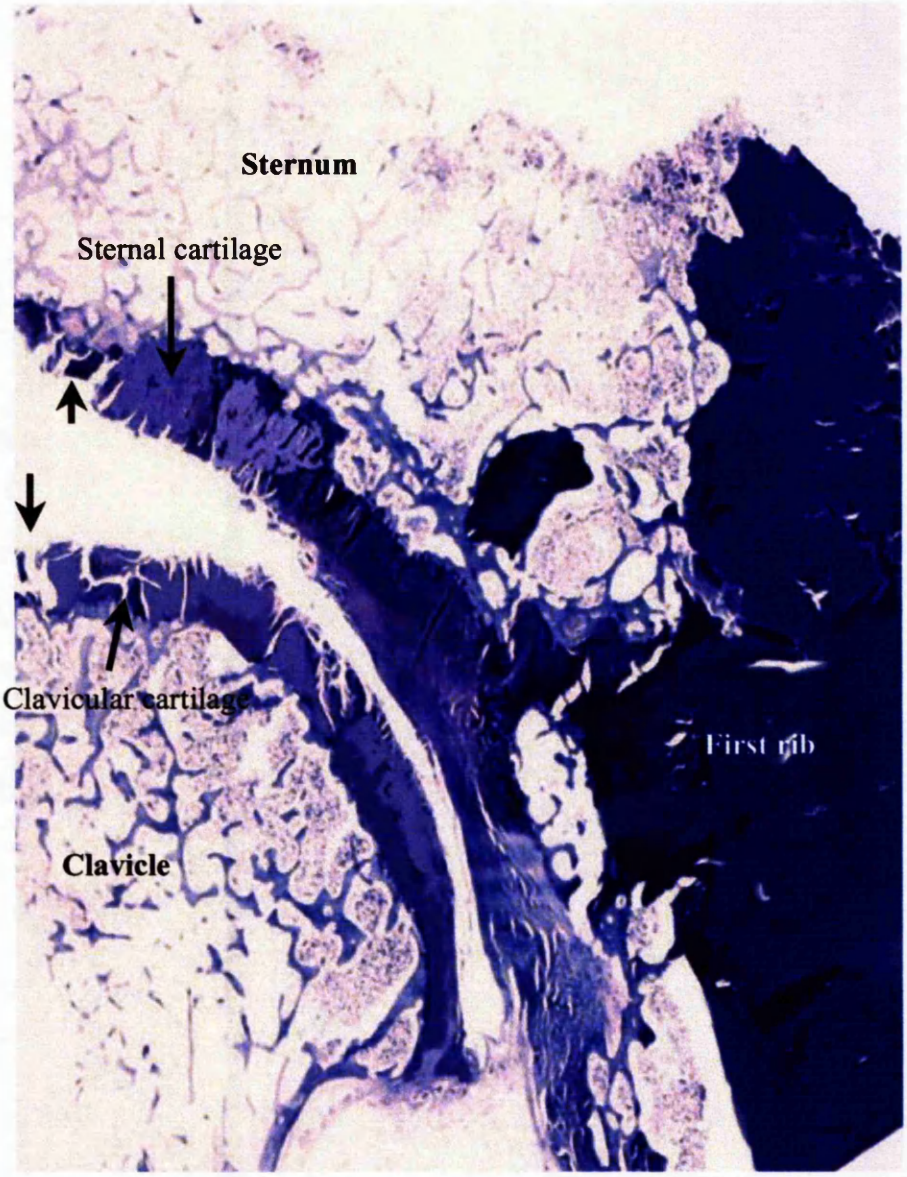


Figure 3.4. Inferior part of the left sternoclavicular joint (Toluidine blue X1). There are established osteoarthritic changes in the articular cartilage (AC) (arrows).

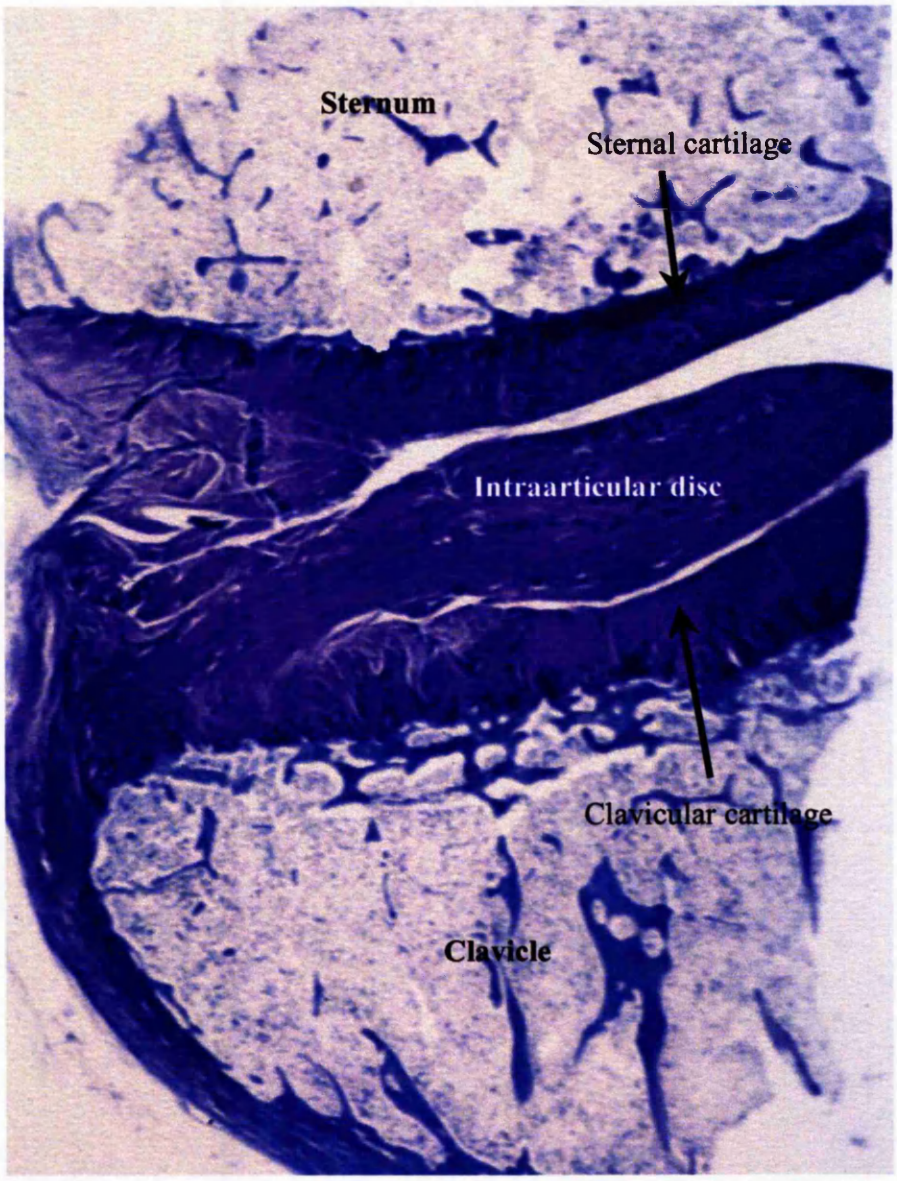


Figure 3.5. Superior part of the left sternoclavicular joint (toluidine blue X1). The normal structures of the sternoclavicular joint are shown.

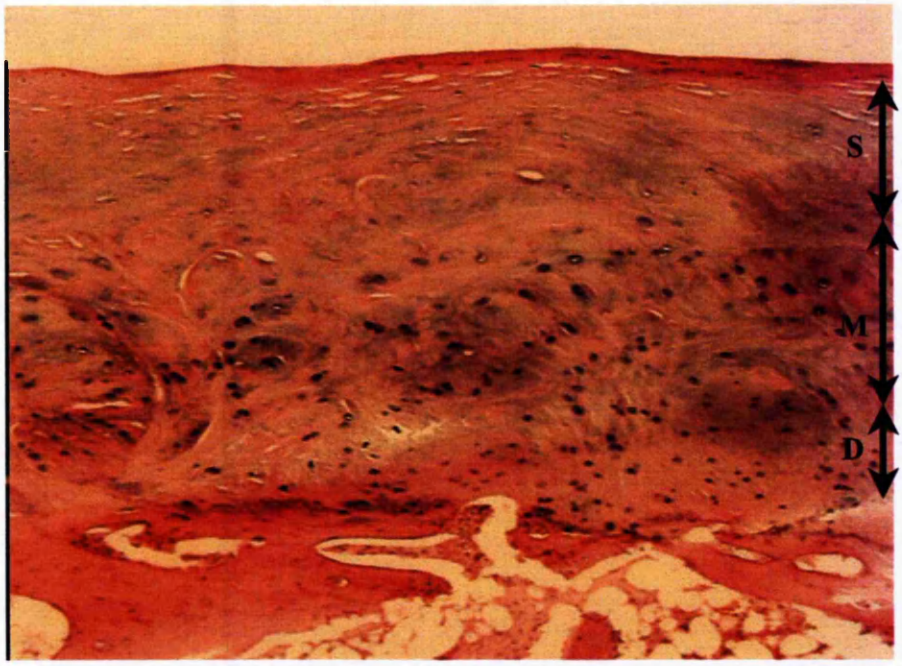


Figure 3.6. Normal clavicular articular cartilage (H&E X10).
The distribution of chondrocytes in various zones is shown (arrows).
S: superficial zone, M:middle zone, D:deep zone.

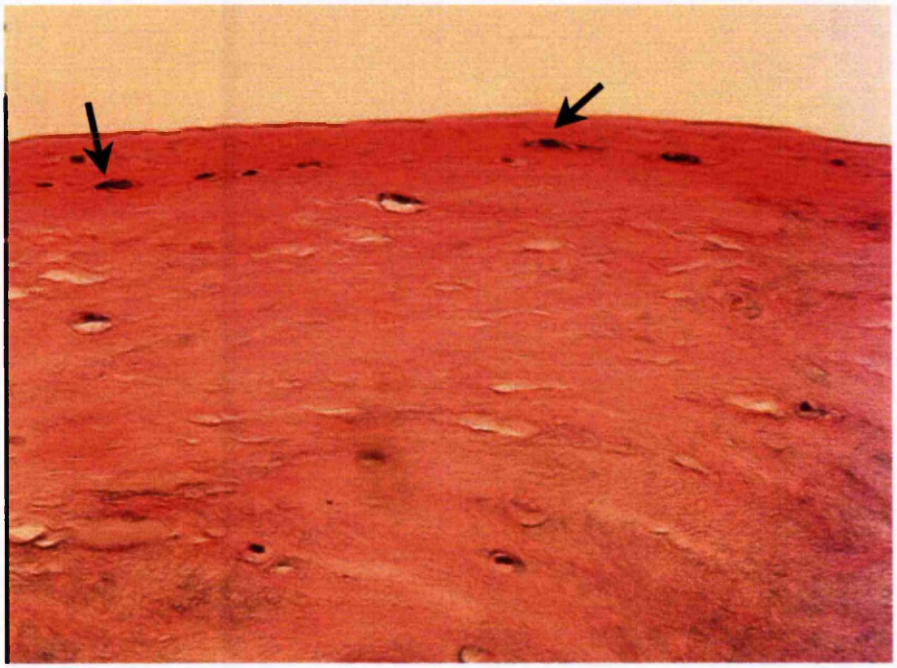


Figure 3.7. Normal superficial clavicular chondrocytes (H&E X20).
These are the flattened cells (arrows).

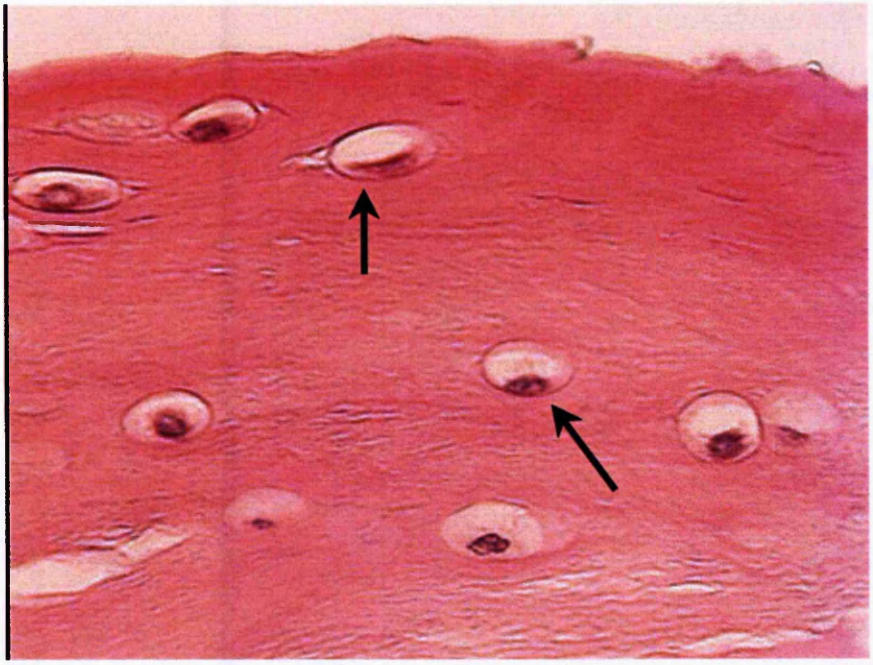


Figure 3.8. Abnormal superficial chondrocytes in mild osteoarthritic clavicular articular cartilage (H&E X40). In osteoarthritis the normally flattened chondrocytes become rounded and hypercellularity is seen (arrows).

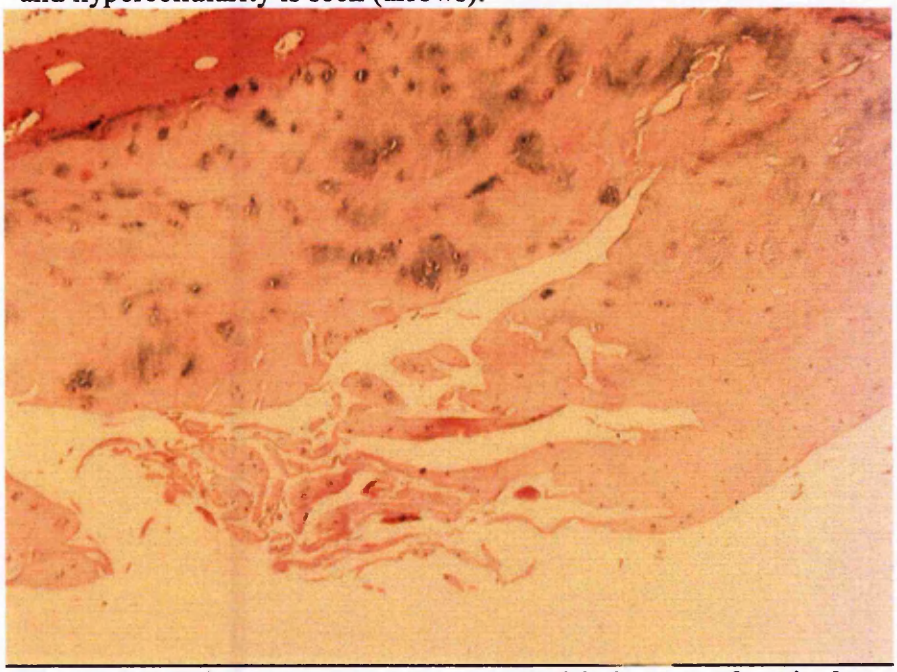


Figure 3.9. Mild to moderate osteoarthritis in sternal articular cartilage (H&E X5). Cleft to radial zone and clones of chondrocytes are seen.

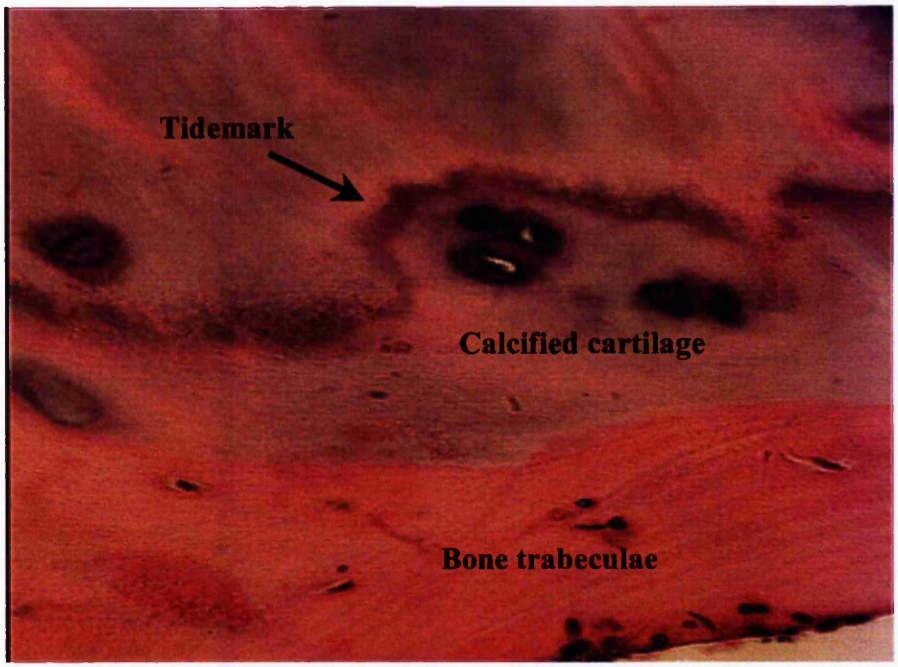


Figure 3.10. Normal tidemark in clavicular articular cartilage (H&E X40). The normal tidemark, calcified cartilage and subjacent bone trabeculae are shown.

Figure 3.11. Normal sternal articular cartilage (H&E X10). Medial and deep zone chondrocytes form group and columns (arrows).

Figure 3.12. Normal intraarticular disc in sternoclavicular joint (H&E X10). Chondrocytes (arrows) are randomly distributed.

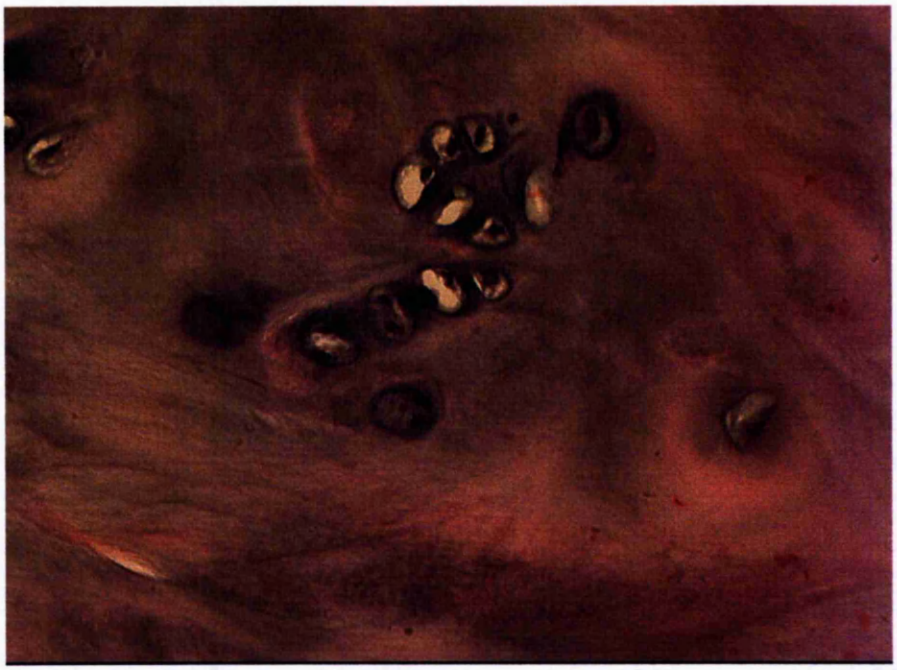


Figure 3.13. Normal chondrocytes in the middle zone of sternal articular cartilage (Toluidine blue X40).

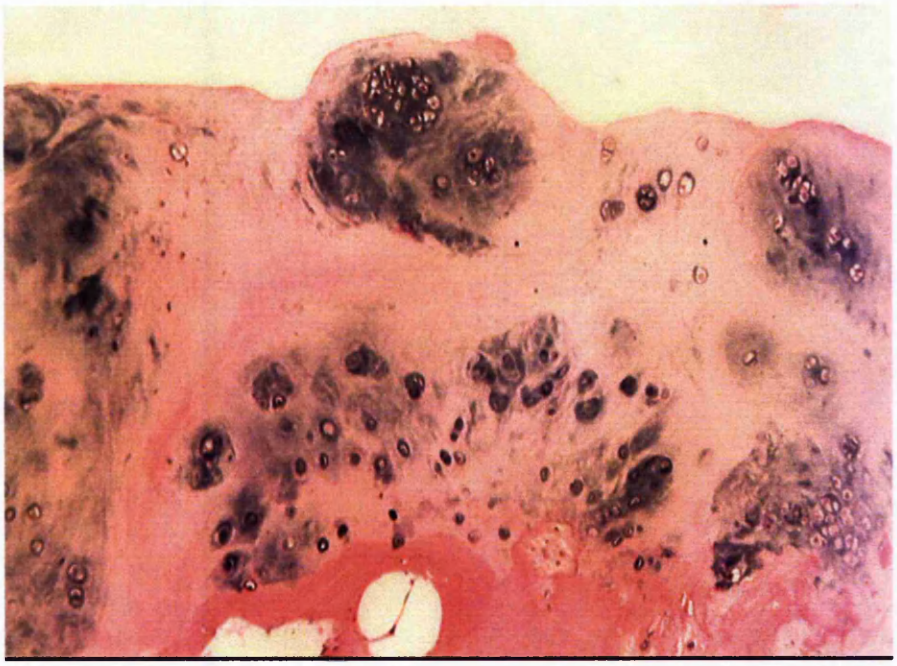


Figure 3.14. Clones of chondrocytes in moderate osteoarthritic clavicular articular cartilage (H&E X10).
Clones of chondrocytes are present mostly in the superficial zone.

Figure 3.15. Chondrocyte clone in severe osteoarthritic clavicular articular cartilage (H&E X40).
The new matrix is stained by haematoxylin instead of eosin.

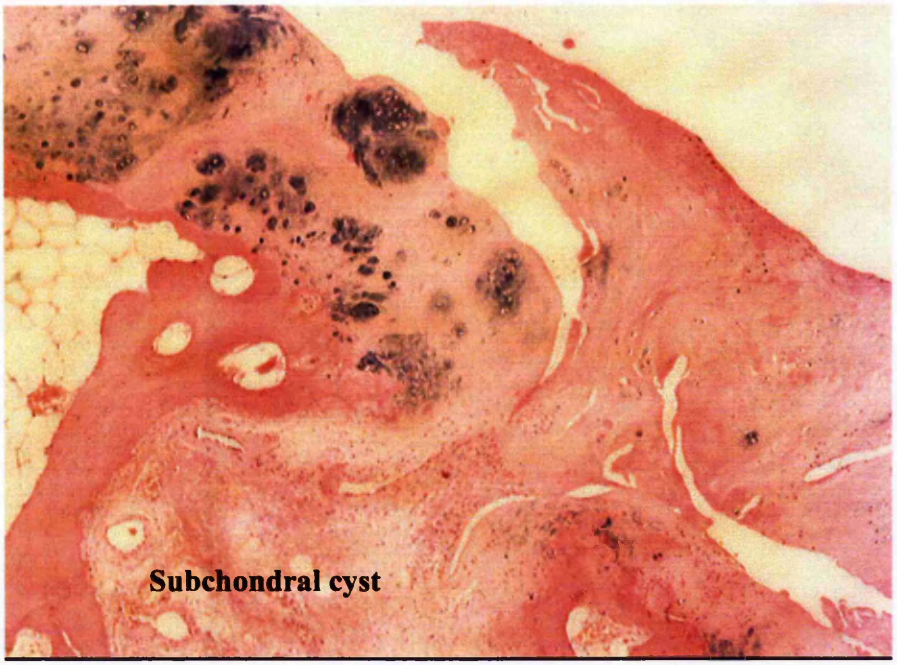


Figure 3.16. Severe OA changes in sternal articular cartilage (H&E X5). Complete disorganisation and clones of chondrocytes are present.

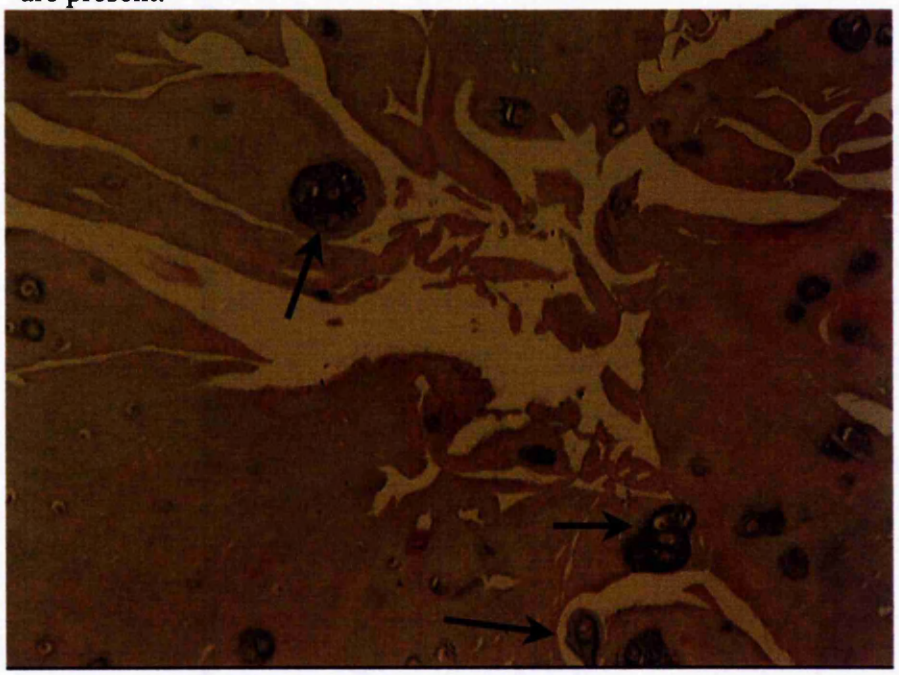


Figure 3.17. Clones of chondrocytes in severe osteoarthritic sternal articular cartilage (H&E X10). Complete disorganisation and clones of chondrocytes (arrows) are present.

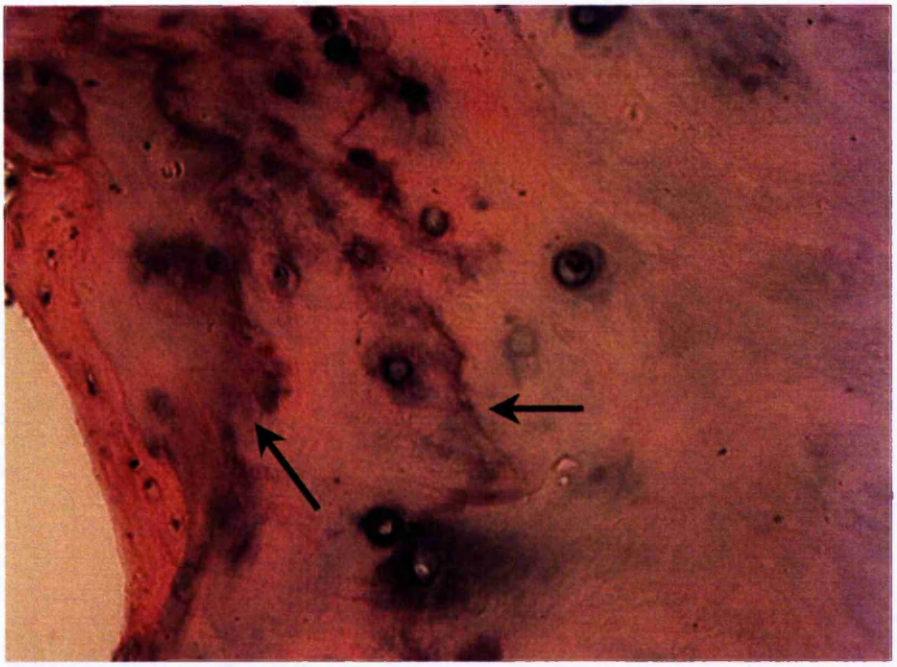


Figure 3.18. Double tidemark in severe osteoarthritic clavicular articular cartilage (H&E X 20). Duplication of the tidemark is seen typically in osteoarthritis (arrows)

Figure 3.19. Multiple tidemarks in severe osteoarthritic clavicular articular cartilage (H&E X20).
Multiple tidemarks may also be a feature of osteoarthritis (arrows).

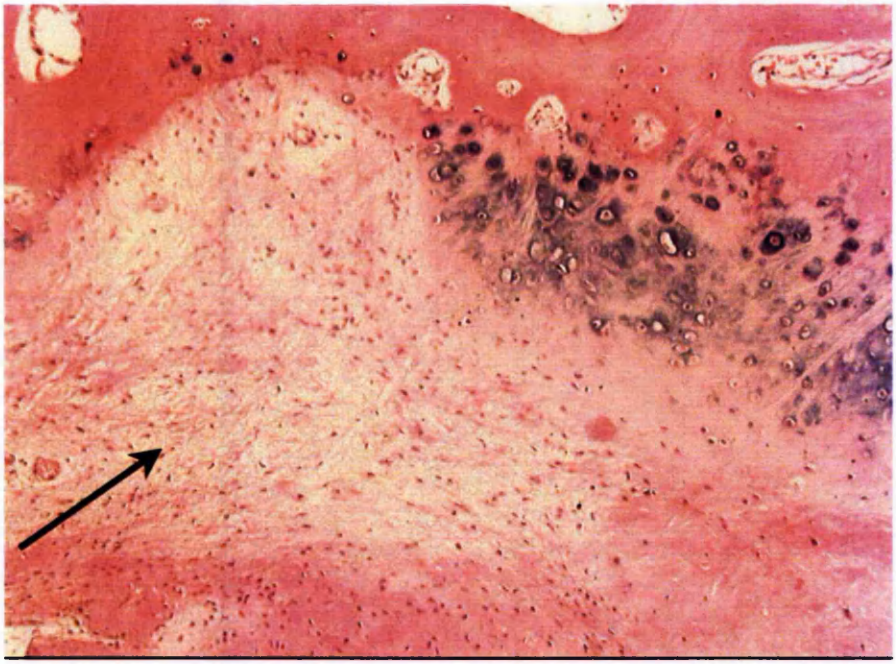


Figure 3.20. Fibrosis in severe osteoarthritic sternal articular cartilage (H&E X10). The articular cartilage is replaced by fibrosis in attempted repair (arrows).

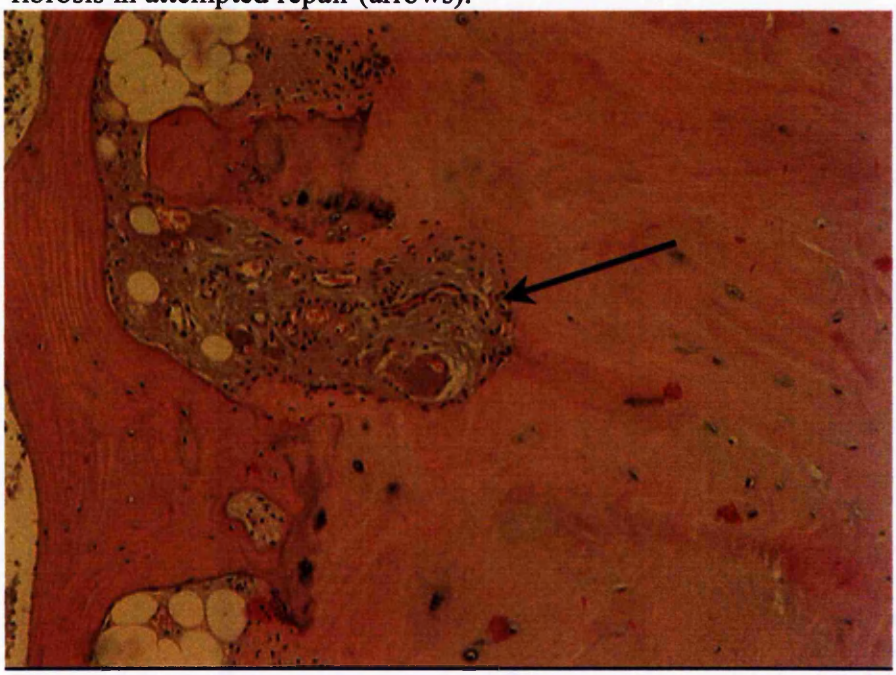


Figure 3.21. Vascular invasion in mild to moderate osteoarthritic sternal articular cartilage (H&E X20).
Tidemark was crossed by a blood vessel (arrow).

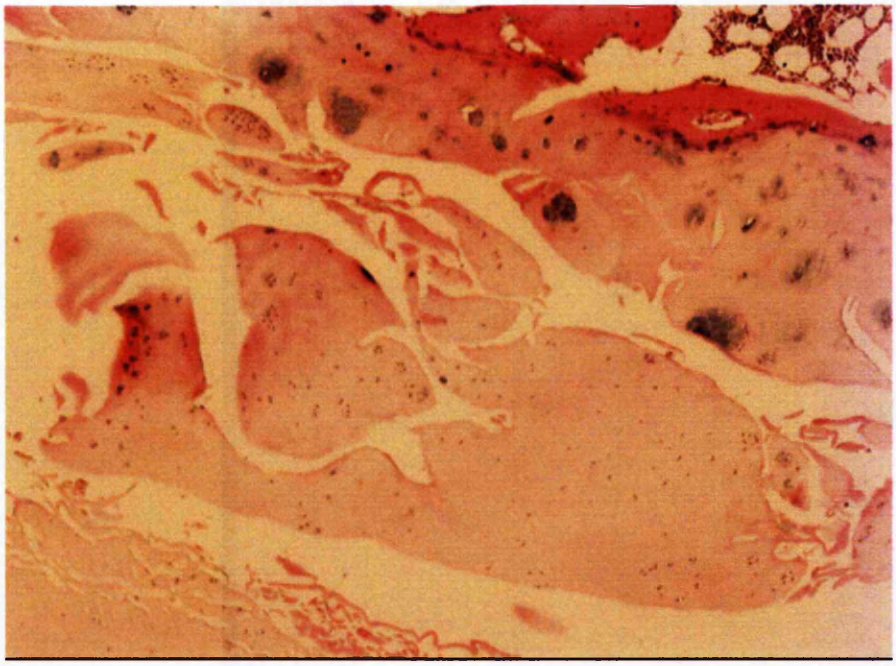


Figure 3.22. Severe osteoarthritic changes in sternal articular cartilage (H&E X20).
Complete disorganisation and clones of chondrocytes are seen.

3.1.3. Histochemical results

3.1.3.1. Toluidine blue staining

Toluidine blue (TB) revealed the distribution of GAGs in cartilage. In normal articular cartilage, chondrocytes were not stained with TB, but the matrix was stained intensely with TB, especially the territorial matrix, but not the surface of the articular cartilage. The deep zone was stained more strongly with TB, but the calcified zone was a very pale purple. Fifteen normal cases were stained intensely with TB. Three normal cases and 14 other cases showed slight reduction of TB staining.

In the matrix of osteoarthritic cartilage, a general reduction of staining by TB was revealed, but the new matrix around the chondrocytes in clones was stained strongly purple. The pericellular matrix was stained moderately with TB. Forty-seven cases showed moderate reduction of TB staining, 32 cases showed severe reduction and nine cases did not stain with TB in 90% of the cartilage.

Using polarised light, areas of superficial fibrillation gave a specific appearance described as 'tiger tail'. This showed as alternating light and dark bands due to changes in the orientation of collagen fibre bundles.

In normal fibrocartilage, the matrix was stained moderately purple, but the chondrocytes were not stained. In osteoarthritic fibrocartilage, the matrix was stained less purple and more patchily than in normal cartilage.

In the first rib, the chondrocytes were not stained, but the territorial matrix was stained very intensely and homogeneously, and the interterritorial matrix was stained moderately.

Examples of these features are seen in Figures 3.24 to 3.31.

Figure 3.23. Normal distribution of the glycosaminoglycans in sternal articular cartilage (Toluidine blue X10).
Normal structure and cells with intense toluidine blue staining are seen.

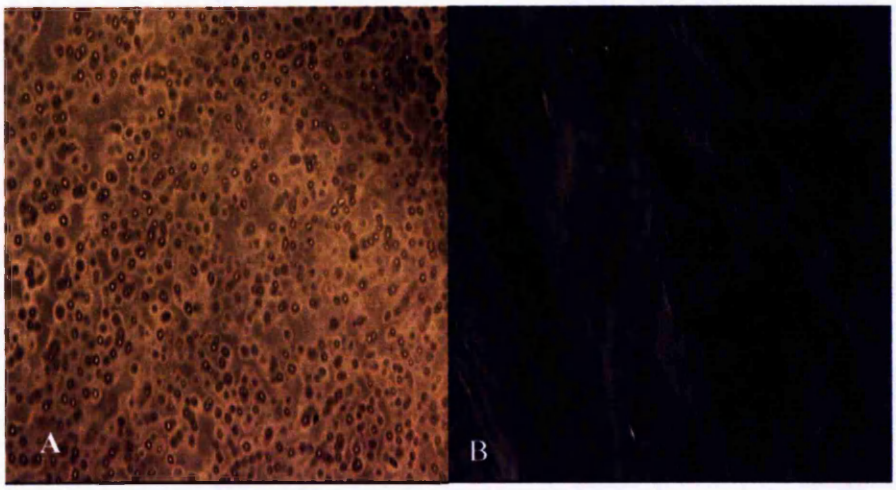


Figure 3.24. Normal fibrocartilage (A) and first rib (B) (Toluidine blue X10).
Distributions of chondrocytes and glycosaminoglycans are different compared to hyaline articular cartilage.

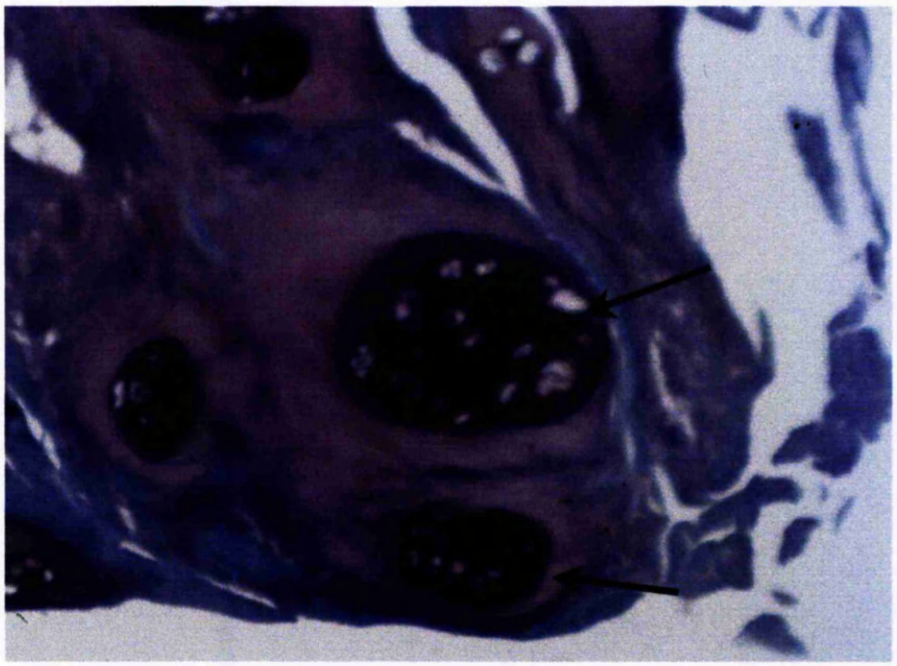


Figure 3.25. Clones of chondrocytes in severe osteoarthritic sternal articular cartilage (arrows) (Toluidine blue X40). Clones of chondrocytes and moderate reduction of TB staining are seen.

Figure 3.26. Fibrillated area of severe osteoarthritic clavicular articular cartilage viewed in polarising light (Toluidine blue X40). "Tiger tail" feature in the fibrillated area (arrows).

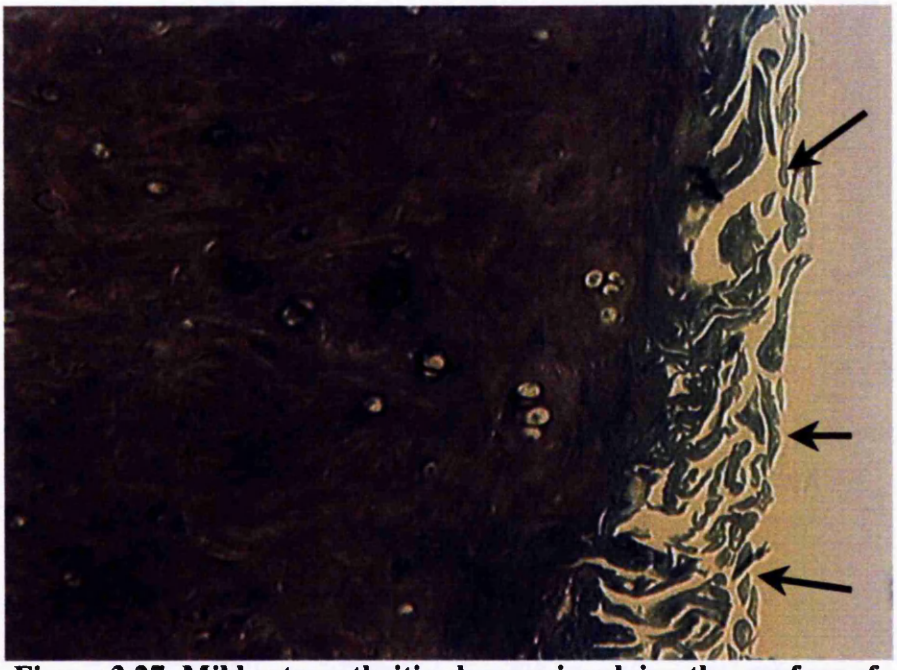


Figure 3.27. Mild osteoarthritic changes involving the surface of sternal articular cartilage (arrows) (Toluidine blue X10). Pannus and surface irregularities with moderate reduction of TB staining are seen.

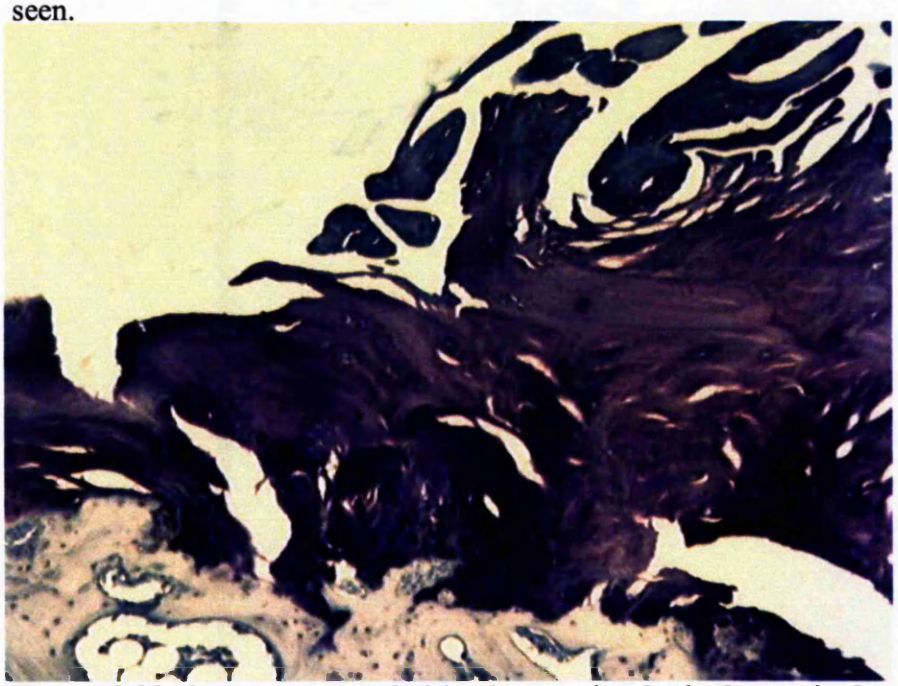


Figure 3.28. Severe osteoarthritic changes in clavicular articular cartilage (Toluidine blue X10). Complete disorganisation with slight reduction of TB staining are seen.

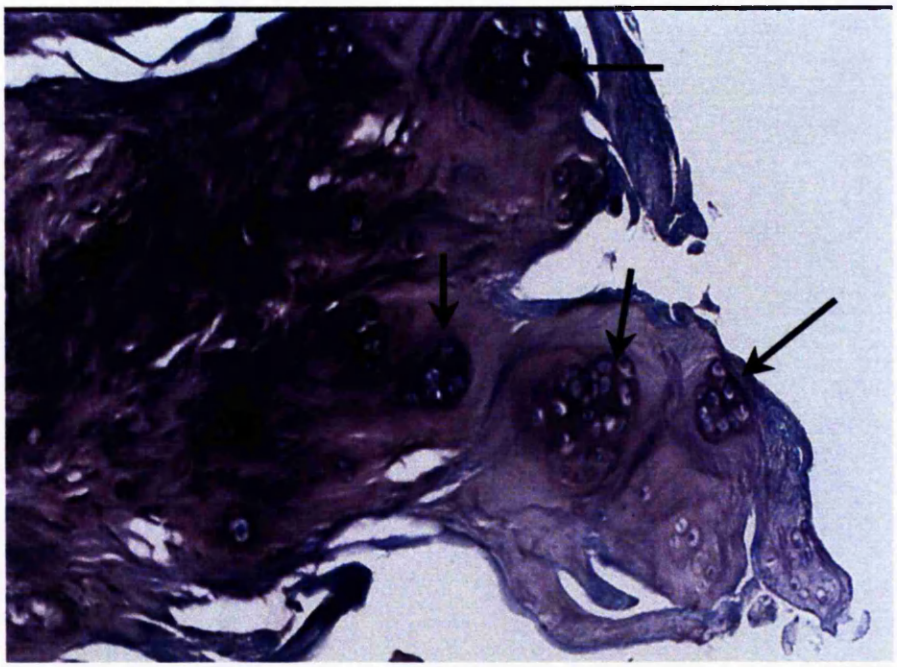


Figure 3.29. Severe osteoarthritic changes in sternal articular cartilage (Toluidine blue X20). Chondrocyte clones , hypocellularity, moderate reduction of TB staining and deep cleft are seen (arrows).

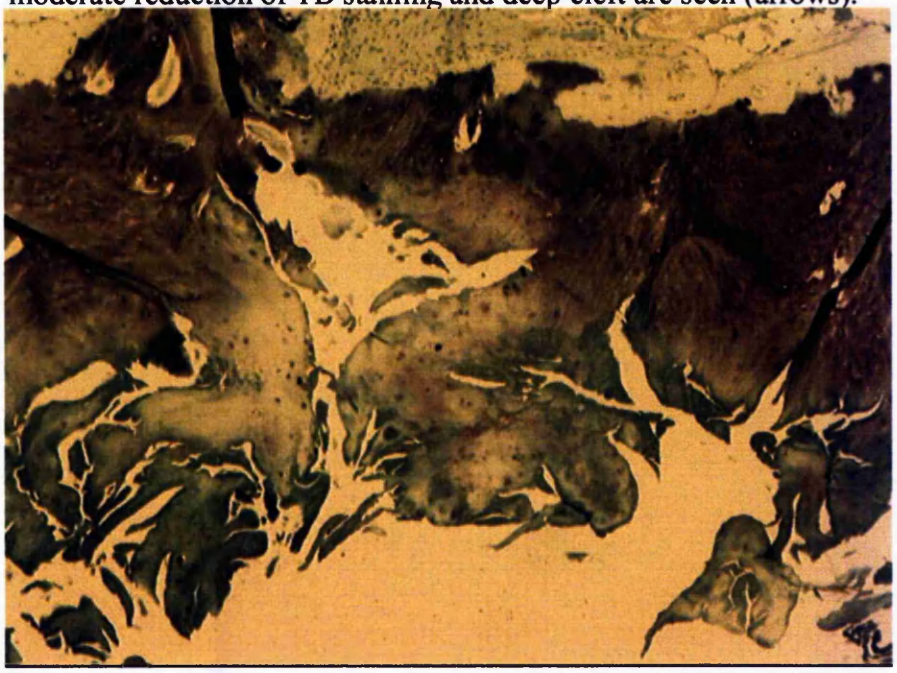


Figure 3.30. Severe osteoarthritic changes in articular cartilage (Toluidine blue X5). Hypocellularity, complete disorganisation, and severe reduction of TB staining are seen.

3.1.3.2. General features

All samples studied showed that both the sternal and clavicular components were covered by a hyaline articular cartilage, with an intra-articular fibrocartilaginous disc. Osteoarthritic changes were seen in 102 cases of 120 cases. In 120 hyaline articular surfaces available for study 86 showed fibrillation of mild to severe degree. Fibrillation was seen in 86 clavicular articular cartilages, 87 intraarticular discs and 54 sternal articular cartilages. Clefts were seen in 47 cases. They were found in 44 clavicular articular cartilages, 46 intraarticular discs and 32 sternal articular cartilages. Twenty-nine cases showed complete disorganisation, which it was in 30 clavicular articular cartilages, 31 intraarticular discs and 17 sternal articular cartilages. There is no significant correlation between age and osteoarthritis at the 0.05 level (2-tailed, Spearman's test). No differences in degenerative changes were found between sexes. The clavicular articular cartilage showed more severe changes than sternal articular cartilage. Changes in the intra-articular disc were slightly more common than in the articular cartilages. Osteoarthritic changes were more severe in the right SCJ than in left and the inferior part of the joints more affected than the superior part. There were significant differences in the severity of OA between right and left SCJs (significant chi-square value less than 0.01) and between superior and inferior part of the joints (significant chi-square value less than 0.01). Thirty of the 44 cases (68%) with severe osteoarthritic changes were of the right sternoclavicular cartilage, but only six cases of 18 normal cases were the right SCJ. Twenty-eight of the 44 cases (63.6%) with severe osteoarthritic changes were in the inferior part of SCJ, while only eight cases of 18 normal were in the inferior part of SCJ. Clonal proliferation of chondrocytes with new cartilage matrix formation (a feature of an attempted repair process) was observed in 65 cases. Subchondral bone cysts were observed in 54 cases. In 100 samples in which the chondro-osseous junction was available for study, subchondral osseous hyperplasia was found in 33, and osteophytosis was observed in a few cases. In 20 samples of the 100, cartilage damage was seen without any related bone changes (Table 3.2).

Table 3.2. Osteoarthritic changes in different parts of the SCJ

No	Age	Sex		Fibrillation			Clefts			Disorganisation			Bone hyperplasia	Cyst
				C Ac	S Ac	Int	C Ac	S Ac	Int	C Ac	S Ac	Int		
1	57	F	RS	+	+	+	+	-	+	-	-	-	-	+
			RI	+	+	+	+	+	+	-	-	-	+	+
			LS	+	-	+	-	-	-	+	-	+	-	-
			LI	+	+	+	-	-	+	-	-	-	+	+
2	53	M	RS	+	+	+	+	-	+	-	-	-	+	-
			RI	+	+	+	+	-	+	-	-	-	+	-
			LS	+	-	+	-	-	-	-	-	-	-	+
			LI	+	+	+	+	-	+	+	+	+	+	+
3	75	M	RS	+	+	+	+	+	+	-	-	-	+	+
			RI	+	+	+	+	-	+	+	-	-	-	+
			LS	+	+	+	+	+	+	-	-	-	+	+
			LI	+	+	+	+	+	+	+	-	-	+	+
4	60	M	RS	+	+	+	-	-	-	+	+	+	-	-
			RI	+	-	+	-	-	-	+	+	+	-	-
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	-	-	-	-	-	-	-	-	-	-	-
5	69	M	RS	+	+	+	-	-	-	+	-	+	+	-
			RI	+	+	+	+	-	+	+	+	+	+	-
			LS	+	+	+	-	+	+	-	-	-	-	+
			LI	+	+	+	-	-	-	-	-	-	-	-
6	68	F	RS	+	+	+	-	-	-	-	-	-	-	+
			RI	+	-	+	-	-	-	+	+	+	-	+
			LS	+	-	+	-	-	-	-	-	-	-	+
			LI	+	-	+	-	-	-	-	-	-	-	+
7	89	F	RS	+	+	+	+	-	+	+	-	+	-	+
			RI	+	-	+	-	-	-	+	+	+	+	+
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	+	-	+	-	-	-	-	-	-	-	+
8	70	M	RS	+	+	+	-	-	-	-	-	-	-	+
			RI	-	-	-	-	-	-	-	-	-	-	-
			LS	+	+	+	+	+	+	+	-	+	+	+
			LI	+	+	+	+	+	+	-	-	-	+	+
9	74	F	RS	+	-	+	-	-	-	-	-	-	-	+
			RI	+	-	+	+	-	-	-	-	-	-	+
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	+	+	+	-	-	-	+	+	+	-	+
10	58	M	RS	+	-	+	-	-	-	-	-	-	-	-
			RI	+	+	+	+	-	-	-	-	-	-	+
			LS	+	-	+	-	+	+	+	-	+	+	+
			LI	+	-	+	-	-	-	+	-	+	+	+
11	86	F	RS	+	+	+	+	+	+	+	-	+	-	+
			RI	+	+	+	+	+	+	+	-	+	+	+
			LS	+	+	+	-	+	+	-	+	-	-	-
			LI	+	-	+	-	-	-	-	-	-	-	-
12	80	M	RS	+	+	+	+	+	+	-	-	-	+	+
			RI	+	+	+	+	+	+	+	-	+	-	+
			LS	+	+	+	-	+	+	-	-	-	+	+
			LI	+	-	+	-	-	-	+	+	+	-	+
13	73	M	RS	+	-	+	-	-	-	-	-	-	-	-
			RI	+	-	+	-	-	-	+	-	+	+	+
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	-	-	-	-	-	-	-	-	-	-	-
14	74	M	RS	+	+	+	+	+	+	-	-	-	+	+
			RI	+	+	+	+	+	+	-	-	-	+	+
			LS	+	+	+	-	-	-	-	-	-	+	+
			LI	+	+	+	-	-	-	-	-	-	-	+

Table 3.3 continued

No	Age	Sex		Fibrillation			Clefts			Disorganisation			Bone hyperplasia	Cyst
				C Ac	S Ac	Int	C Ac	S Ac	Int	C Ac	S Ac	Int		
15	53	M	RS	+	+	+	+	+	+	-	-	-	+	+
			RI	+	-	+	+	+	+	-	-	-	+	+
			LS	+	-	+	-	-	-	-	-	-	-	+
			LI	+	-	+	+	-	+	+	+	+	+	-
16	78	F	RS	+	+	+	-	-	-	-	-	-	-	+
			RI	+	+	+	+	+	+	+	+	+	-	+
			LS	+	-	+	+	+	+	-	-	-	-	+
			LI	+	+	+	+	+	+	-	-	+	+	+
17	23	F	RS	-	-	-	-	-	-	-	-	-	-	-
			RI	-	-	-	-	-	-	-	-	-	-	-
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	-	-	-	-	-	-	-	-	-	-	-
18	35	M	RS	-	-	-	-	-	-	-	-	-	-	-
			RI	-	-	-	-	-	-	-	-	-	-	-
			LS	+	-	+	-	-	-	-	-	-	-	-
			LI	+	+	+	+	-	+	-	-	-	-	+
19	83	M	RS	+	+	+	+	-	+	+	-	+	-	+
			RI	+	+	+	+	-	+	-	-	-	-	-
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	+	+	+	-	-	-	-	-	-	-	-
20	92	F	RS	+	+	+	-	-	-	+	-	+	-	-
			RI	-	-	-	-	-	-	-	-	-	-	-
			LS	-	-	-	-	-	-	-	-	-	-	-
			LI	-	-	-	-	-	-	-	-	-	-	-
21	89	F	RS	+	+	+	-	-	-	-	-	-	-	-
			RI	+	+	+	-	-	-	-	-	-	+	+
			LS	+	-	+	-	-	-	-	-	-	-	-
			LI	+	-	+	-	-	-	-	-	-	-	-
22	59	M	RS	+	-	+	+	+	+	-	-	-	+	-
			RI	+	+	+	-	-	-	+	+	+	+	-
			LS	+	-	+	+	+	+	+	-	+	+	+
			LI	+	+	+	-	-	-	-	-	-	-	-
23	56	F	RS	+	-	+	+	-	+	+	-	+	-	+
			RI	+	-	+	+	-	+	-	-	-	-	-
			LS	+	+	+	+	-	-	+	-	-	+	+
			LI	+	+	+	+	-	+	-	-	-	-	-
24	81	M	RS	+	-	+	+	-	+	-	-	-	+	+
			RI	+	+	+	+	+	+	-	-	-	-	+
			LS	+	-	+	+	+	+	-	-	-	-	+
			LI	+	+	+	+	+	+	+	+	+	-	+
25	65	F	LS	+	+	+	-	+	-	-	-	-	-	-
			LI	+	+	+	-	-	-	-	-	-	-	-
26	47	M	LS	-	-	-	-	-	-	-	-	-	-	-
			LI	+	-	+	-	-	-	-	-	-	+	-

RS: right superior, RI: right inferior, LS: left superior, LI: left inferior, F: female, M: male

C Ac: clavicular articular cartilage, S Ac: sternal articular cartilage, Int: intraarticular disc.

3.1.3.3. Alcian blue staining

Alcian blue (AB) staining showed different staining patterns with the three concentrations of magnesium chloride used. Each concentration also showed a difference in staining with distance from the articular cartilage surface. In the presence of a little electrolyte (0.05M) all polyanions took up the stain intensely. Therefore articular cartilage, fibrocartilage and first rib matrices were stained homogenously blue and chondrocytes also showed a strong reaction. The subchondral bone showed reaction as well.

As the magnesium chloride concentration was raised to 0.5M, there was less staining in the matrix of cartilage and subchondral bone. Strong staining was only seen in the middle zone of articular cartilage. The matrix of the fibrocartilage was stained moderately and homogeneously. In subchondral bone only a very weak reaction was found. The territorial matrix of the first rib was stained strongly, but there was only weak staining of the interterritorial matrix and cells.

At a concentration of 0.9M magnesium chloride, the matrix of the middle zone was stained moderately and matrix of the deep zone strongly. The matrix of the fibrocartilage was stained moderately and homogeneously with AB. No reaction was seen in subchondral bone. The territorial matrix of the first rib was stained strongly, but the interterritorial matrix and the cells showed no reaction.

In osteoarthritic cartilage the staining of the matrix, at all concentrations of electrolyte, was decreased especially in the middle zone. The matrices of the clones of chondrocytes were stained strongly in the higher electrolyte concentrations of 0.5M and 0.9M.

Examples of these features are seen in Figures 3.32.-3.33.

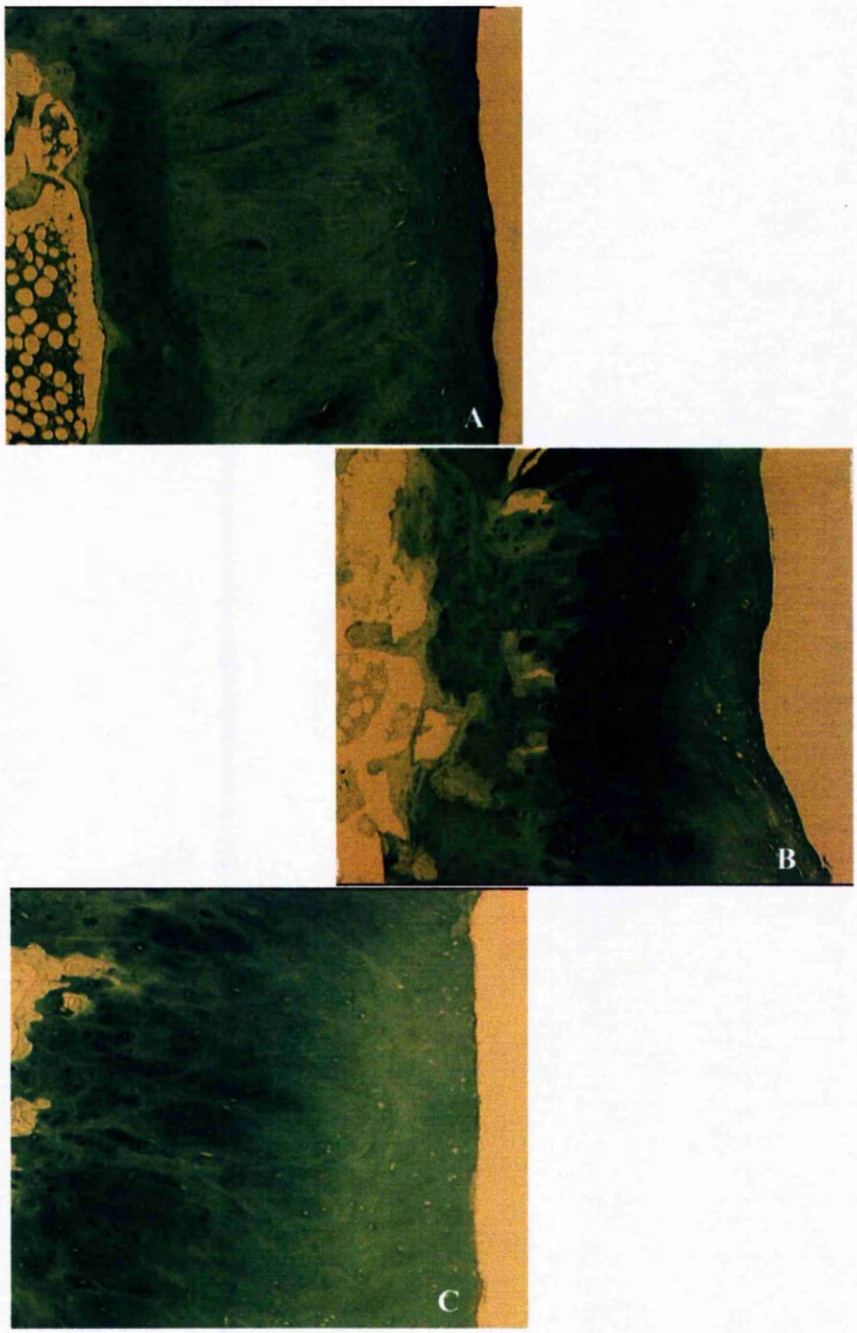


Figure 3.31. Normal articular cartilages (Alcian blue X5).
A: Alcian blue staining of the normal articular cartilage with (A) 0.05M (B)0.5M (C)0.9M concentration of $MgCl_2$. (A) All polyanions stain the same. (B) strong staining was seen only in middle zone. (C) middle zone stained moderately and deep zone strongly.

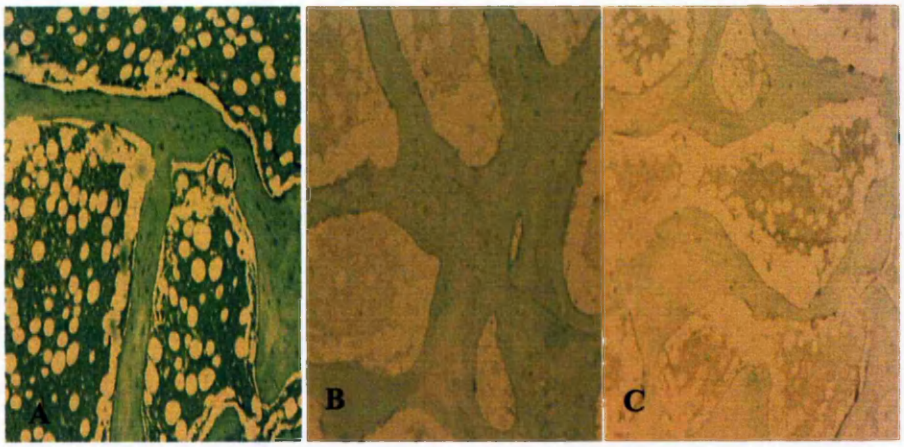


Figure 3.32. Normal subchondral bone (Alcian blue X10).
A: uniform staining with 0.05M concentration of $MgCl_2$.
B: reduction of staining with concentration 0.5M.
C: slight staining with concentration 0.9M.

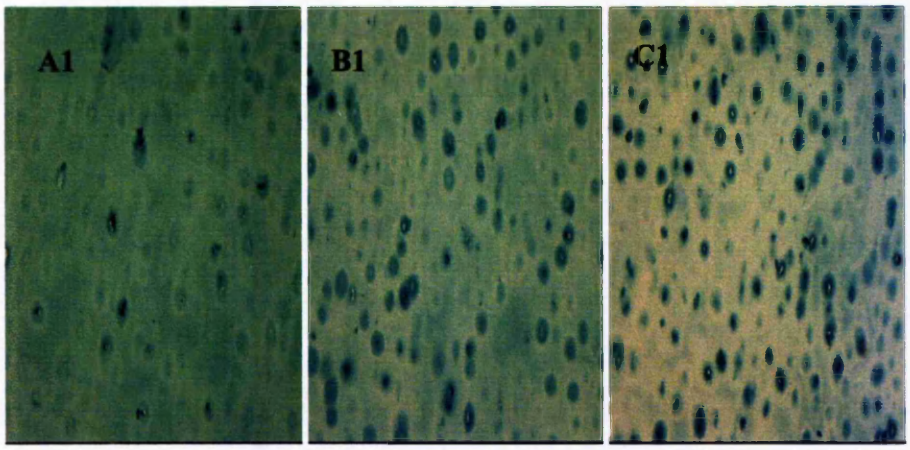


Figure 3.33. Normal first rib (Alcian blue X10).
A1: uniform staining with concentration 0.05M $MgCl_2$.
B1: territorial matrix strongly and interterritorial matrix moderately stained in concentration 0.5M.
C1: territorial matrix strongly and interterritorial matrix weakly stained in concentration 0.9M.

3.1.3.4. Picro sirius red for collagen distribution

Picro sirius red (PSR) showed the distribution of collagen in articular cartilage. In normal cases, collagen fibrils arched superficially and were compressed to run parallel to the articular surface. The staining was stronger in pericellular areas. These features were seen in 28 cases. The tidemark was stained similarly in all samples (normal and OA).

The staining pattern of collagen was changed in osteoarthritic cartilage. A mixed pattern of pale and intense staining was found in 74 cases of osteoarthritic cartilage. The fibrillated surface was very pale and the new matrix of the chondrocytes in clones was less stained than the matrix of the 'old' cartilage. Twenty-eight cases of severe OA showed very pale staining matrices, 19 severe cases showed irregular staining and three cases showed normal staining. Thirty-nine cases of mild to moderate OA showed pale staining matrices, sixteen cases showed irregular staining and seven cases showed normal staining.

Normal fibrocartilage stained with PSR, but was not homogeneous in all cases. In osteoarthritic fibrocartilage irregularity of the collagen staining was seen. The first rib was stained less than articular cartilage and fibrocartilage with PSR.

Examples of these features are seen in Figures 3.34 to 3.37.

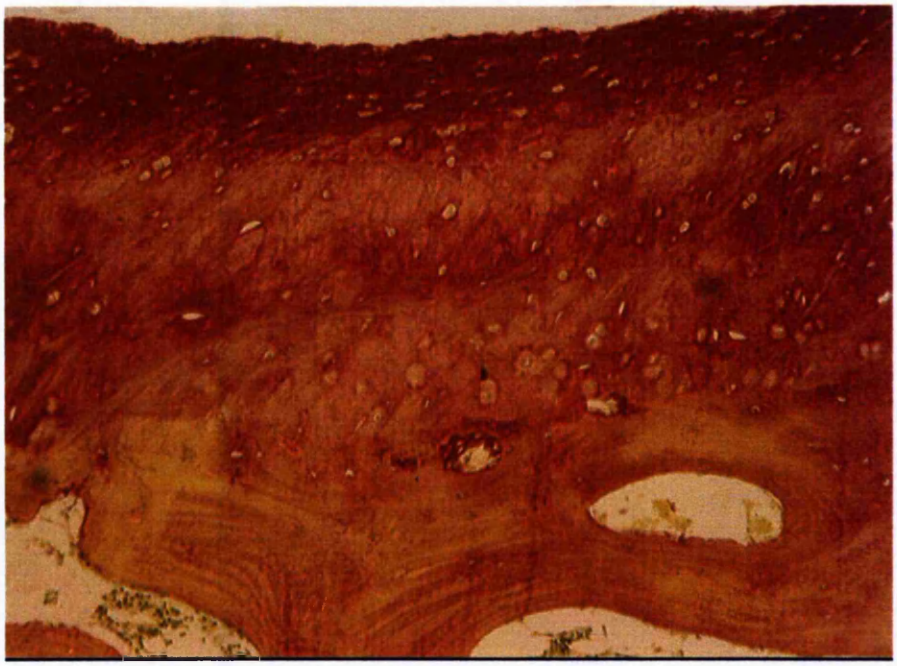


Figure 3.34. Normal sternal articular cartilage (Picro sirius red X10).

It shows normal distribution of the collagen in the zones.

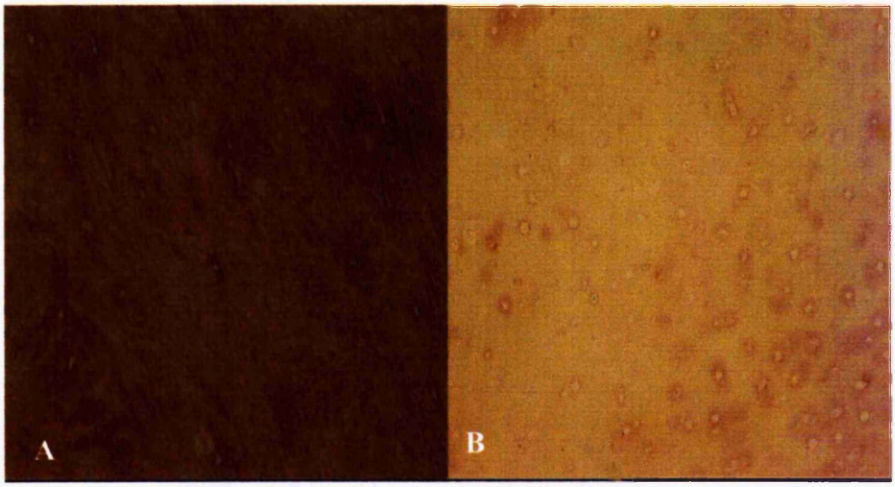


Figure 3.35. Normal fibrocartilage (A) and first rib (B) (Picro sirius red X10).

It shows the distribution of the collagen in two types of cartilage.

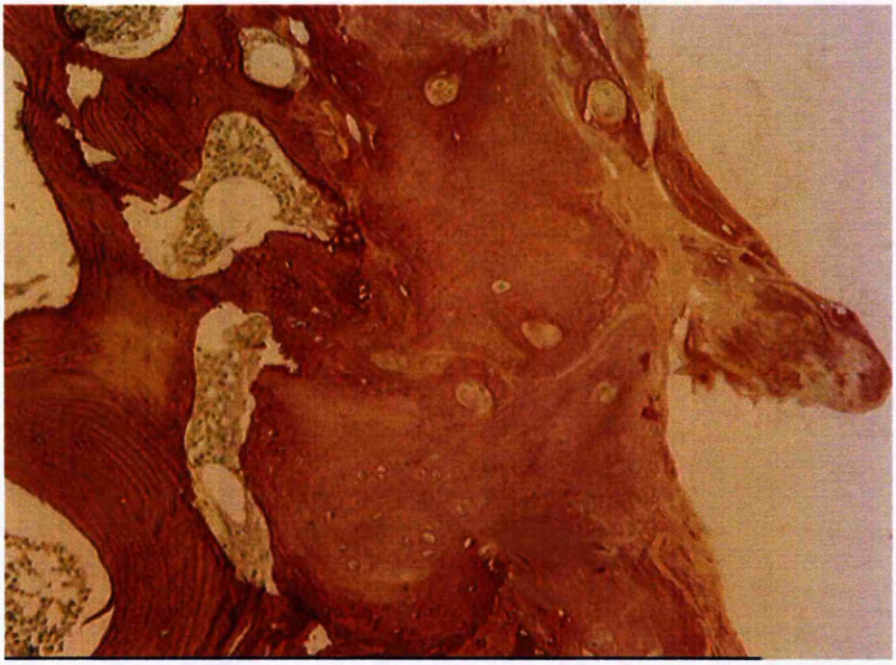


Figure 3.36. Mild to moderate osteoarthritic changes in clavicular articular cartilage (Picro sirius red X5).
The distribution of the collagen is changed in osteoarthritis.

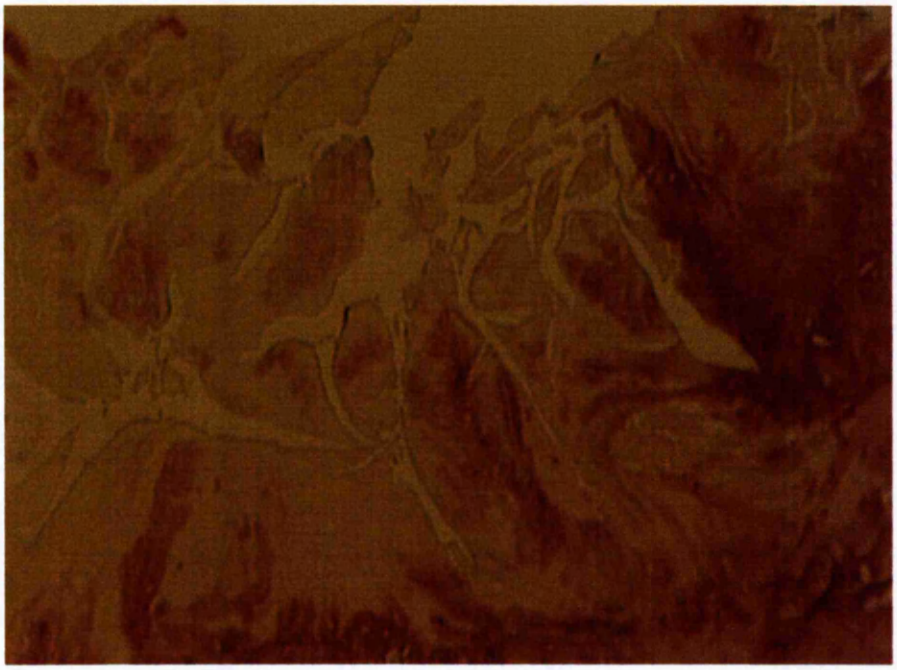


Figure 3.37. Severe osteoarthritic changes in sternal articular cartilage (Picro sirius red X10).
It shows collagen loss especially in the superficial area.

3.1.3.5. Alkaline Congo red staining

Using alkaline Congo red (ACR), amyloid deposits were seen as separate round or linear microdepositions. ACR stained amyloid light pink to red, depending upon the concentration of the amyloid. In positive cases, amyloid was noted to be close to the surface of the cartilage and to run in deposits lying parallel to the surface of the articular cartilage and fibrocartilage. Deposition was found in the deeper part of the articular cartilage or fibrocartilage as well. Amyloid deposits were also observed adjacent to deep cracks in severe osteoarthritis. Microdepositions were found in fibrocartilage, mostly superficial, and in synovial tissue and capsule as well. All microdepositions had an apple green birefringence in polarized light. In this study amyloid deposits were found in 99 clavicular cartilages, 97 discs and 78 sternal cartilages. There were localised bilaterally and no differences were found between the amounts in the left and the right joints. Eighteen cases were negative, which half of the cases were left joints.

According to the results of this study, there was a significant correlation between age and amyloidosis at the 0.01 level (2-tailed, Spearman's test) while they did not show a spatial correlation between OA and the occurrence of amyloid. Correlation between osteoarthritis and amyloid depositions was not significant at the 0.05 level (2-tailed, Spearman's test). However, a relationship between severity of OA and the amount of amyloid deposited cannot be excluded, as 14 out of 23 cases (60.8%) of the heaviest deposits were found in the older subjects with severe OA. Amyloid deposits were present in areas which stained lightly or intensely with toluidine blue (details are given in Table 3.3).

Two patterns of amyloid deposition were found in cartilage. The first was a diffuse deposition without a marked border, which was usually weak to moderate in intensity and present in the superficial zone. The second pattern was condensed with a sharp border, which was intensely stained and usually found in the deep zone.

Examples of these features are seen in Figures 3.38 to 3.43.

Table 3.3. Correlation between OA changes and amyloid deposition in SCJ

No	Age	Sex		G OA	Amyloid
1	57	F	RS	M&M	-
			RI	S	-
			LS	M&M	-
			LI	S	-
2	53	M	RS	M&M	+
			RI	S	+
			LS	M&M	+
			LI	S	-
3	75	M	RS	M&M	+++
			RI	S	++
			LS	M&M	++
			LI	S	++
4	60	M	RS	M&M	+
			RI	M&M	+
			LS	N	++
			LI	N	++
5	69	M	RS	M&M	+++
			RI	S	++
			LS	M&M	+
			LI	S	++
6	98	F	RS	S	+++
			RI	S	+++
			LS	M&M	++
			LI	M&M	++
7	89	F	RS	S	++
			RI	S	++
			LS	N	++
			LI	M&M	++
8	70	M	RS	S	++
			RI	N	++
			LS	M&M	+
			LI	S	++
9	74	F	RS	M&M	-
			RI	S	++
			LS	N	++
			LI	M&M	+
10	58	M	RS	M&M	-
			RI	M&M	-
			LS	S	+
			LI	M&M	+
11	86	F	RS	S	+++
			RI	S	+
			LS	M&M	-
			LI	M&M	-
12	80	M	RS	M&M	+++
			RI	S	+++
			LS	M&M	++
			LI	S	+++
13	73	M	RS	M&M	+
			RI	S	++
			LS	N	++
			LI	N	++
14	74	M	RS	S	+
			RI	S	+
			LS	M&M	+
			LI	M&M	+
15	53	M	RS	S	+
			RI	S	-
			LS	M&M	+
			LI	S	+
16	78	F	RS	M&M	+++
			RI	S	++
			LS	M&M	+++
			LI	S	++

17	23	F	RS	N	-
			RI	N	-
			LS	N	-
			LI	N	-
18	35	M	RS	N	++
			RI	N	++
			LS	M&M	-
			LI	M&M	+
19	83	M	RS	S	+++
			RI	S	+++
			LS	N	++
			LI	M&M	++
20	92	F	RS	M&M	++
			RI	N	++
			LS	N	++
			LI	N	++
21	89	F	RS	M&M	+
			RI	S	+
			LS	M&M	+
			LI	M&M	+
22	59	M	RS	S	+
			RI	S	+
			LS	M&M	++
			LI	M&M	++
23	56	F	RS	S	+++
			RI	M&M	+
			LS	M&M	+
			LI	S	+++
24	81	M	RS	M&M	+++
			RI	S	+++
			LS	M&M	++
			LI	S	+++
25	65	F	LS	M&M	+
			LI	M&M	+
26	47	M	LS	N	+
			LI	M&M	-
27	88	F	RS	M&M	+++
			RI	S	+
			LS	M&M	++
			LI	M&M	++
28	90	F	RS	M&M	+++
			RI	S	+++
			LS	M&M	++
			LI	M&M	++
29	92	M	RS	M&M	++
			RI	M&M	++
			LS	M&M	++
			LI	S	+++
30	81	F	RS	M&M	-
			RI	S	+
			LS	M&M	+
			LI	S	++
31	89	F	RS	S	+++
			RI	S	+++
			LS	M&M	+
			LI	M&M	+

RS: right superior, RI: right inferior, LS: left superior, LI: left inferior, F: female, M: male
N: normal, M&M: mild to moderate, S: severe

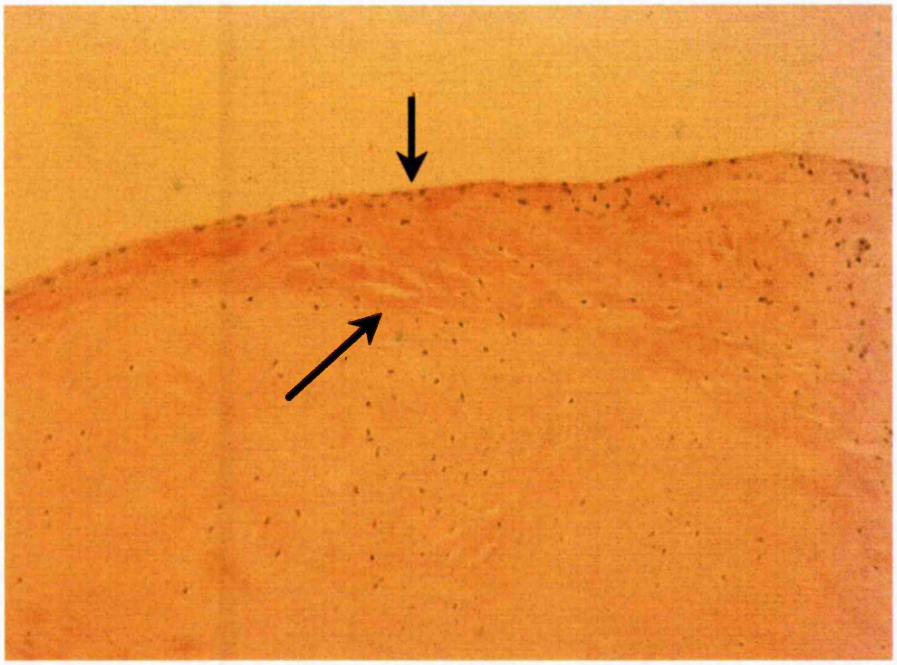


Figure 3.38. Amyloid deposits in the superficial zone of the sternal articular cartilage (arrows) (Alkaline Congo red X10).

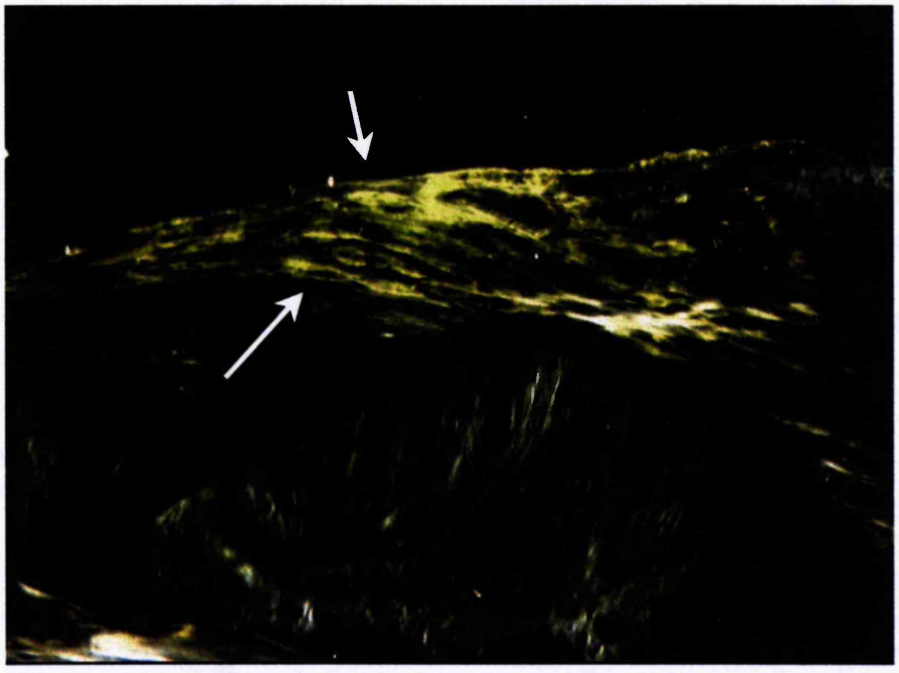


Figure 3.39. Apple green birefringence of amyloid deposits under polarising light (Alkaline Congo red X10).
Amyloid deposits in the surface of sternal articular cartilage are seen (arrows).

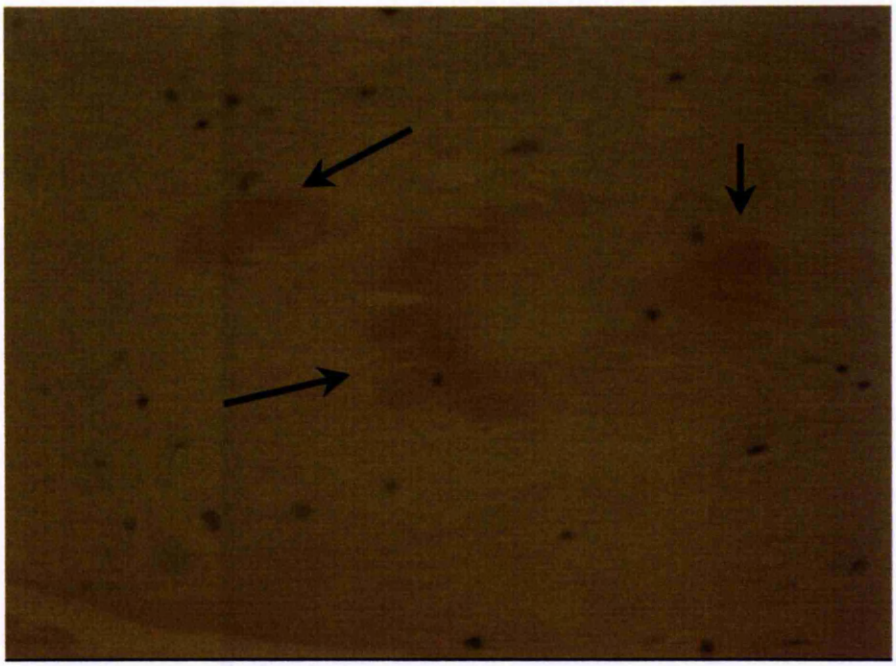


Figure 3.40. Amyloid deposits within clavicular articular cartilage (Alkaline Congo red X20). The depositions are arrowed.

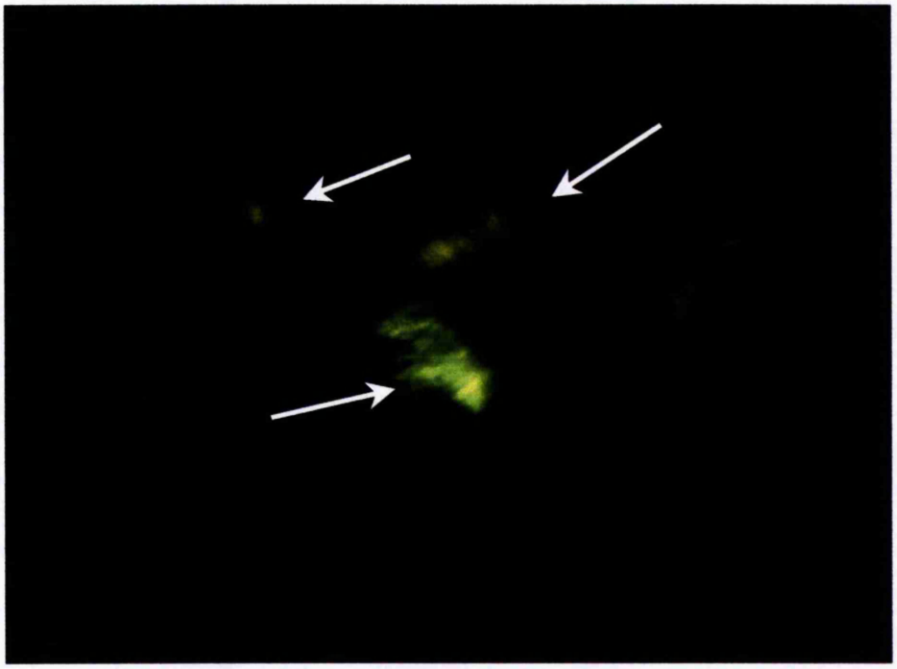


Figure 3.41. Apple green birefringence of amyloid deposits under polarising light in clavicular articular cartilage (arrows) (Alkaline Congo red X20).

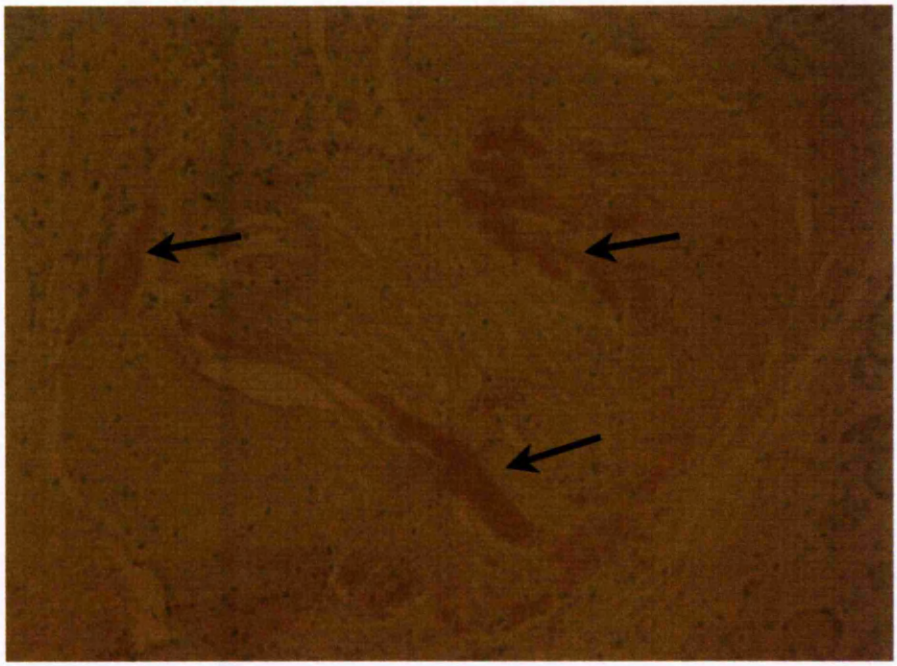


Figure 3.42. Diffuse amyloid deposition in sternal articular cartilage (Alkaline Congo red X10). The depositions are arrowed.

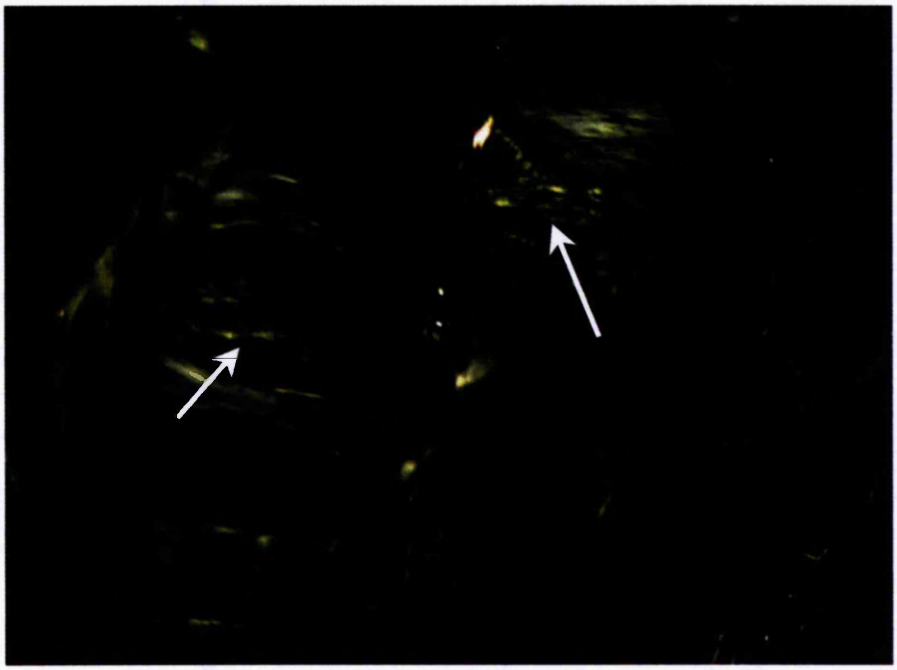


Figure 3.43. Apple green birefringence of the diffuse amyloid depositions under polarising light in sternal articular cartilage (arrows) (Alkaline Congo red X10).

3.1.4. Immunohistochemistry

All sections, positive or negative with ACR, were tested with five antibodies. These were anti AA-amyloid, anti β_2-microglobulin, anti κ-light chain, anti λ-light chain and anti P-component antibody. All amyloid deposits stained with anti P-component antibody. None stained with anti AA monoclonal antibody. Ninty-nine cases were stained with anti β_2-microglobulin antibody, 86 with anti λ antibody and 78 with anti κ antibody (all monoclonal antibodies). These results indicate that mixed patterns of immunoreactivity were present. There was no relationship between age, sex and the types of the depositions.

The results of these staining reactions in SC joints are summarised in Table 3.4.

3.1.4.1. Distribution of the antibody staining in SCJ

Different parts of articular cartilage were positive with four antibodies. Anti β2-microglobulin stained cells and their pre-cellular area of 99 cases. These were located in the superficial zone and occasionally in the medial zone. In 21 cases with high concentrations of amyloid deposits, matrices in the superficial zones were weakly, and in 8 cases were mildly positive. β2-Microglobulin antibodies stained the cytoplasm of cells of clones of chondrocytes as well.

Anti λ-light chain antibody stained the interterritorial matrix of the 86 superficial zone, which in 23 cases the interterritorial matrix in the medial zone was stained as well. In all positive cases the interterritorial matrix was stained, but the territorial matrix was weakly positive in 32 cases. The new matrix in clones of chondrocytes was positive in 19 cases.

Anti κ-light chain antibody stained the interterritorial matrix in the 78 superficial area, and in 34 cases chondrocytes were stained with anti κ-light chain as well.

Anti P-component antibody stained cells, their pre-cellular area, and the matrix around them in 102 cases. This positivity was found in the superficial zone and occasionally in the medial zone.

Amyloid deposits were not only found in the articular cartilage and fibrocartilage, but also in synovium and joint capsule. In synovium, positive anti β2-microglobulin reactions were present around blood vessels. Anti λ and κ light chain positive reactions were diffusely present in all parts of the synovium. In capsule and synovium, anti β2-microglobulin was positive in 61% of cases, anti λ light chain in 58% and anti κ light chain 45%. In these sections, the positive antibodies staining were present in both amyloid deposits (Congo red positive area) and non-amyloid deposits (Congo red negative area) area. Examples of these features are seen in Figures 3.44 to 3.47.

3.1.4.2. Anti CD68

In 37% of cases with amyloid deposits large mononuclear cells in the synovial and joint capsule stained positively with anti CD68. These presumed macrophages were located close to amyloid deposits. No macrophages were found in articular cartilage and fibrocartilage.

Examples of these features are seen in Figure 3.48.

Table 3.4. Details of anti amyloid immunohistochemical staining in SCJ

No	Age	Sex		ACR	Anti β2-M	Anti κ	Anti λ	Anti AA	Anti P-com
1	57	F	RS	-	-	-	-	-	-
			RI	-	-	-	-	-	-
			LS	-	-	-	-	-	-
			LI	-	-	-	-	-	-
2	53	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	-	-	-	-	-	-
3	75	M	RS	+	+	+	+	-	+
			RI	+	+	-	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
4	60	M	RS	+	+	-	+	-	+
			RI	+	+	+	-	-	+
			LS	+	+	+	+	-	+
			LI	+	+	-	-	-	+
5	69	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
6	98	F	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
7	89	F	RS	+	+	-	+	-	+
			RI	+	+	-	-	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
8	70	M	RS	+	+	+	+	-	+
			RI	+	+	-	+	-	+
			LS	+	+	+	-	-	+
			LI	+	+	+	-	-	+
9	74	F	RS	-	-	-	-	-	-
			RI	+	+	-	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
10	58	M	RS	-	-	-	-	-	-
			RI	-	-	-	-	-	-
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
11	86	F	RS	+	+	+	-	-	+
			RI	+	+	+	+	-	+
			LS	-	-	-	-	-	-
			LI	-	-	-	-	-	-
12	80	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	-	+	-	+
			LI	+	+	-	+	-	+
13	73	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	-	-	+
			LI	+	+	-	-	-	+
14	74	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
15	53	M	RS	+	+	+	+	-	+
			RI	-	-	-	-	-	-
			LS	+	+	+	-	-	+
			LI	+	+	-	-	-	+

Table 3.4 continued

No	Age	Sex		ACR	Anti β2-M	Anti κ	Anti λ	Anti AA	Anti P-com
16	78	F	RS	+	+	-	-	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
17	23	F	RS	-	-	-	-	-	-
			RI	-	-	-	-	-	-
			LS	-	-	-	-	-	-
			LI	-	-	-	-	-	-
18	35	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	-	-	-	-	-	-
			LI	+	+	+	+	-	+
19	83	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
20	92	F	RS	+	+	-	+	-	+
			RI	+	+	-	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
21	89	F	RS	+	+	+	-	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
22	59	M	RS	+	-	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	-	+	-	+
			LI	+	+	-	+	-	+
23	56	F	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	-	+	-	+
			LI	+	+	+	+	-	+
24	81	M	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	-	-	+
			LI	+	+	-	+	-	+
25	65	F	LS	+	+	+	+	-	+
			LI	+	+	+	-	-	+
26	47	M	LS	+	+	+	+	-	+
			LI	-	-	-	-	-	-
27	88	F	RS	+	+	+	+	-	+
			RI	+	+	+	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	-	+	-	+
28	90	F	RS	+	+	+	-	-	+
			RI	+	+	-	+	-	+
			LS	+	-	-	+	-	+
			LI	+	+	+	+	-	+
29	92	M	RS	+	+	+	-	-	+
			RI	+	+	-	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
30	81	F	RS	-	-	-	-	-	-
			RI	+	+	+	+	-	+
			LS	+	+	-	+	-	+
			LI	+	+	+	+	-	+
31	89	F	RS	+	+	+	+	-	+
			RI	+	-	-	+	-	+
			LS	+	+	+	+	-	+
			LI	+	+	+	+	-	+
			Total	85%	82.5%	65.8%	70.8%	0%	85%

RS: right superior, RI: right inferior, LS: left superior, LI: left inferior, F: female, M: male, ACR: alkaline Congo red, Anti β2-M: anti β2-microblubolin, Anti κ: anti κ light chain, Anti λ: anti λ light chain, Anti AA: anti AA amyloid, Anti P-com: anti P Component

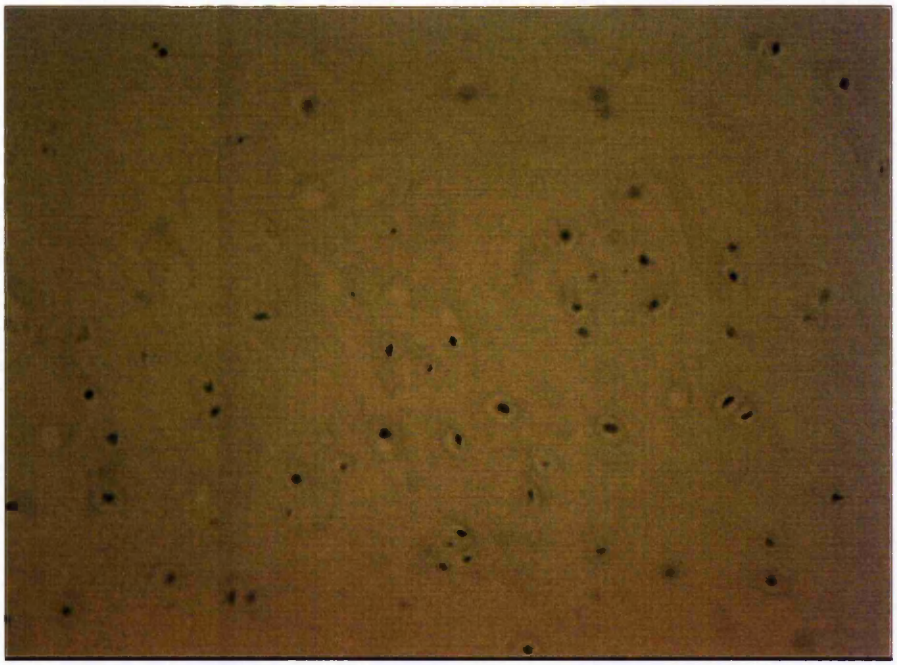


Figure 3.44. Negative reaction for AA amyloid in sternal articular cartilage (Immunohistochemistry with anti AA amyloid X20).

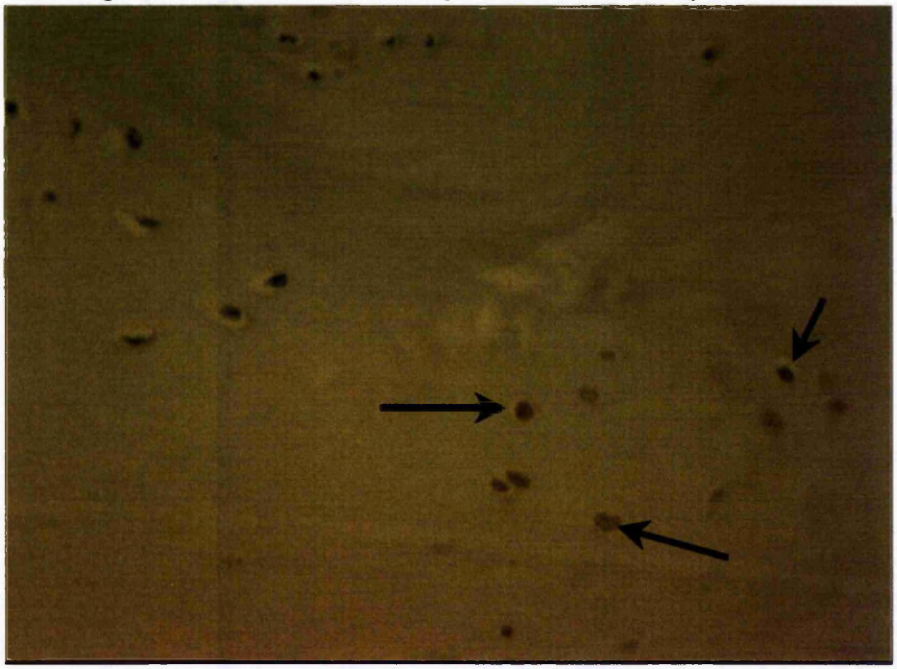


Figure 3.45. Positive β-2microglobulin cells in sternal articular cartilage (arrows) (Immunohistochemistry with anti β2-microglobulin X20).

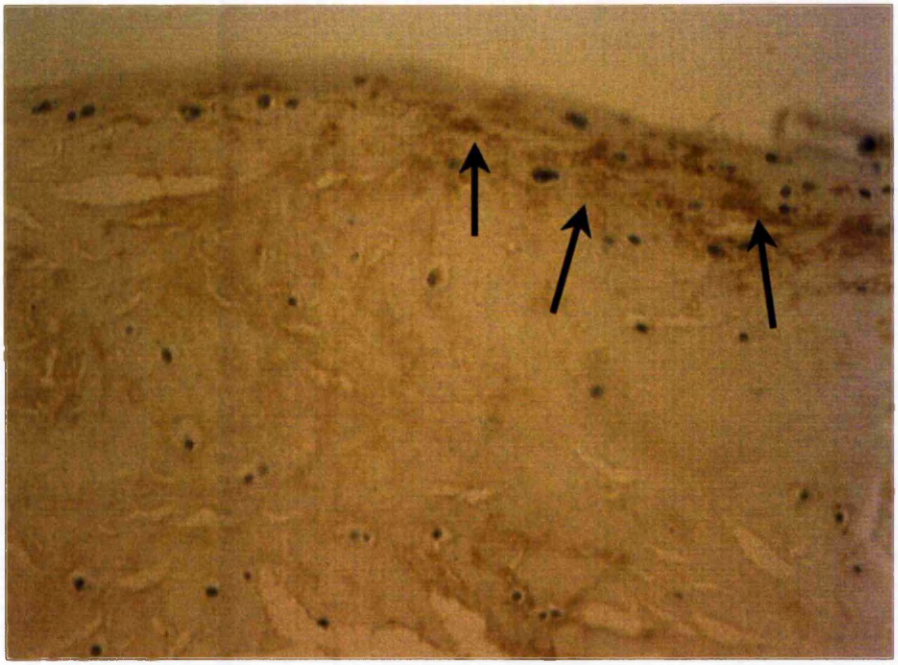


Figure 3.46. Positive λ light chain matrix staining in sternal articular cartilage (arrows) (Immunohistochemistry with anti λ light chain X10).

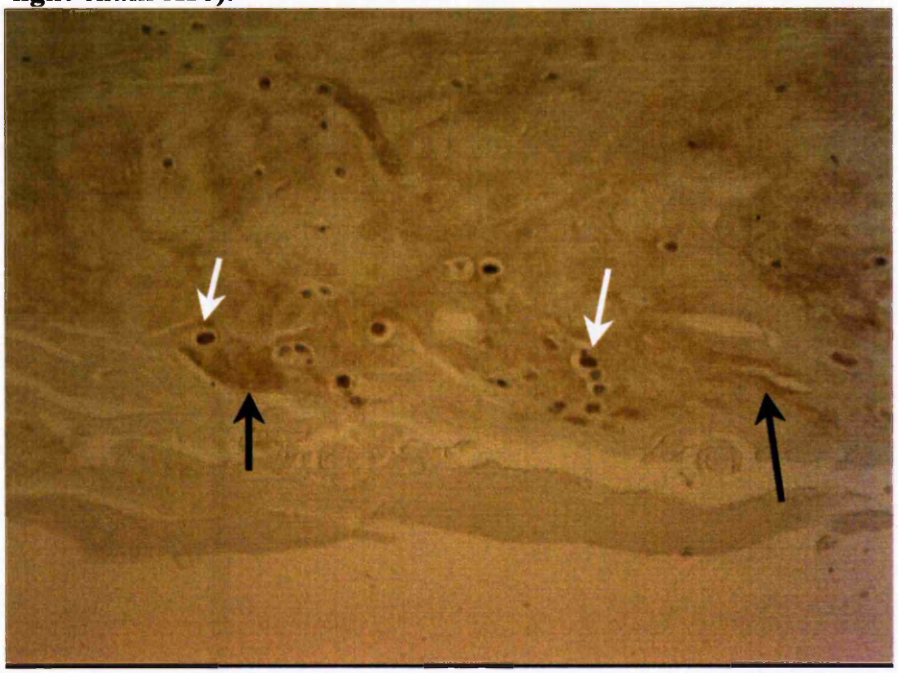


Figure 3.47. Positive κ light chain staining of matrix (black arrows) and cells (white arrows) in sternal articular cartilage (Immunohistochemistry with anti κ light chain X10).

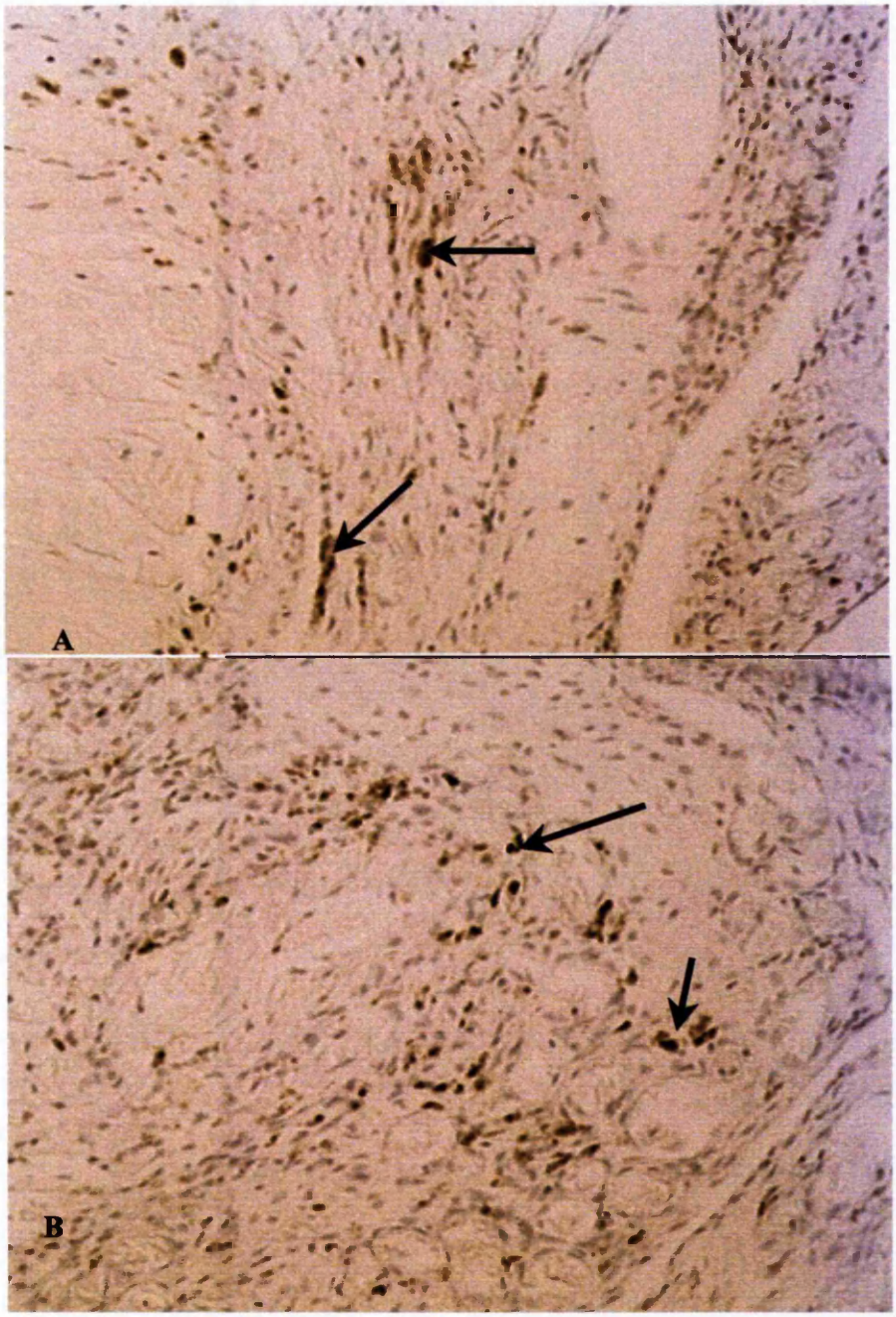


Figure 3.48. Macrophages are stained by CD68 antibody in capsule (A) and synovium (B) of the sternoclavicular joints (arrows) (X10).

3.1.5. Lectin histochemical studies

The lectin-binding pattern qualitatively describes the topographical location of certain sugar residues. In this study lectin staining was applied to both normal and OA cartilages to define the location of these glycan structures histologically. Lectins are classified into groups according to specific carbohydrate binding. The results of using 14 lectins on normal, mild to moderate and severe OA sternoclavicular joints are explained below.

The results were evaluated using a grade-scoring (ranking) method in which 0 represent none, 1 weak, 2 moderate, 3 strong and 4 very strong, intense staining.

3.1.5.1. Mannose and complex N-glycan binding lectins

HHA

Articular cartilage

Matrix: Superficial matrix of normal articular cartilage was stained weakly to moderately. Matrix of the medial and deep parts showed only weak reactions with HHA. In mild to moderate OA, the superficial part of the matrix was bound by HHA strongly. The medial part of the matrix was stained weakly and the deep part of the matrix was negative. In severe OA, matrix was stained weakly. Two different reactions were seen between the matrix of the clones of chondrocytes and HHA: 40% of them showed a strong reaction with HHA, but others were negative.

Chondrocytes: In the superficial and medial zone of normal and osteoarthritic articular cartilage, chondrocytes were stained very strongly, but non-homogeneously (dark brown punctate). Weak binding was seen in the deep part of the cartilage between chondrocytes and HHA. Chondrocytes of 40% of the clones showed weak to moderate reaction with HHA.

Fibrocartilage

Matrix: In normal fibrocartilage and severe OA, there was a weak binding with HHA, but in mild to moderate OA it was moderate.

Chondrocytes: In normal and osteoarthritic fibrocartilage, chondrocytes showed a non-homogeneous very strong reaction.

Subchondral bone

Bone trabeculae were stained weakly and osteocytes showed no reaction with HHA.

Synovium and capsule

Synovium and capsule showed a weak reaction with HHA.

Summary of the HHA lectin staining is shown in appendix C (Table C1). Examples of these features are seen in Figures 3.49 –3.50.

PSA

Articular cartilage

Matrix: The territorial matrix of normal articular cartilage and in severe OA showed a moderate reaction with this lectin, but in mild to moderate OA it was strong. Interterritorial matrix in normal SCJ was stained very strongly and strongly in osteoarthritic SCJ. Matrix of the clones of chondrocytes was stained moderately to strongly.

Chondrocytes: In normal and osteoarthritic articular cartilage, chondrocytes were stained strongly, although in normal articular cartilage, the deep chondrocytes were stained weakly. Chondrocytes of the clones showed no reaction with PSA.

Fibrocartilage

Matrix: In normal and osteoarthritic fibrocartilage matrix showed moderate reactions with PSA.

Chondrocytes: In normal and osteoarthritic fibrocartilage, 50% of the chondrocytes moderately bound PSA.

First rib

Matrix: No staining was seen in rib matrix with PSA.

Chondrocytes: The chondrocytes were not stained with PSA.

Subchondral bone

Bone trabeculae showed moderate to strong reactions, but osteocytes were negative.

Synovium and capsule

Synovium and capsule were stained moderately to strongly.

Summary of the PSA lectin staining is shown in appendix C (Table C2). Examples of these features are seen in Figures 3.51 –3.52.

LCA

Articular cartilage

Matrix: In normal articular cartilage the superficial part of the matrix was stained weakly. In osteoarthritic articular cartilage the superficial part of the matrix showed moderate to strong binding to LCA. The territorial matrix of the medial part was stained strongly. Fifty percent of the clones showed moderate to strong reactions with LCA.

Chondrocytes: In normal and osteoarthritic cartilage, chondrocytes failed to bind to LCA. Chondrocytes of the clones showed no reaction with LCA.

Fibrocartilage

Matrix: In normal fibrocartilage and mild to moderate OA the matrix was stained moderately. In severe OA there was weak binding between matrix and LCA.

Chondrocytes: In normal fibrocartilage and mild to moderate OA, the majority of the chondrocytes were stained moderately, but in severe OA chondrocytes were not stained by LCA.

First rib

Matrix: The matrix of the first rib showed no reaction with LCA.

Chondrocytes: There were no binding sites on chondrocytes for LCA.

Subchondral bone

Bone trabeculae were stained weakly, but osteocytes showed no reaction.

Synovium and capsule

Synovium and capsule were bound weakly by LCA

Summary of the LCA lectin staining is shown in appendix C (Table C3). Examples of these features are seen in Figures 3.53 –3.54.

E-PHA

Articular cartilage

Matrix: The matrices of the superficial and middle zones of normal and osteoarthritic cartilage showed moderate to strong reactions with e-PHA. Matrix of the deep zone showed no reaction. Matrix of the clones was stained moderately to strongly.

Tidemark of the osteoarthritic cartilage was stained strongly with e-PHA.

Chondrocytes: In the superficial and middle zones of normal and osteoarthritic cartilage, chondrocytes were stained moderately to strongly. The chondrocytes of 60% of the clones were moderately positive.

Fibrocartilage

Matrix: The matrix of normal and osteoarthritic fibrocartilage was stained moderately with e-PHA.

Chondrocytes: In normal and osteoarthritic fibrocartilage, chondrocytes showed moderate reactions with e-PHA.

First rib

Matrix: There were no binding sites for e-PHA in the matrix of the first rib.

Chondrocytes: The chondrocytes were not stained by e-PHA.

Subchondral bone

Bone trabeculae were stained moderately to strongly and osteocytes showed only a weak reaction.

Synovium and capsule

Synovium and capsule were stained weakly with e-PHA.

Summary of the e-PHA lectin staining is shown in appendix C (Table C4). Examples of these features are seen in Figures 3.55 –3.56.

L-PHA

Articular cartilage

Matrix: The matrix of normal articular cartilage was negative with l-PHA. The superficial part of the matrix in mild to moderate and severe osteoarthritis showed strong binding to l-PHA. No reaction was seen between the matrix of the clones and l-PHA.

Tidemark was stained strongly with l-PHA.

Chondrocytes: In normal and osteoarthritic articular cartilage, chondrocytes failed to show any reaction with l-LPHA. Chondrocytes of the clones showed no reaction.

Fibrocartilage

Matrix: In normal fibrocartilage, matrix was stained moderately, but in osteoarthritic fibrocartilage, it was only weakly positive.

Chondrocytes: In normal and mild to moderate OA, chondrocytes were stained weakly, and in severe OA chondrocyte staining was negative with l-PHA.

First rib

Matrix: The matrix of the first rib was not stained by l-PHA.

Chondrocytes: There were no binding sites for l-PHA on these chondrocytes.

Subchondral bone

Bone trabeculae were stained weakly and osteocytes showed no reaction.

Synovium and capsule

Synovium was stained moderately, but capsule showed no reaction with l-PHA.

Summary of the l-PHA lectin staining is shown in appendix C (Table C5). Examples of these features are seen in Figures 3.57 –3.58.

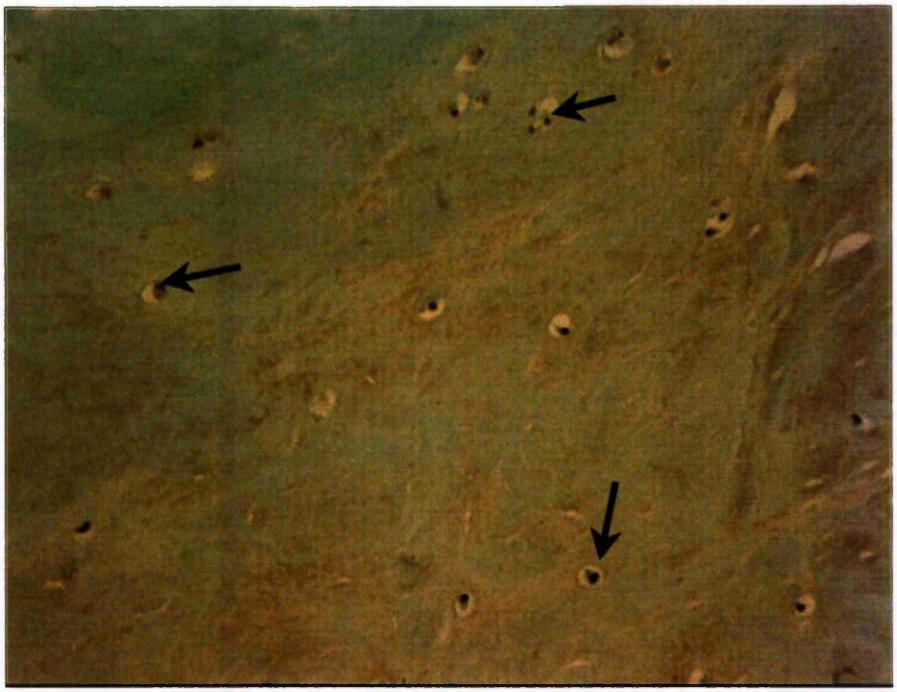


Figure 3.49. HHA lectin staining of normal articular cartilage (X10). Matrix was stained weakly to moderately and chondrocytes showed strong reaction (arrows).

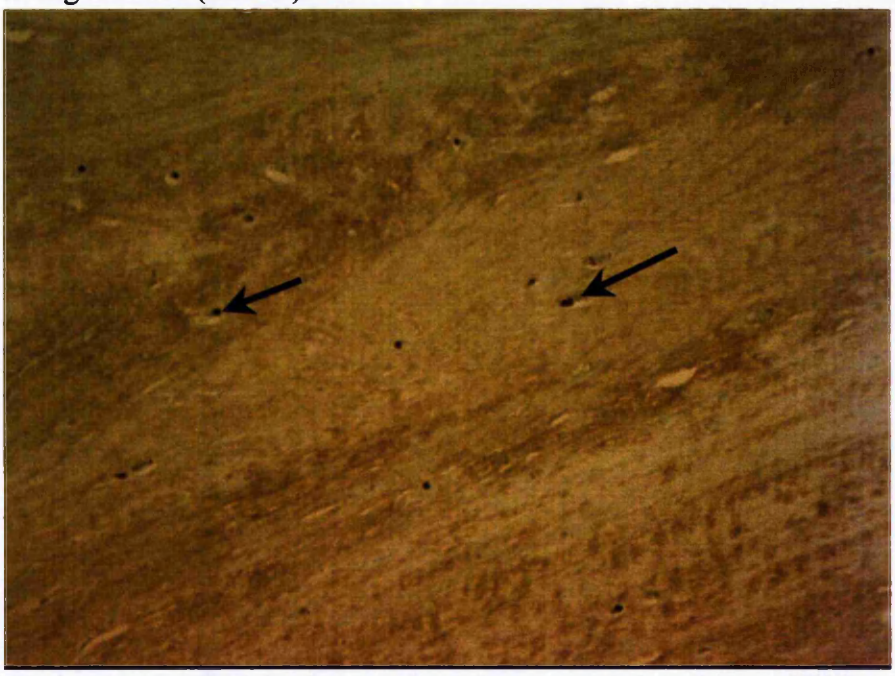


Figure 3.50. HHA lectin staining of normal fibrocartilage (X10). Matrix was stained moderately and chondrocytes showed strong reaction (arrows).

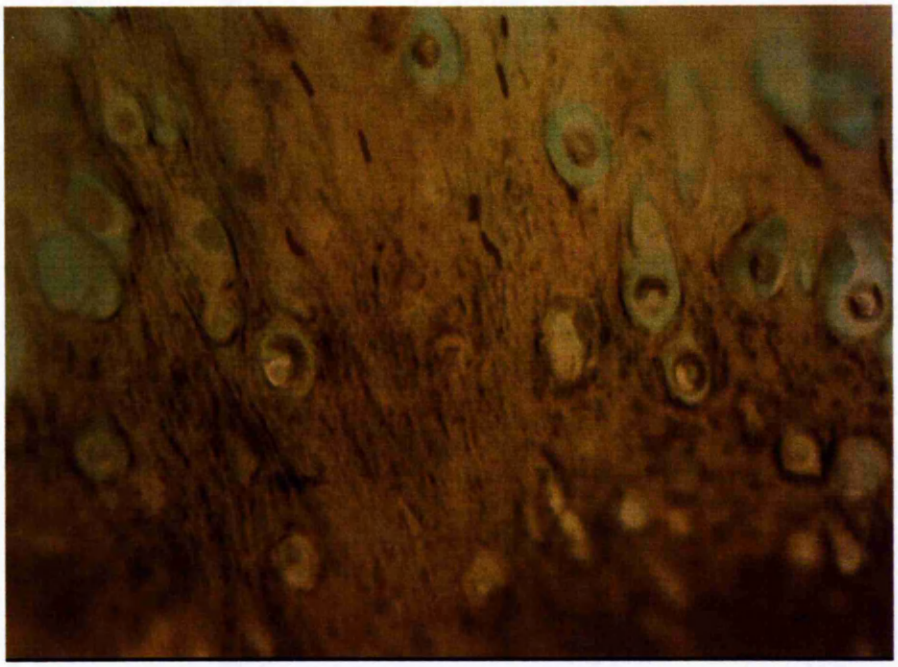


Figure 3.51. PSA lectin staining of mild to moderate osteoarthritic cartilage (X20). Matrix and chondrocytes showed strong reactions with PSA.

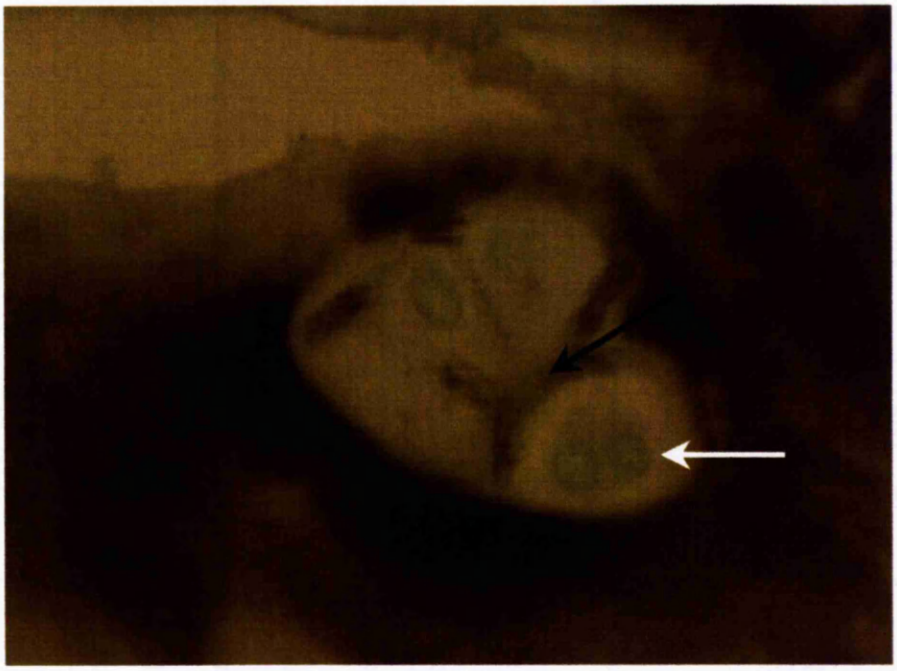


Figure 3.52. PSA lectin staining of chondrocyte clones (X40). Matrix (black arrow) of the clones showed a reaction but chondrocytes (white arrow) did not.

Figure 3.53. LCA lectin staining of the superficial part of osteoarthritic articular cartilage (X10).
Matrix stained moderately to strongly but chondrocytes were negative.

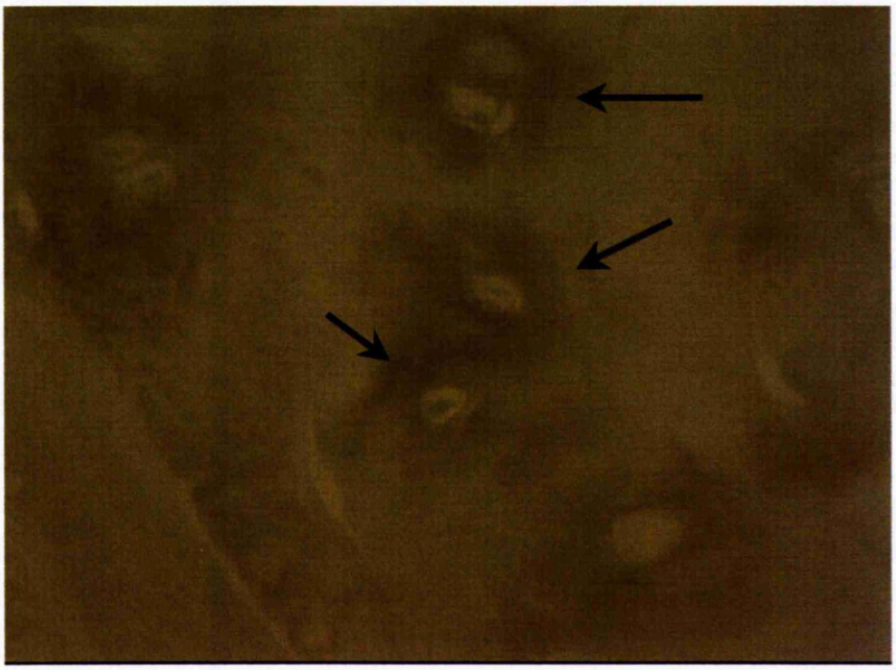


Figure 3.54. LCA lectin staining of the medial zone of osteoarthritic articular cartilage (X20). Territorial matrix was stained strongly (arrows).

Figure 3.55. E-PHA lectin staining of osteoarthritic cartilage (X20). Matrix and chondrocytes were stained moderately to strongly.

Figure 3.56. E-PHA lectin staining of the tidemark in normal articular cartilage (arrow) (X20).

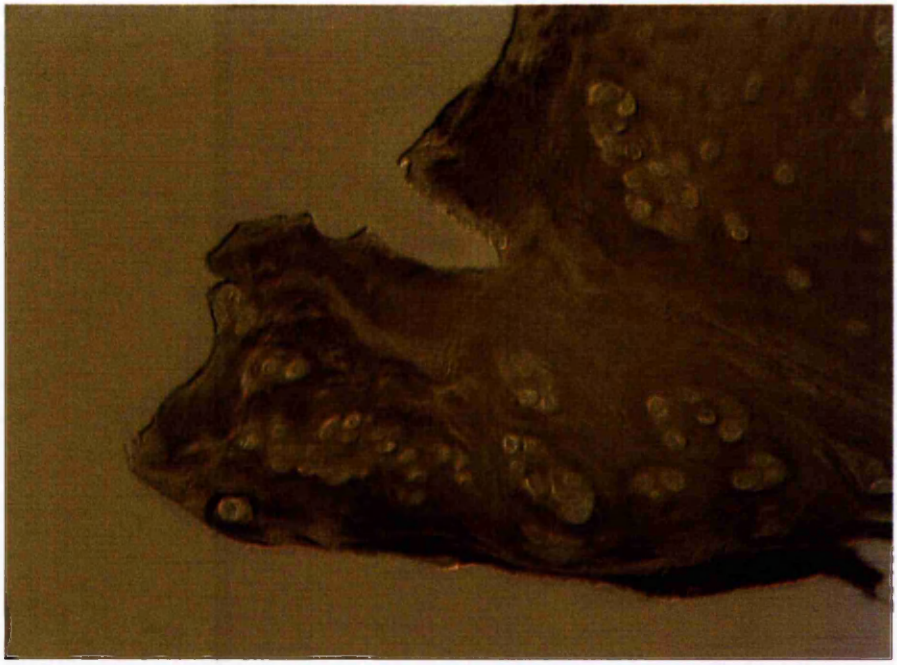


Figure3. 57. L-PHA lectin staining of the superficial zone of osteoarthritic articular cartilage (X20).
Matrix was stained strongly but chondrocytes were negative.

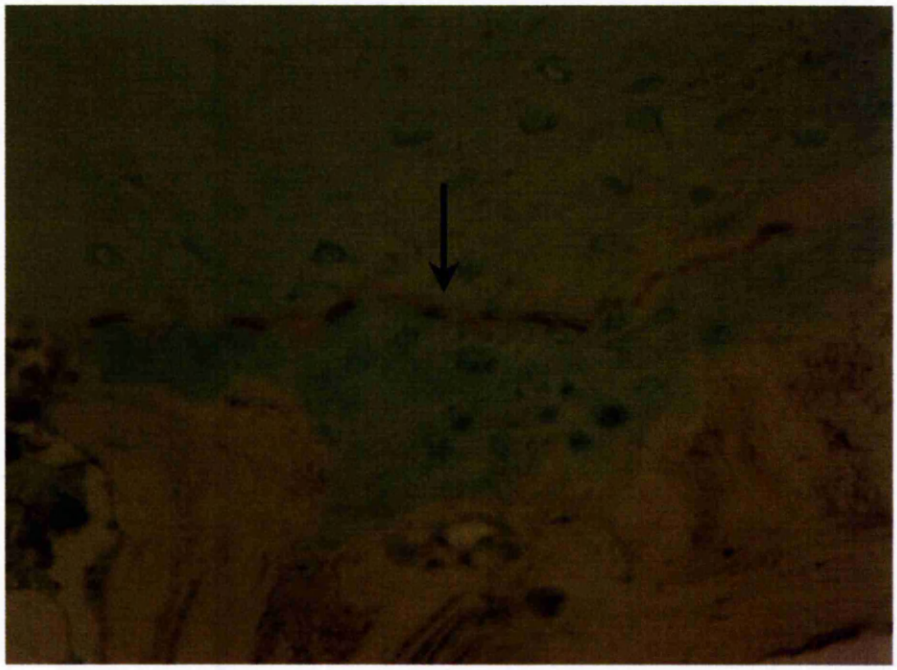


Figure 3.58. L-PHA lectin staining of the tidemark in normal articular cartilage (arrow) (X20).

3.1.5.2. Fucose-binding lectins

UEA-I

Articular cartilage

Matrix: In normal SCJ the matrix of all zones was weakly positive with UEA-I. In mild to moderate OA, superficial matrix showed a very strong reaction with UEA-I, but in severe OA there were weak reactions. Matrix of the clones was negative.

Tidemark was positive with UEA-I.

Chondrocytes: In normal and osteoarthritic SCJ, chondrocytes did not show any reaction with UEA-I. Chondrocytes of the clones showed a moderate reaction.

Fibrocartilage

Matrix: The matrix in normal and mild to moderate OA was stained moderately and in severe OA, it was stained weakly.

Chondrocytes: In fibrocartilage, chondrocytes showed no binding sites for UEA-I.

Subchondral bone

Bone trabeculae showed weak reaction and osteocytes were negative.

Synovium and capsule

Synovium and capsule were stained moderately with UEA-I.

Summary of the UEA-I lectin staining is shown in appendix C (Table C6). Examples of these features are seen in Figures 3.59 –3.60.

Figure 3.59. UEA-I lectin staining of normal articular cartilage (X40). Only the matrix stained weakly.

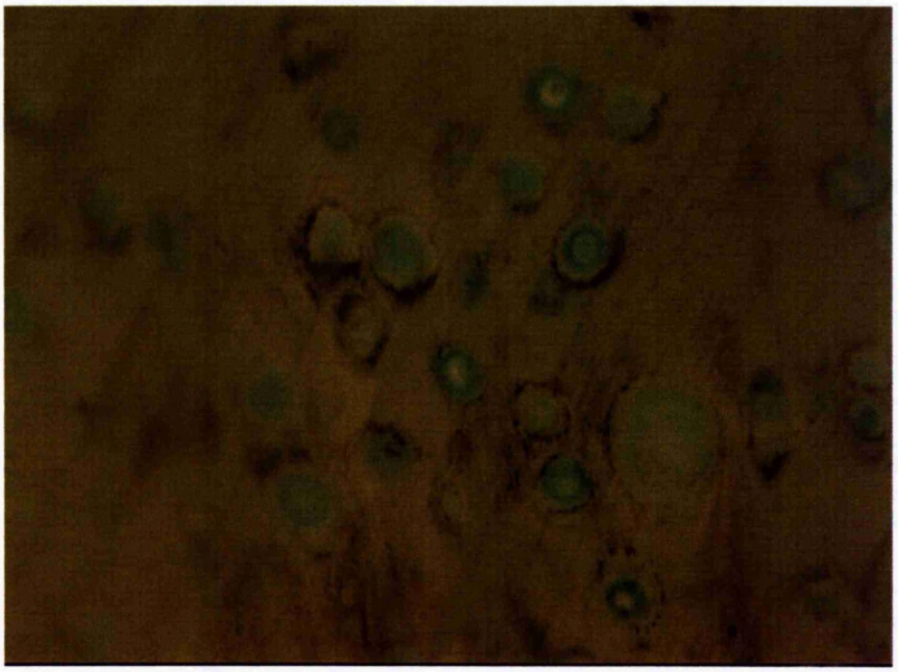


Figure 3.60. UEA-I lectin staining of mild to moderate osteoarthritic articular cartilage (X40). Matrix showed a strong reaction but chondrocytes were negative.

3.1.5.3. N-Acetylneuraminyl-binding lectin

MAA

Articular cartilage

Matrix: In the surface of normal SCJ, the matrix was stained moderately, but in medial and deep zones it was weakly stained. In osteoarthritic articular cartilage, the matrix showed a moderate to strong reaction with MAA. Matrix of the clones showed no reaction.

Tidemark of the osteoarthritic cartilage was stained moderately with MAA.

Chondrocytes: In the surface of normal SCJ, chondrocytes were stained moderately, but in medial and deep zones only 20% of them showed a weak reaction. In the surface of osteoarthritic SCJ, chondrocytes were stained weakly, but in medial and deep zones 30% of them showed strong reactions with MAA. Chondrocytes of the clones were stained moderately to strongly.

Fibrocartilage

Matrix: Normal fibrocartilage matrix was stained weakly, and osteoarthritic fibrocartilage matrix was stained moderately.

Chondrocytes: Like matrix, chondrocytes in normal fibrocartilage showed only weak reactions with MAA and in osteoarthritic fibrocartilage they were bound moderately to MAA.

Subchondral bone

Bone trabeculae were stained weakly to moderately and osteocytes were stained strongly.

Synovium and capsule

Synovium and capsule were stained moderately to strongly.

Summary of the MAA lectin staining is shown in appendix C (Table C7). Examples of these features are seen in Figures 3.61 –3.62.

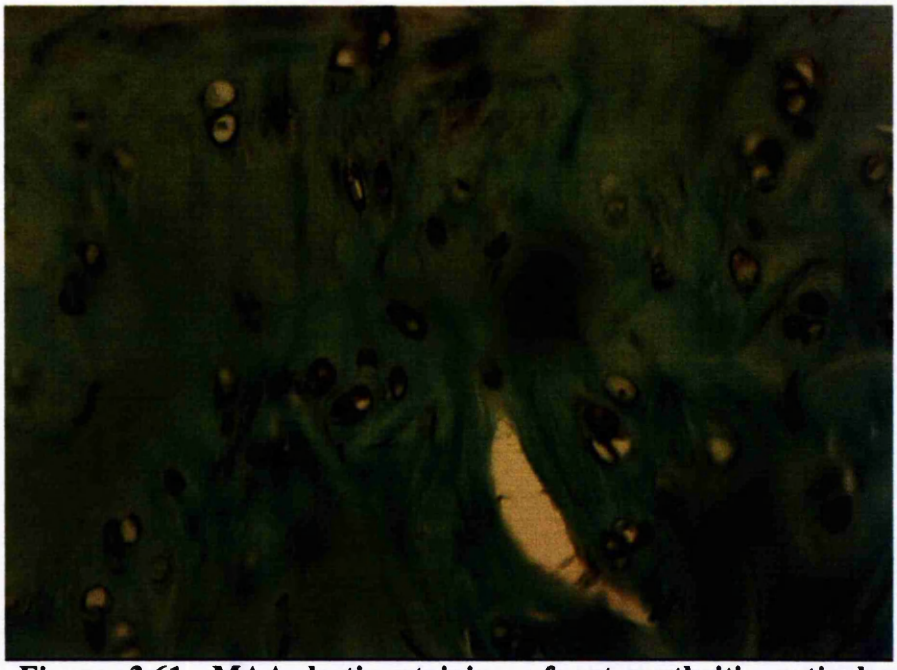


Figure 3.61. MAA lectin staining of osteoarthritic articular cartilage (X20).
Both matrix and chondrocytes were stained.

Figure 3.62. MAA lectin staining of the tidemark in normal articular cartilage (arrow) (X10).

3.1.5.4. β-Galactose-binding lectins

ECA

Articular cartilage

Matrix: The superficial matrix in normal cartilage and osteoarthritic cartilage was stained weakly to moderately. The interterritorial matrix showed more reaction than territorial matrix in normal articular cartilage. The matrix of mild to moderate OA in medial and deep zones was stained strongly, although the matrix of severe OA showed weak reactions with ECA. Matrix of the clones was not stained.

Chondrocytes: In the superficial area of normal and mild to moderate OA, chondrocytes were stained moderately, but in medial and deep zones of these areas, 30% of them were stained very strongly with ECA. 40% of the chondrocytes in severe OA showed strong reactions with ECA. Chondrocytes of the clones showed no reaction with ECA.

Fibrocartilage

Matrix: The matrix of normal and osteoarthritic fibrocartilage was stained weakly with ECA.

Chondrocytes: In normal fibrocartilage, chondrocytes were stained weakly. In mild to moderate OA, they showed moderate reactions with ECA, but chondrocytes of severe OA failed to show any reaction with ECA.

Subchondral bone

Bone trabeculae showed a weak reaction and osteocytes were negative.

Synovium and capsule

Synovium and capsule showed moderate to strong reactions with ECA.

Summary of the ECA lectin staining is shown in appendix C (Table C8). Examples of these features are seen in Figures 3.63 –3.64.

PNA

Articular cartilage

Matrix: In normal and severe OA, the matrix was stained weakly, but in mild to moderate OA there were strong reactions between interterritorial matrix and PNA. Matrix of the clones showed no reaction.

Chondrocytes: PNA showed no reactions with chondrocytes of normal and osteoarthritic cartilage. Chondrocytes of the clones were negative.

Fibrocartilage

Matrix: The matrix of fibrocartilage in normal and osteoarthritic cartilages showed weak reactions with PNA.

Chondrocytes: No reaction was seen between PNA and chondrocytes of normal and osteoarthritic fibrocartilage.

First rib

Matrix: There were no binding sites on the matrix for PNA.

Chondrocytes: No staining of the chondrocytes was observed.

Subchondral bone

Bone trabeculae showed a weak reaction and osteocytes were negative.

Synovium and capsule

Summary of the PNA lectin staining is shown in appendix C (Table C9). Synovium and capsule showed no reaction with PNA.

Examples of these features are seen in Figures 3.65 –3.66.

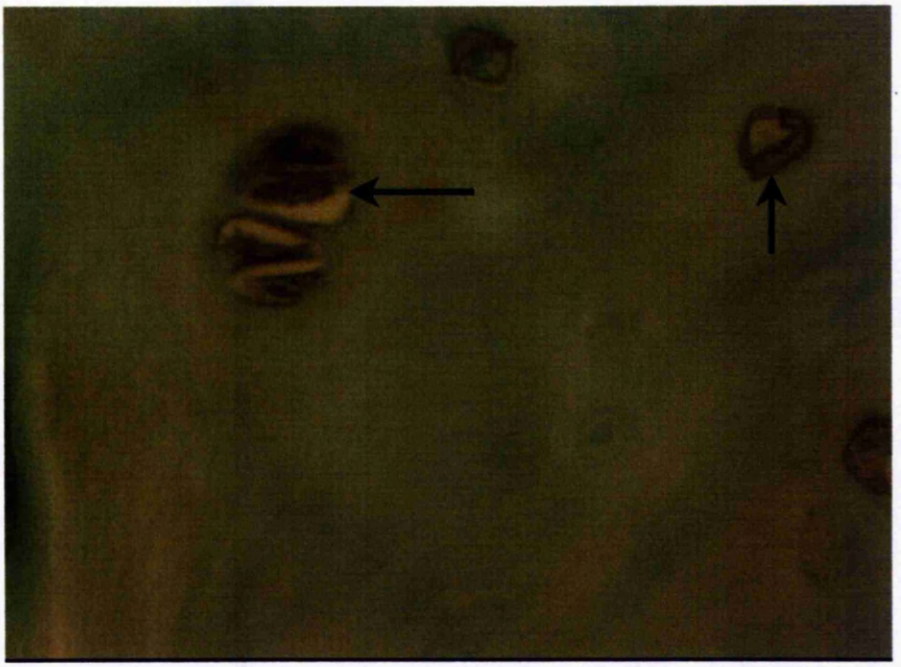


Figure 3.63. ECA lectin staining of cartilage in severe OA (X40). Matrix stained weakly but chondrocytes stained strongly (arrows).

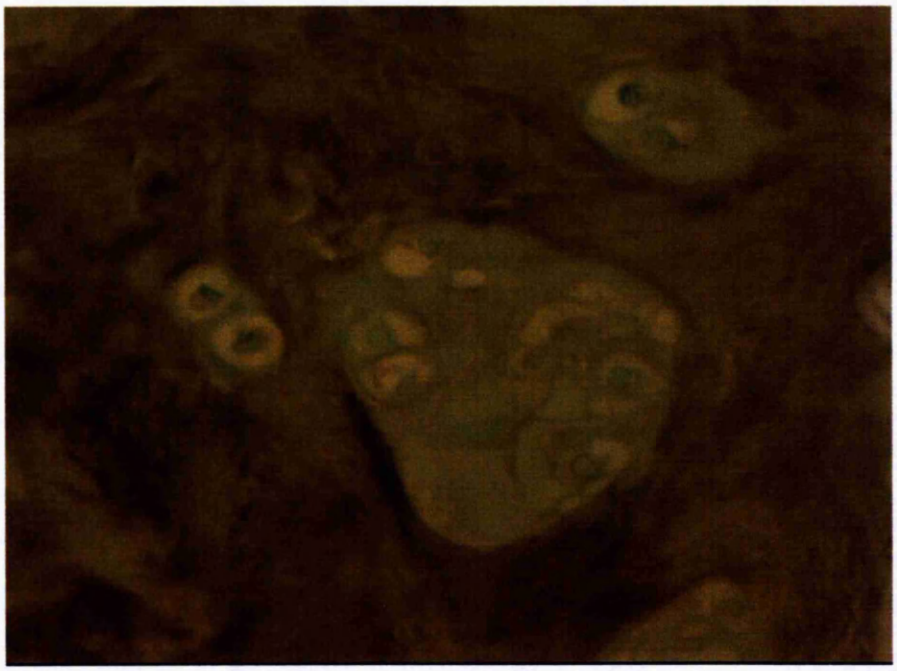


Figure 3.64. ECA lectin staining of clones of chondrocytes in mild to moderate OA (X40). Clones of chondrocytes are not stained strongly, but original matrix was stained strongly.

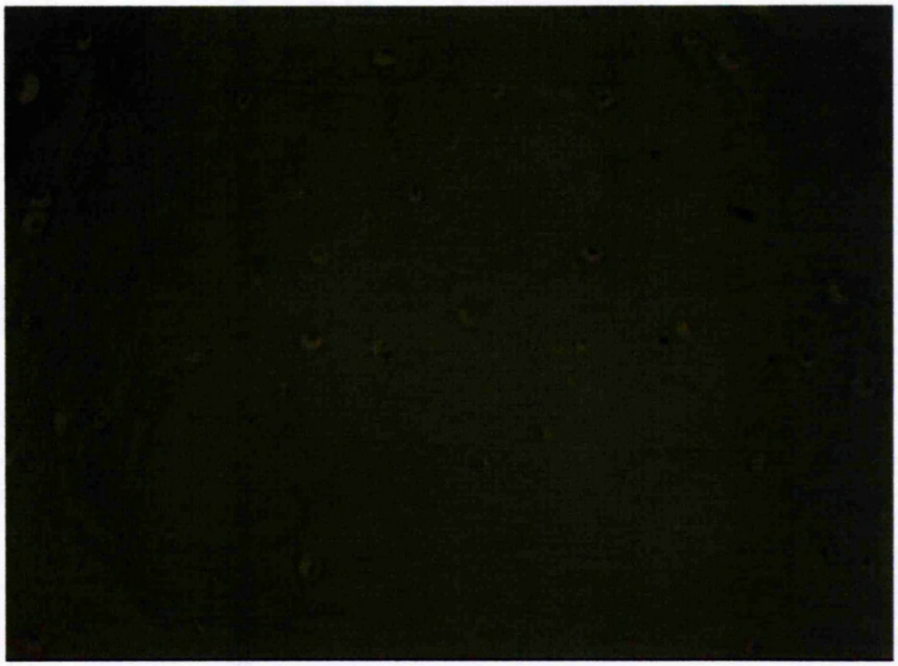


Figure 3.65. PNA lectin staining of normal articular cartilage (X10). Only the matrix stained weakly.

Figure 3.66. PNA lectin staining of mild to moderate osteoarthritic cartilage (X10). Matrix stained strongly, but chondrocytes were negative.

3.1.5.5. N-acetylgalactosamine-binding lectins

DBA

Articular cartilage

Matrix: Only the interterritorial matrix of normal SCJ showed moderate reactions with DBA. Other parts of normal and osteoarthritic matrix failed to show any reaction. Matrix of the clones showed no reaction with DBA.

Chondrocytes: Chondrocytes in normal and osteoarthritic cartilage showed no reaction with DBA. Chondrocytes of the clones were negative as well.

Fibrocartilage

Matrix: No staining was seen in the matrix of normal and osteoarthritic fibrocartilage.

Chondrocytes: No reaction was seen between chondrocytes of normal and osteoarthritic fibrocartilage and DBA.

First rib

Matrix: There was no reaction between the matrix of the first rib and DBA.

Chondrocytes: The chondrocytes showed no reaction with DBA.

Subchondral bone

Bone trabeculae were stained weakly and osteocytes were negative.

Synovium and capsule

No reaction was seen between synovium and capsule with DBA.

Summary of the DBA lectin staining is shown in appendix C (Table C710). Examples of these features are seen in Figures 3.67 –3.68.

VVA

Articular cartilage

Matrix: VVA had only a weak reaction with the surface of mild to moderate osteoarthritic cartilage. Normal matrix and other osteoarthritic parts showed no reaction. Matrix of the clones was not stained with VVA.

Chondrocytes: No reaction was seen between chondrocytes of normal and osteoarthritic SCJ with VVA. Chondrocytes of the clones showed no reaction.

Fibrocartilage

Matrix: The matrix of normal and osteoarthritic fibrocartilage was negative for VVA.

Chondrocytes: Both matrix and chondrocytes of normal and osteoarthritic cartilage showed no reaction with VVA.

First rib

Matrix: The matrix of the first rib failed to stain with VVA.

Chondrocytes: There was no reaction between chondrocytes and VVA.

Subchondral bone

No reaction was seen between bone trabeculae and osteocytes with VVA.

Synovium and capsule

Synovium showed no reaction with VVA, but capsule was stained weakly.

Summary of the VVA lectin staining is shown in appendix C (Table C11). An example of this feature is seen in Figure 3.69.

MPA

Articular cartilage

Matrix: In normal SCJ, the matrix was stained weakly. In the matrix of osteoarthritic cartilage, only the interterritorial matrix of severe OA showed a weak reaction with MPA. Matrix of the clones showed no reaction.

Chondrocytes: Chondrocytes of normal and osteoarthritic articular cartilage had no binding sites for MPA. Chondrocytes of the clones showed no reaction.

Fibrocartilage

Matrix: In normal fibrocartilage, the matrix was stained weakly. Osteoarthritic matrix had no reaction with MPA.

Chondrocytes: Only normal fibrocartilage chondrocytes showed a moderate reaction with MPA, and others showed no reaction.

First rib

Matrix: There were no binding sites in the matrix for MPA.

Chondrocytes: No staining by MPA was seen on the chondrocytes.

Subchondral bone

Bone trabeculae and osteocytes were stained weakly.

Synovium and capsule

Both synovium and capsule were negative.

Summary of the MPA lectin staining is shown in appendix C (Table C12). An example of this feature is seen in Figure 3.70.

HPA

Articular cartilage

Matrix: No reaction was seen between the matrix of normal and osteoarthritic SCJ and HPA. Matrix of the clones showed no reaction with HPA.

Chondrocytes: Chondrocytes of normal and osteoarthritic SCJ had no binding sites for HPA. Chondrocytes of the clones showed no reaction.

Fibrocartilage

Matrix: No reaction was seen between normal and osteoarthritic matrix of fibrocartilage and HPA.

Chondrocytes: Chondrocytes of normal and osteoarthritic fibrocartilage showed no reaction with HPA.

First rib

Matrix: There was no reaction between the matrix of first rib and HPA.

Chondrocytes: The chondrocytes showed no reaction with HPA.

Subchondral bone

No reaction was seen between bone trabeculae and osteocytes with HPA.

Synovium and capsule

Synovium and capsule had no biding site for HPA.

Summary of the HPA lectin staining is shown in appendix C (Table C13). An example of this feature is seen in Figure 3.71.

WFA

Articular cartilage

Matrix: The matrix of normal and osteoarthritic SCJ showed no reaction with WFA.

Chondrocytes: No reaction was seen between normal and osteoarthritic chondrocytes and WFA.

Fibrocartilage

Matrix: No binding sites for WFA were found in matrix of normal and osteoarthritic fibrocartilage.

Chondrocytes: Chondrocytes of normal and osteoarthritic fibrocartilage showed no reaction with WFA.

First rib

Matrix: The matrix of the first rib showed no reaction with WFA.

Chondrocytes: There were no binding sites on chondrocytes for WFA.

Subchondral bone

Bone trabeculae showed weak to moderate reaction with WFA, but osteocytes were negative.

Synovium and capsule

Synovium showed no reaction with WFA, but capsule was stained moderately.

Summary of the WFA lectin staining is shown in appendix C (Table C14). An example of this feature is seen in Figure 3.72.

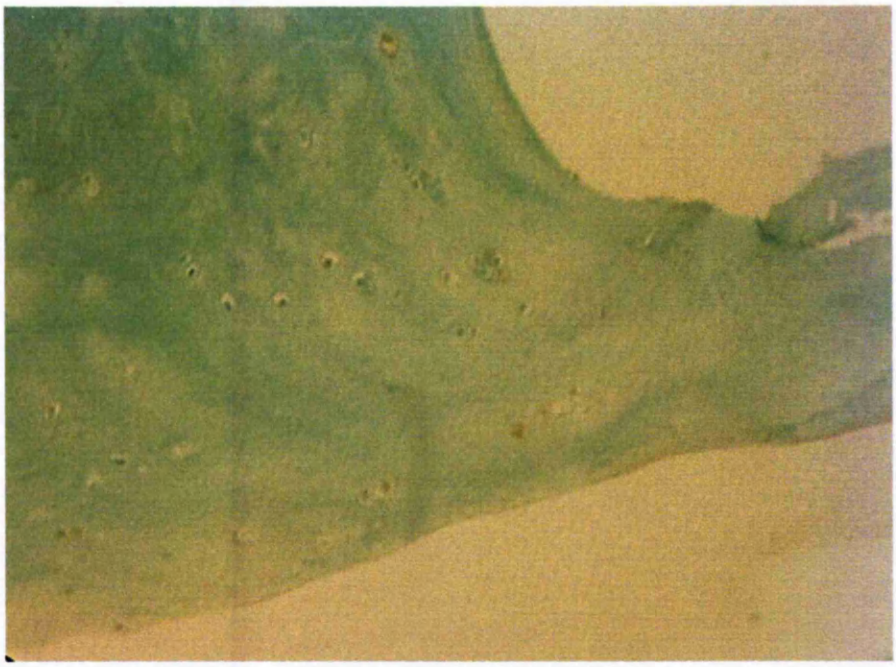


Figure 3.67. DBA lectin staining of osteoarthritic articular cartilage (X10). No staining of the matrix and chondrocytes was seen.

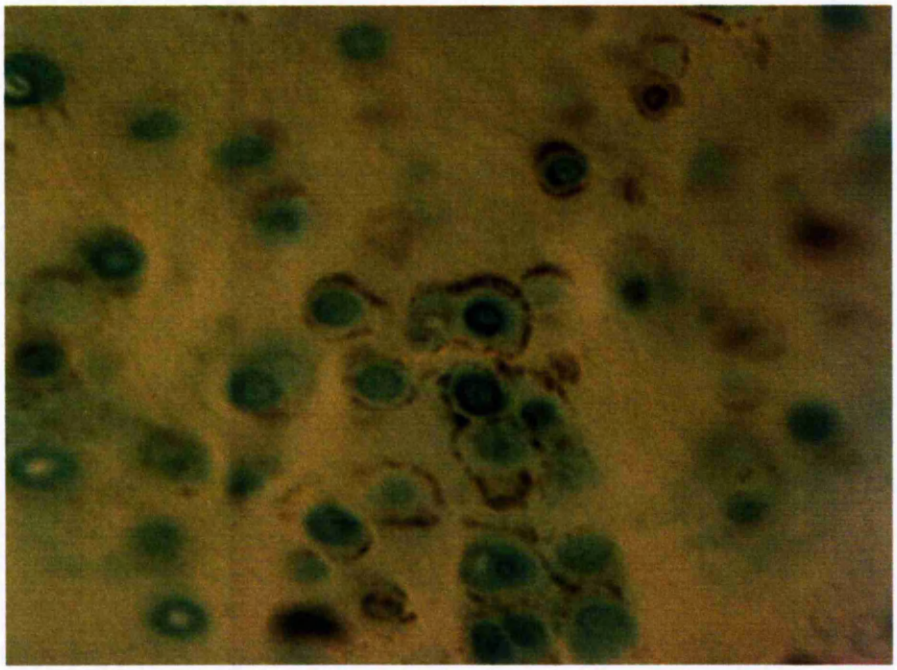


Figure 3.68. DBA lectin staining of normal articular cartilage (X40). Perilacunar matrix was stained moderately to strongly.

Figure 3.69. VVA lectin staining of mild to moderate osteoarthritic cartilage (X10). Only weak staining of the matrix was seen.

Figure 3.70. MPA lectin staining of normal articular cartilage (X10). Only the matrix showed a weak reaction.

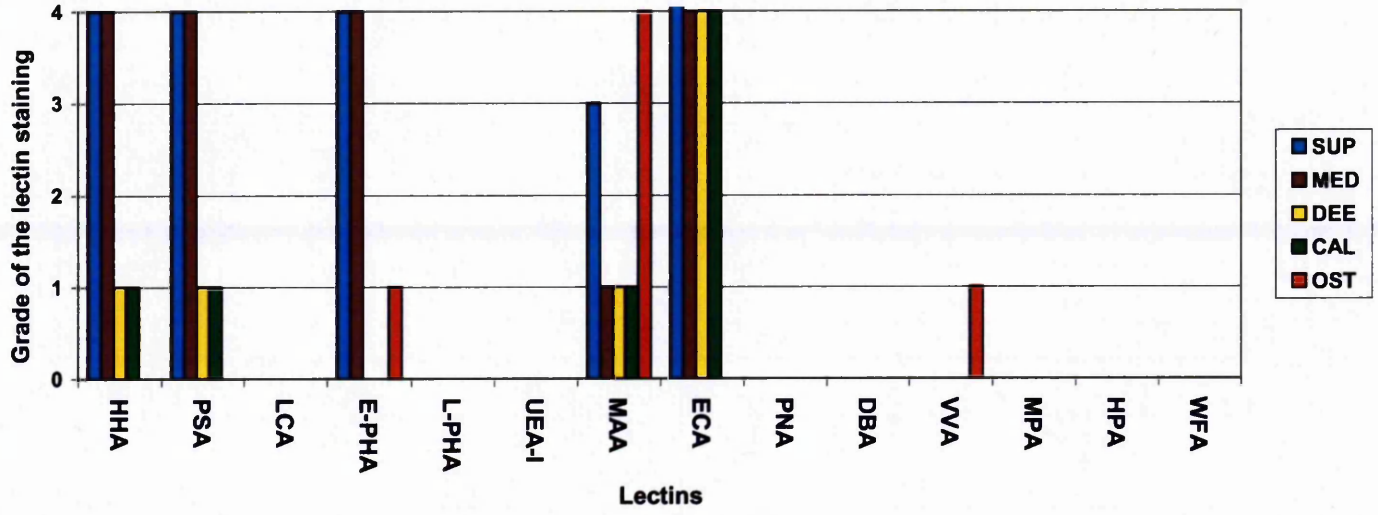
Figure 3.71. HPA lectin staining of articular cartilage (X10).
No reaction between HPA and articular cartilage was seen.

Figure 3.72. WFA lectin staining of articular cartilage (X10).
No reaction between WFA and articular cartilage was seen.

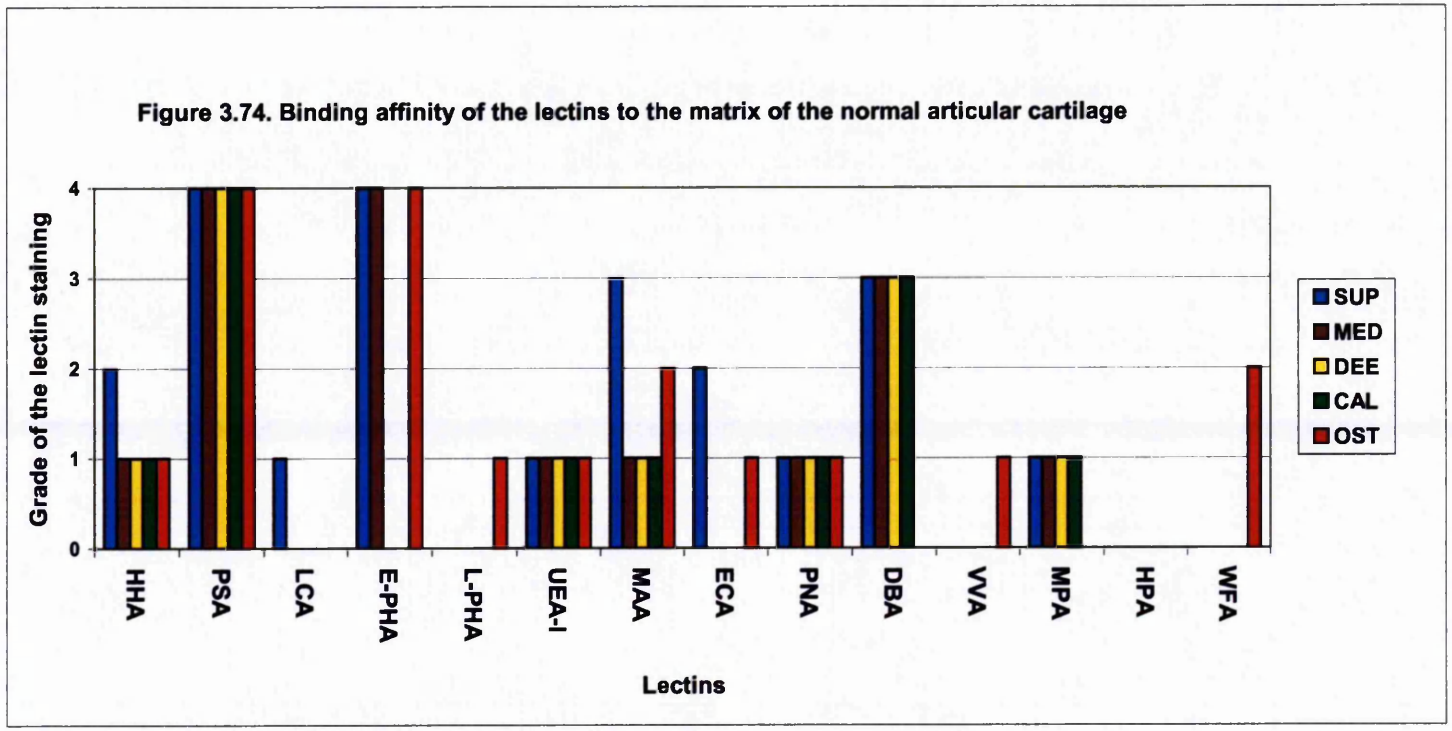
3.1.5.6. Overall summary

For all the cell and matrix staining reactions of normal and osteoarthritic articular cartilage the intensities of the lectin staining reactions for each lectin were averaged and recorded in a number of glycoprofiles. These are shown in Figures 3.95 to 3.100. In the articular cartilage glycoprofiles comparisons of cells and matrix in the superficial, medial, deep and calcified zones and subchondral bone were made.

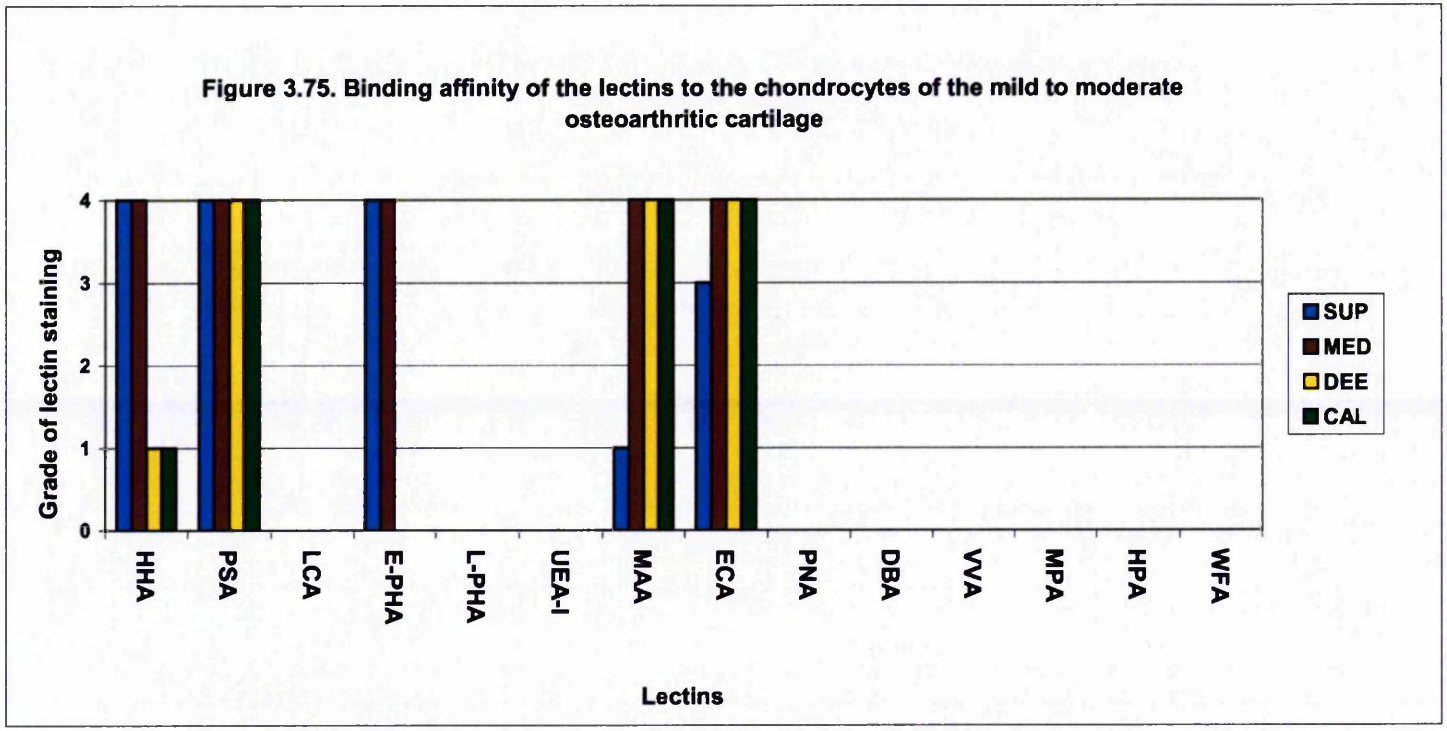
Figure 3.73. Binding affinity of the lectins to the chondrocytes of the normal articular cartilage



SUP: superficial chondrocytes, MED: medial chondrocytes, DEE: deep chondrocytes, CAL: calcified zone chondrocytes, OST:osteocytes.

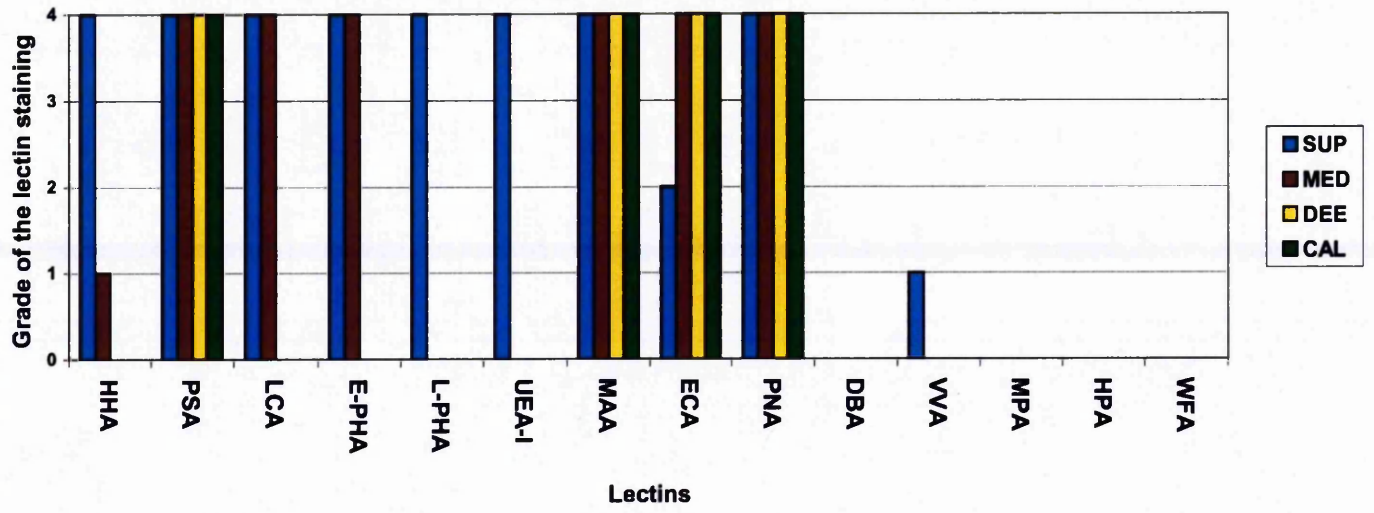


SUP: superficial zone, MED: medial zone, DEE: deep zone, CAL: calcified zone, OST: osteoid.



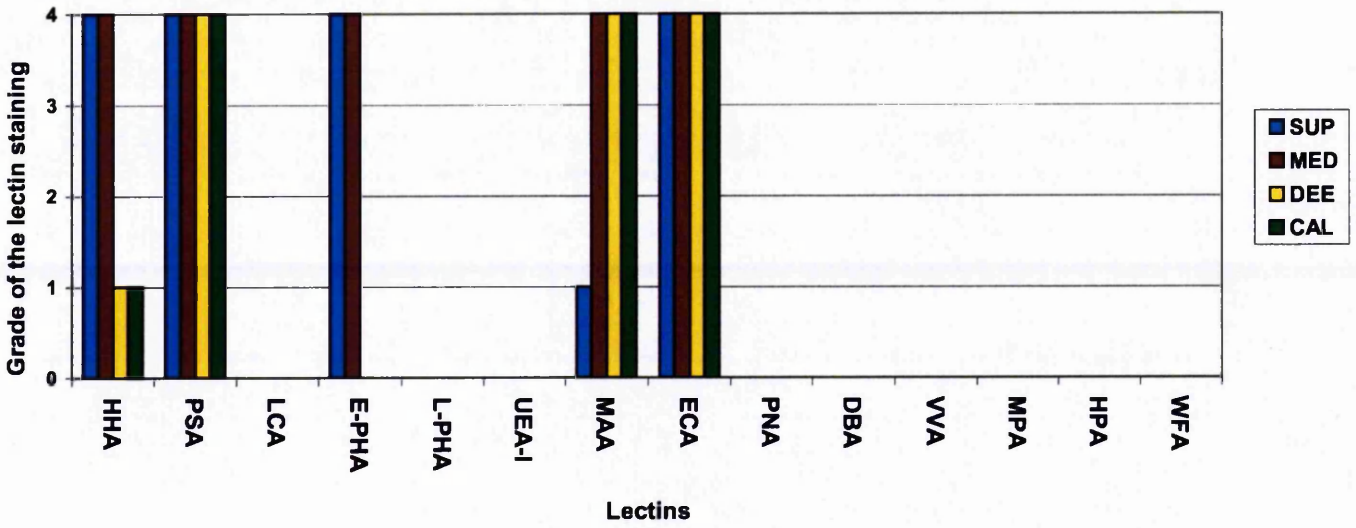
SUP: superficial chondrocytes, MED: medial chondrocytes, DEE: deep chondrocytes, CAL: calcified zone chondrocytes.

Figure 3.76. Binding affinity of the lectins to the matrix of the mild to moderate osteoarthritic cartilage



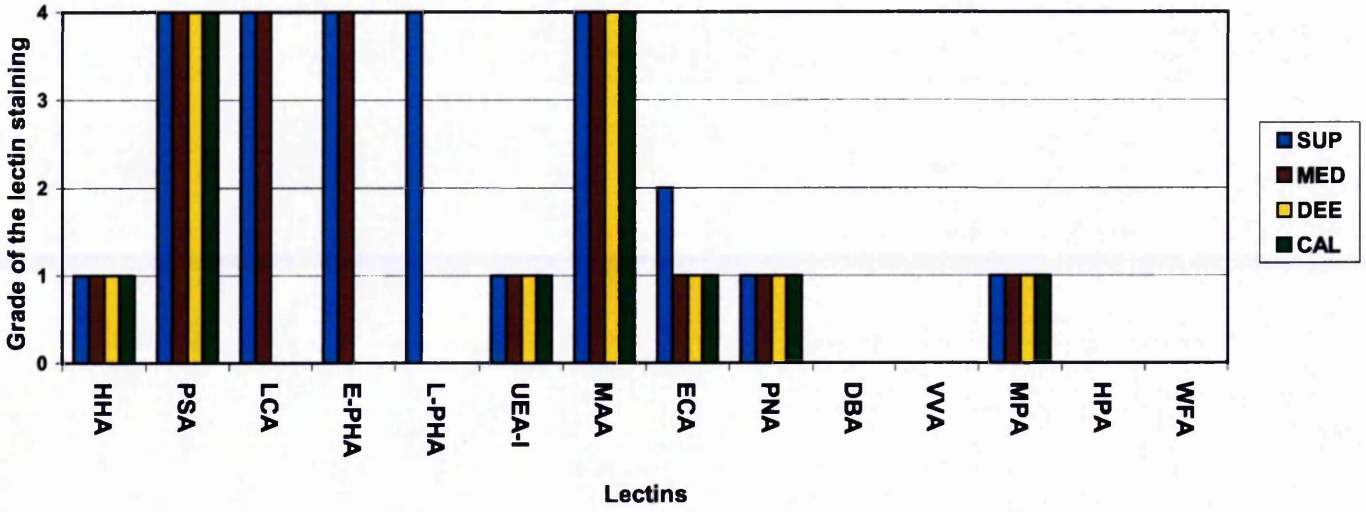
SUP: superficial zone, MED: medial zone, DEE: deep zone, CAL: calcified zone

Figure 3.77. Binding affinity of the lectins to the chondrocytes of the severe osteoarthritic cartilage



SUP: superficial chondrocytes, MED: medial chondrocytes, DEE: deep chondrocytes, CAL: calcified zone chondrocytes.

Figure 3.78. Binding affinity of the lectins to the matrix of the severe osteoarthritic cartilage



SUP: superficial zone, MED: medial zone, DEE: deep zone, CAL: calcified zone

Section 2

3.2 Costochondral junction

Seventy-five costochondral junctions (stillbirth and infant) were investigated by a variety of techniques. All costochondral junctions were stained by H&E, toluidine blue and picro sirius red. Lectin histochemistry, using 16 different lectins, was also performed on the costochondral junctions.

3.2.1 Microanatomy

Using H&E staining, samples were classified into two groups. One group had normal cartilage growth plate features and the second group showed abnormal features. Normal samples contained a well-formed growth plate bordered on one end by chondral apophyseal cartilage and the other end by subchondral bone.

In the normal cartilage growth plate, the reserve cartilage adjacent to the proliferative zone was almost indistinguishable from the reminder of the apophyseal cartilage, except that the reserve cells were slightly elongated with their long axis parallel to the cartilage-bone interface. Cartilage canals, comprised of vascular and perivascular fibrous connective tissue, were sometimes seen in the apophyseal cartilage. The cells in the reserve zone were spherical in outline, existed singly, or in pairs occasionally, and were relatively few in number compared with the number of cells in other zones. They were separated from each other by more extracellular matrix than cells in the other zones.

In the proliferative zone, the spherical, single or paired cells in the reserve zone give way to flattened chondrocytes, which were organised into elongated clusters separated by septa. The individual clusters, which were two or four cells wide and five to ten cells deep, showed variation in cell features so that cells at the same distance from the cartilage/bone interface might be not all be at the same stage of differentiation.

The cells in the hypertrophic zone were in groups like the proliferative zone, but they were larger than the proliferating cells. The matrix around them was less than in other areas. A few lacunae near to the osteochondral interface were empty.

All cases were divided into two groups according to their PM records. First group had normal record and second had reports of various skeletal abnormalities. The diagnostic slides were checked by a pathologist.

The microanatomy was altered in some of the abnormal cases as shown by changes in the arrangement of the cells or by changes in the cell populations. Others of the abnormal cases did not show any microanatomical change.

In thanatophoric dwarfism, the reserve zone was hypercellular with less matrix between the cells. In the proliferative zone no cell columns were seen, and the size of the cells was greater than normal. In the hypertrophic zone not all cells became hypertrophic and cells of mixed size were seen. In the subchondral tissue only a few bone trabeculae were present. In achondrogenesis type I, the resting cells were vacuolated. No cell columns were seen in the proliferative zone and in the hypertrophic zone there was a mixture of cell sizes. In homozygous achondroplasia, the resting cells looked normal, but the proliferative cells were round and were not all in columns. The size of cells in the hypertrophic zone was no bigger than that of proliferative cells. The resting zone of the rachitic growth plate showed no differences from normal. The proliferative zone contained irregular cell columns extending into other zones. In addition, there was obvious expansion of the hypertrophic zone. In Campomelic dysplasia and Beckwith's syndrome the features of the growth plate appeared normal.

3.2.2 Histochemistry

3.2.2.1 Toluidine blue staining

This stain demonstrated glycosaminoglycans (GAGs) in the cartilage growth plate. The pericellular matrix was stained very intensely with TB. However, TB stained the entire matrix in the cartilage growth plate moderately to strongly. In the proliferative and hypertrophic zones, territorial and interterritorial matrices, which were spatially related to the chondrocyte clusters rather than to individual cells, and cells within a cluster shared the same staining, which was intense blue, for territorial or interterritorial matrices. TB stained the matrix in the reserve zone less than matrices of other zones.

Abnormal growth plate showed not much difference.

3.2.2.2 Picro sirius red

The territorial matrix in all cartilage growth plate zones was stained intensely by PSR. In the proliferative and hypertrophic zones, a band of slightly more intense staining was often observed at the inner border of the territorial matrix. This band surrounded the clusters of chondrocytes in these zones. Interterritorial matrix in proliferative and hypertrophic zones was stained more intensely with PSR than the territorial matrix.

Hypertrophic chondrocytes were stained with PSR. The cartilage matrix septa of the lower growth plate and the new bone trabeculae were also stained by PSR.

Abnormal growth plates did not show not much difference from normal.

Examples of these features are seen in Figures 3.73 to 3.77.

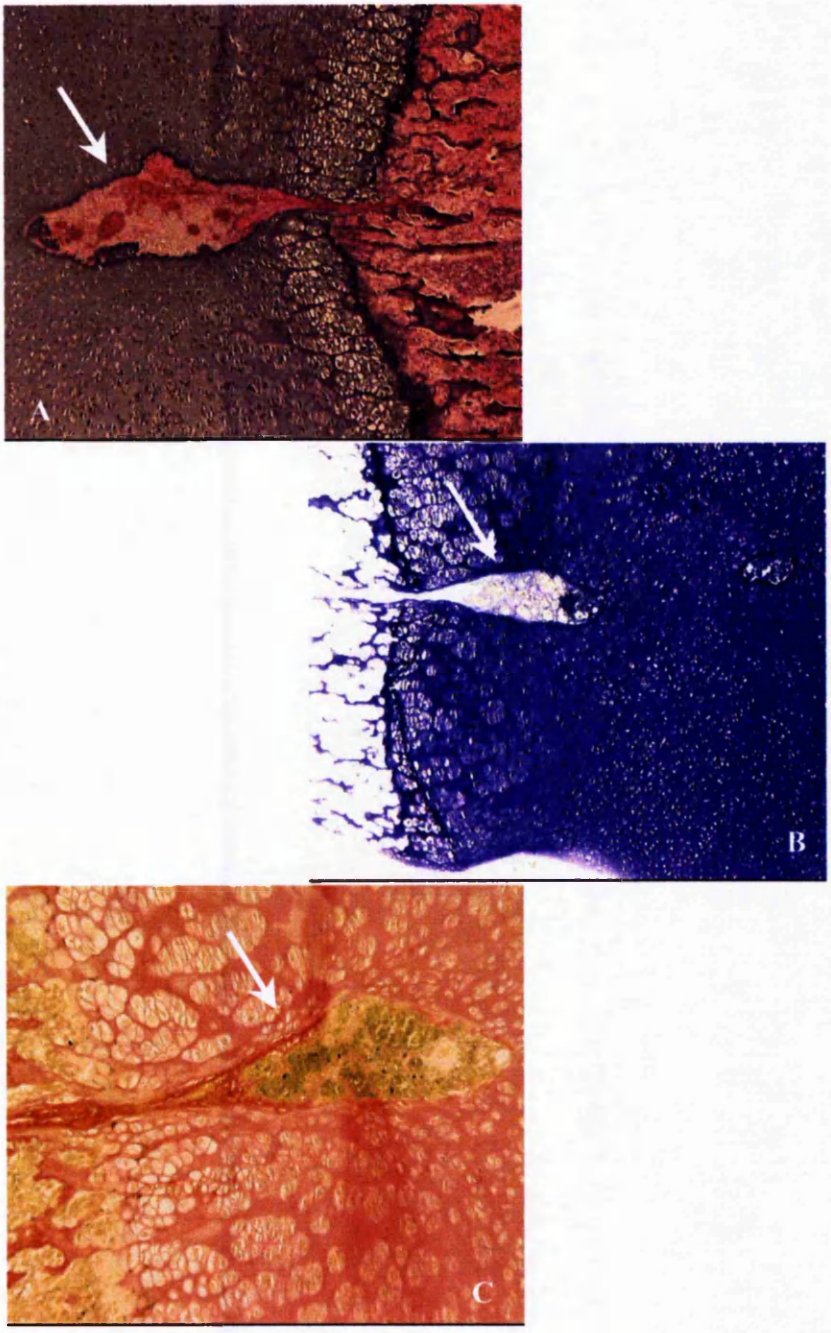


Figure 3.79. Cartilage canal in normal growth plate (arrows) (A: H&E X5) (B:Toluidine blue X5) (C: Picro sirius red X10)

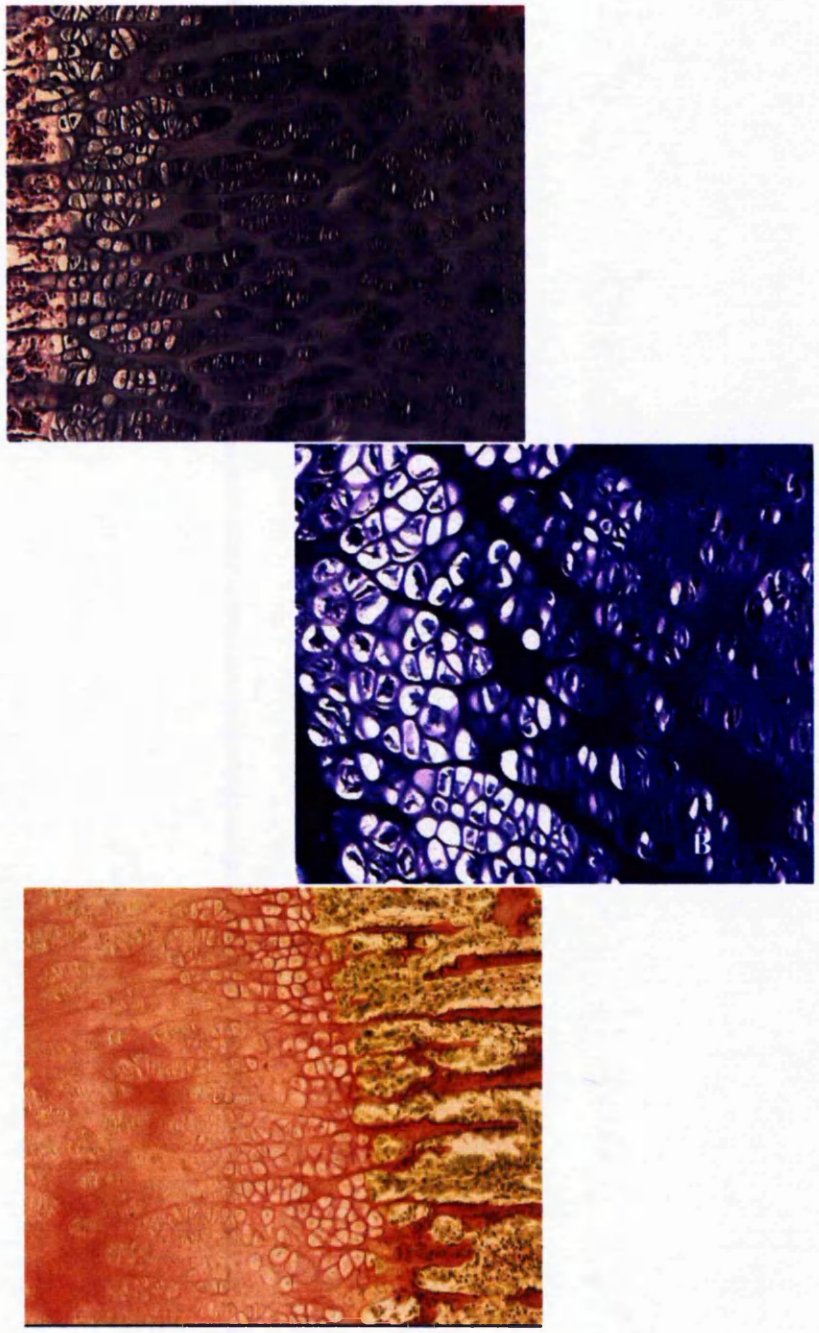


Figure 3. 80. Normal proliferative and hypertrophic zones of growth plate. (A: H&E X5) (B: Toluidine blue X10) (C:Picro sirius red X5).

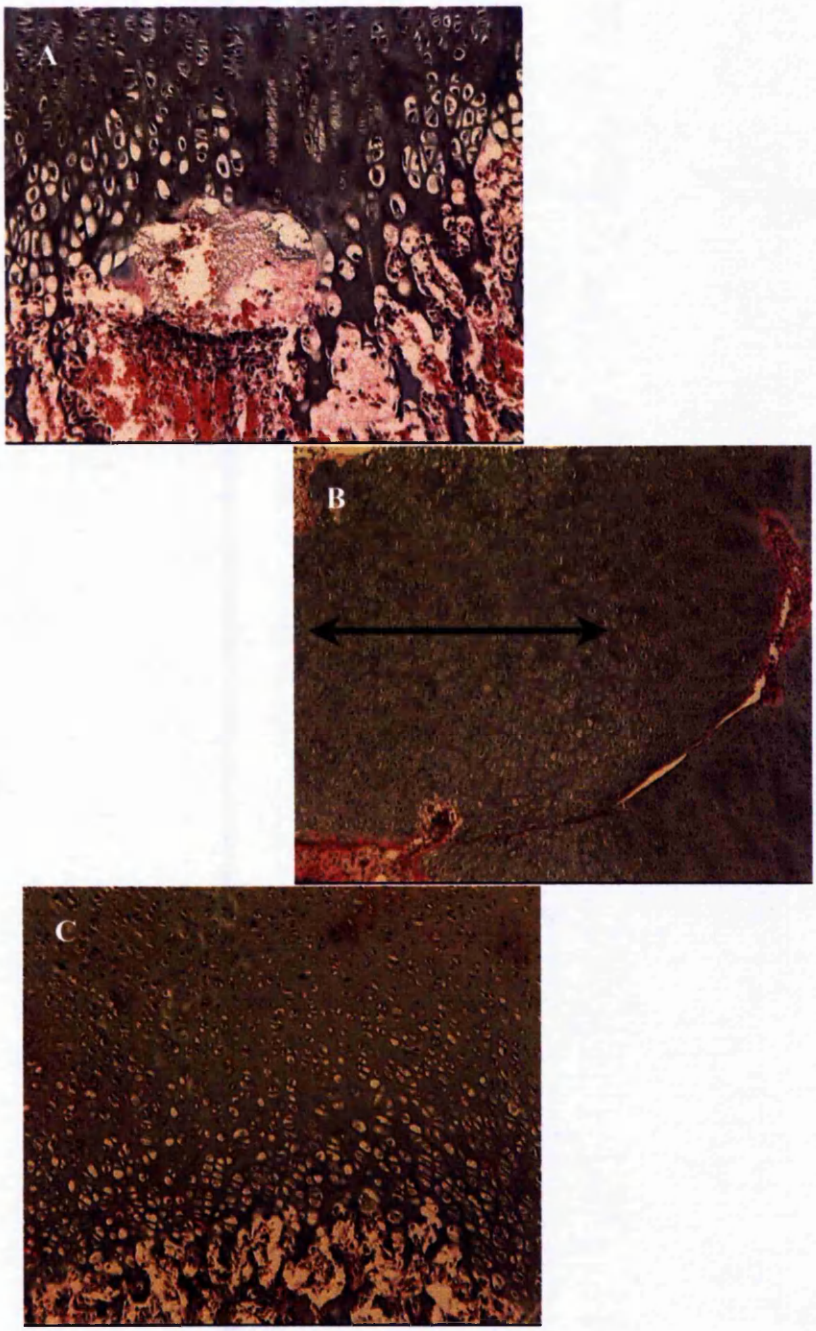


Figure 3.81. Abnormal growth plates (H&E)
A: proliferative and hypertrophic zones in achondroplasia (X5).
B: expansion of the rachitic hypertrophic zone (arrow) (X5).
C: proliferative and hypertrophic zones in thanataphoric dysplasia (X5).

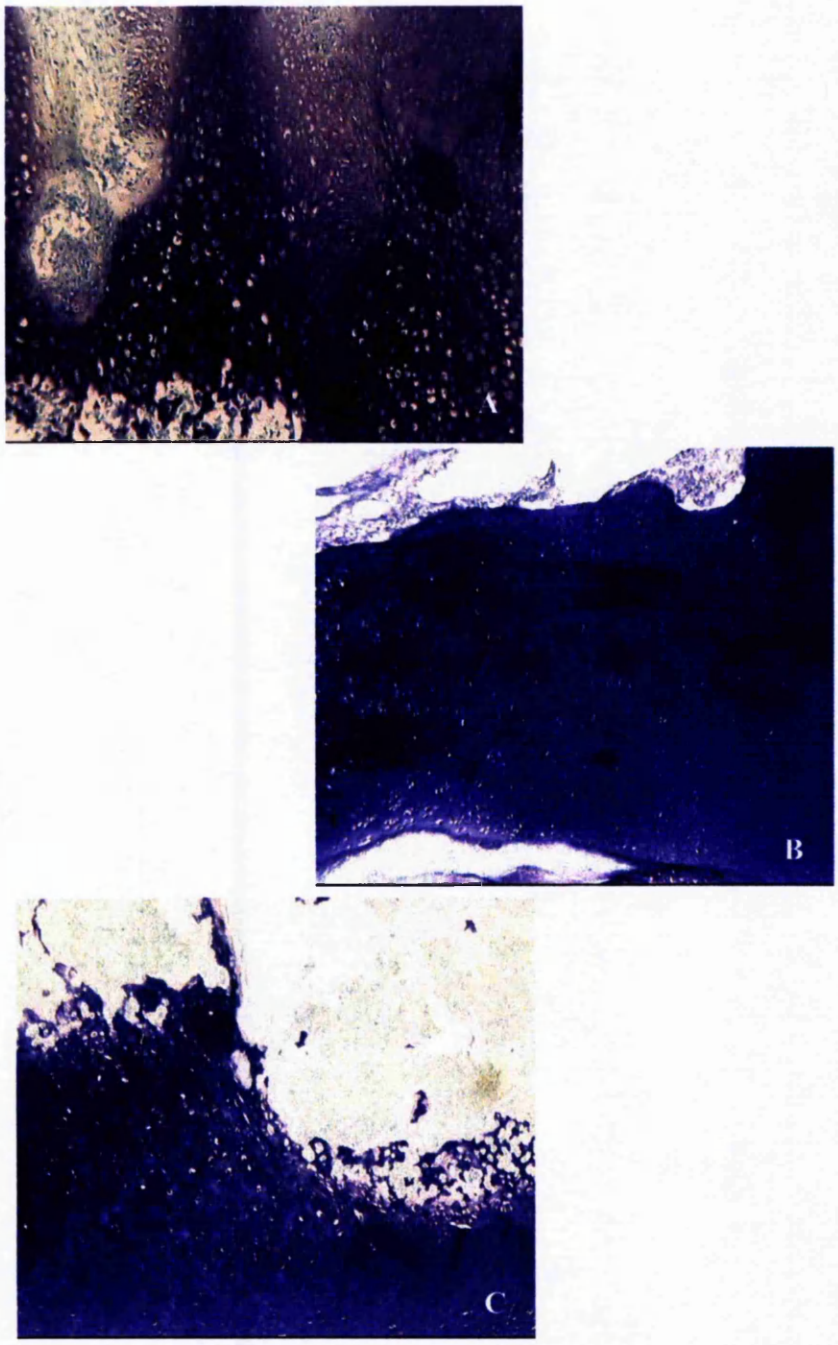


Figure 3.82. Abnormal growth plate (Toluidine blue).
A: growth plate of thanataphoric dysplasia (X10). B: homogeneous toluidine blue staining of the abnormal growth plate (X5).
C: proliferative and hypertrophic zones in achondrogenesis (X5).

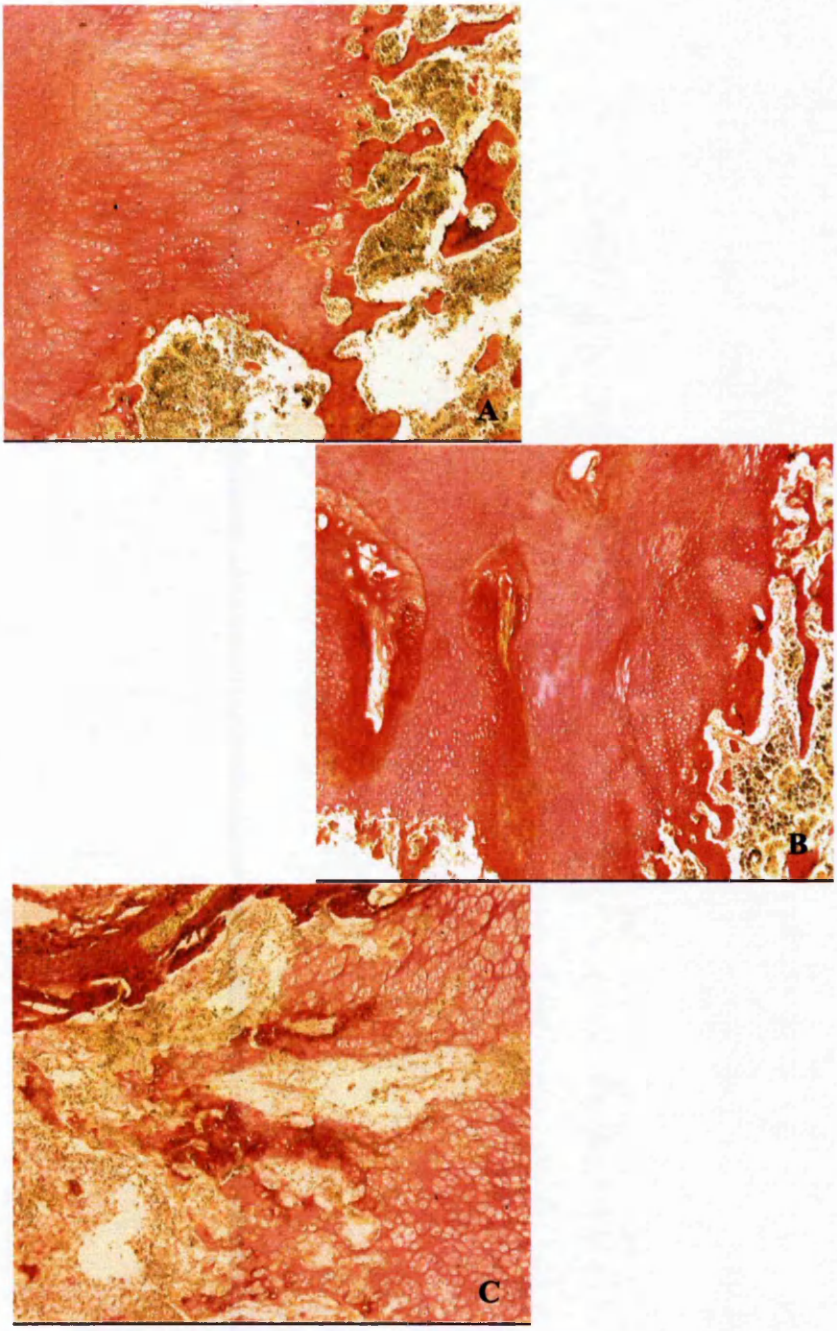


Figure 3.83. Abnormal growth plates (Picro sirius red)
A: proliferative and hypertrophic zones in achondrogenesis (X5).
B: proliferative and hypertrophic zones in thanatophoric dysplasia (X5).
C: abnormal costochondral junction in achondroplasia (X10).

3.2.3. Lectin histochemistry

3.2.3.1. Mannose and complex N-glycan binding lectins

HHA

Matrix: HHA showed no strong reactions with territorial and interterritorial matrix of normal and abnormal growth plate.

Chondrocytes: Normal chondrocytes were stained mostly strongly with HHA. HHA was a strong marker for the chondrocytes of thanatophoric dwarfism, short limb dwarfism, Beckwith's syndrome and calcium deposition abnormalities (e.g. rickets), but chondrocytes of achondroplasia, achondrogenesis and Campomelic dysplasia showed no reaction with HHA.

After β-elimination:

Matrix: A weak to moderate reaction was seen in the matrix of the normal resting zone of the growth plate after β-elimination with HHA. No changes were seen in the matrix of other zones.

Chondrocytes: All chondrocytes of the normal growth plate showed moderate to strong reaction after β-elimination with HHA. The pericellular areas of the resting chondrocytes were stained moderately with HHA after β-elimination.

Pre-treatment with hyaluronidase:

Matrix: Only the interterritorial matrix of the normal resting zone was stained weakly with HHA after pre-treatment with hyaluronidase.

Chondrocytes: No change was seen in chondrocytes after pre-treatment with hyaluronidase.

Examples of these features are seen in Figures 3.78- 3.79.

Table 3.5. Summary of HHA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	4p	-	-	-	1-3p	-	-	-	1	-	-	-
2		3p	-	-	-	3p	-	-	-	3p	-	-	-
3		4	4	-	1	4	4	-	-	4	-	-	-
4		4	-	-	1-2	3-4	-	-	1-2	3-4	-	-	-
5		1p	-	-	-	-	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	-	-	-	-	-	-	-	-	-	-	-
8		4	4	-	1	3-4	-	-	-	4	-	-	-
9		3p	-	-	-	3	-	-	-	3p	-	-	-
10		3p	-	-	-	2-4	-	-	-	2-4	-	-	-
11		1	1	1	1	1	1	1	1	1	1	1	1
12		-	-	-	-	-	-	-	-	3p	-	-	-
13		3p	3	-	-	-	-	-	-	-	-	-	-
14	CD	2p	-	-	1	3-4	-	-	2	2-3	1	-	2
15		3p	-	-	-	4p	-	-	-	4p	-	-	-
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	1	-	-	-	1	-	-	1	1
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		4	-	-	1	4	-	-	-	4	-	-	-
21	SA	-	-	-	-	3p	-	-	-	3p	-	-	-
22		1p	-	-	-	2-3	-	-	-	1-2	-	-	-
23		-	-	-	-	0-2	0-2	-	-	1-4	-	-	-
24	ACD	2-4	-	-	1	3-4	-	-	1	3-4	1	-	-
25		3-4	-	-	-	3	-	-	-	3	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix,
p: punctuate staining

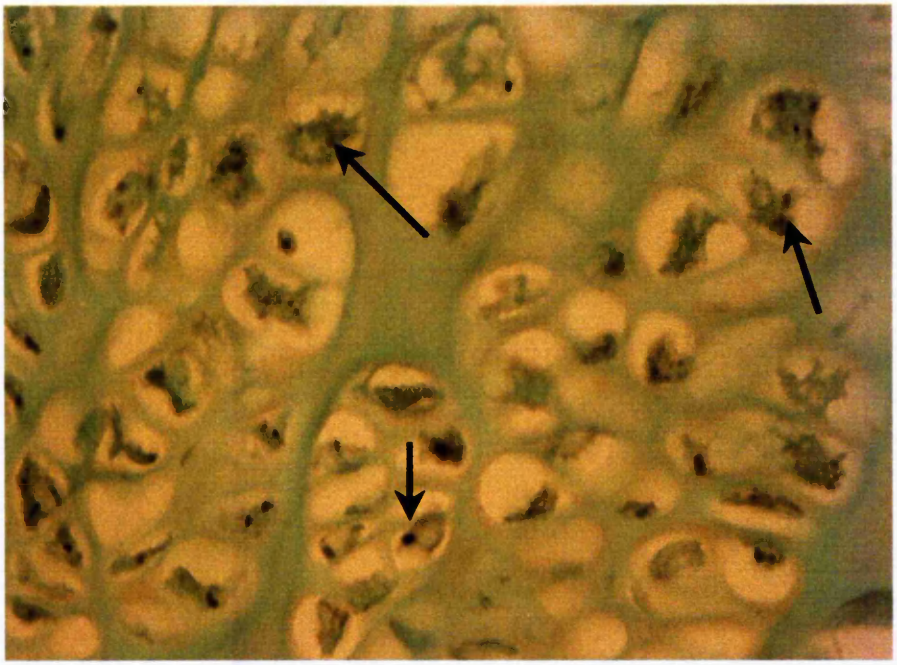


Figure 3.84. HHA lectin staining of the normal hypertrophic zone (X20). Chondrocytes showed a strong brown punctuate staining (arrows).

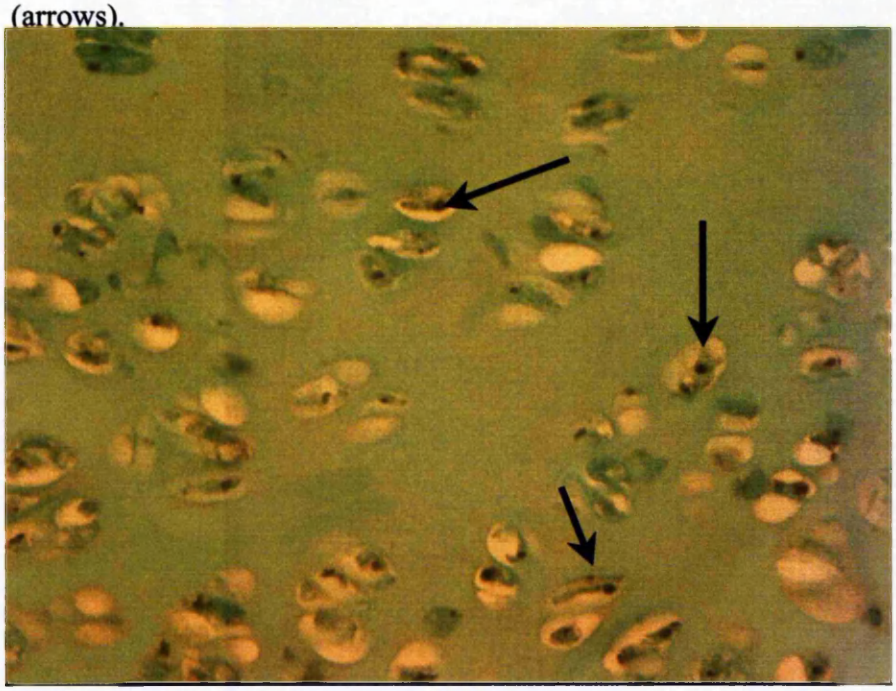


Figure 3.85. HHA lectin staining of the thanatophoric proliferative zone (X20). Abnormal chondrocytes showed a brown punctuate staining (arrows).

PSA

Matrix: Territorial matrix of the hypertrophic zone of normal growth plate stained mostly weakly to strongly with PSA and, in the proliferative zones, territorial matrix stained weakly to moderately. Pericellular matrix of the normal hypertrophic cells was also stained.

Interterritorial matrices of normal cases were stained weakly with PSA. Interterritorial matrix of abnormal growth plates was stained weakly to moderately with PSA and the interterritorial matrices of proliferative and hypertrophic zones of thanatophoric dysplasia and short limb dwarfism were stained strongly with PSA.

Chondrocytes: The normal resting chondrocytes were stained strongly by PSA. Chondrocytes of normal proliferative and hypertrophic zones were mostly weakly stained by PSA.

Chondrocytes of the resting zone from cases with abnormal calcium depositions were stained strongly with PSA. In proliferative and hypertrophic zones of abnormal cases mostly chondrocytes were stained.

Examples of these features are seen in Figures 3.80 -3.81.

Table 3.6. Summary of PSA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	1-2	2-3	-	1	-	-	1-2	1	-	-	-	1
2		0-4	-	-	-	0-3	-	-	-	-	-	2	1
3		3-4	2-3	-	1	-	-	-	2-3	0-1	3	3	3
4		0-3	-	-	-	0-3	-	-	-	0-3	3	3	-
5		-	-	-	-	-	-	-	1	-	2	0-2	0-2
6		1	1	-	1	1	1	-	-	1	1	-	1
7		-	-	-	-	-	-	-	-	-	-	-	-
8		0-1	-	-	1	0-1	-	-	-	0-1	-	-	-
9		0-2	-	-	-	0-1	-	-	1-2	1	1	-	-
10		-	-	-	-	0-1	-	-	1-2	1	1	1	-
11		2	-	-	1	2	-	1	1	2	1	1	1
12		-	-	2-3	1	-	-	2	-	-	2	2	0-1
13		-	1	2-3	2	0-1	1	1	1	0-1	2	1	1
14	CD	-	-	-	1	0-1	-	-	3	0-1	-	-	3
15		-	-	-	1	0-1	-	-	2-3	0-1	-	-	2-3
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	0-1	0-1	-	-	0-1	1	-	-	0-1
19	GD	0-2	1	-	0-2	1-2	-	-	0-2	1	2	-	0-2
20		1-2	-	-	1	0-1	-	1	2	2	-	2	1-2
21	SA	-	-	-	2	0-3	-	-	1	1-3	-	1	1
22		-	-	-	1	-	-	-	1	1-2	-	-	1
23		0-1	-	-	1	1-3	-	-	1	1-2	-	-	1
24	ACD	2-3	-	-	0-1	0-1	-	-	0-1	0-3	-	-	-
25		1-3	-	-	1	1-2		2	1	0-2	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

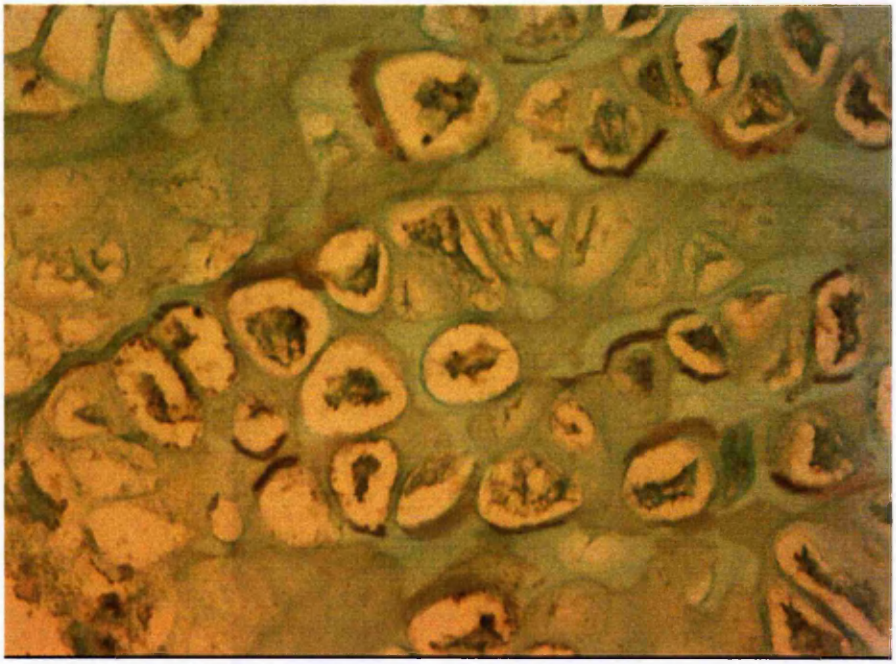


Figure 3.86. PSA lectin staining of the normal hypertrophic zone (X20).
Territorial matrix was strongly and chondrocytes were weakly stained.

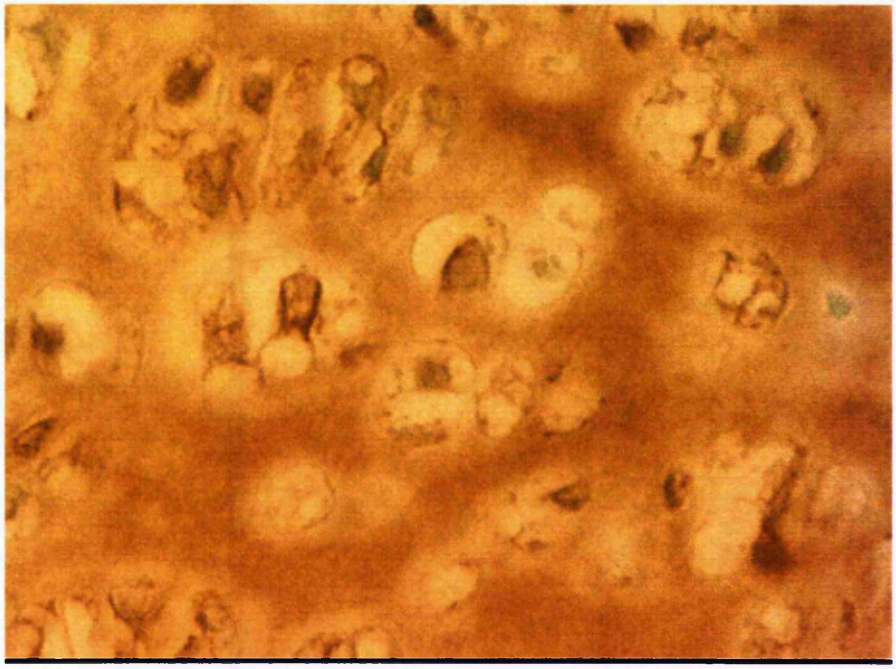


Figure 3.87. PSA lectin staining of the thanatophoric proliferative zone (X20). Matrix and most of the chondrocytes were stained strongly.

E-PHA

Matrix: Interterritorial matrix of normal growth plate showed only mild reactions with e-PHA. However, the interterritorial matrices in abnormal cases were stained moderately to strongly with e-PHA.

Territorial matrices of all cases showed no reaction with e-PHA.

Chondrocytes: The resting chondrocytes of normal growth plate were stained strongly with e-PHA and proliferative and hypertrophic chondrocytes of normal growth plate showed strong reactions with e-PHA.

None of the chondrocytes of the abnormal cases were stained strongly with e-PHA, except the chondrocytes of Beckwith's syndrome, which were stained very strongly with e-PHA.

After β-elimination:

Matrix and chondrocytes: No changes were seen in the staining of any part of the normal growth plate after β-elimination.

Pre-treatment with hyaluronidase:

Matrix: Interterritorial matrix of all zones of the normal growth plate showed a weak reaction with e-PHA after pre-treatment with hyaluronidase.

Chondrocytes: Pre-treatment with hyaluronidase did not change the reaction between chondrocytes of the normal growth plate and e-PHA.

Examples of these features are seen in Figures 3.82- 3.83.

Table 3.7. Summary of e-PHA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	4	-	-	1	1	-	-	-	1	-	-	-
2		-	-	-	-	3	-	-	-	-	-	-	-
3		4	4	-	1	4	4	-	-	4	-	-	-
4		2-3	-	-	2	2-4	-	-	2	3-4	-	-	2
5		-	-	-	-	-	-	-	-	-	-	-	-
6		-	2	-	0-1	1	2	-	0-1	0-1	2	-	0-1
7		0-3	-	-	0-1	-	-	-	-	-	-	-	-
8		1-3	-	-	-	1-3	-	-	-	3	-	-	-
9		3-4	-	-	2	3-4	2-3	-	2	2-3	1-2	-	2
10		0-3	-	-	1	3-4	-	-	-	3-4	-	-	-
11		-	-	-	1-2	1	-	-	1-2	0-1	1-2	-	1-2
12		-	-	-	1	-	-	-	1	-	-	-	1
13		4	4	-	2	-	-	-	-	1	1	-	1
14	CD	0-2	-	-	3	1-2	-	-	3-4	1-2	-	-	3-4
15		2	-	-	2	1	-	-	2-3	1-2	-	-	2-3
16		0-2	-	-	2-3	-	-	-	2-3	-	-	-	1-3
17		2	-	-	2-3	2	-	-	2-3	2	-	-	2-3
18		1	-	-	0-2	1-2	-	-	0-2	1	-	-	0-2
19	GD	1	-	-	0-2	1-2	-	-	0-2	1-2	-	-	0-2
20		4	-	-	2-3	3-4	-	-	2-3	4	-	-	2-3
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		?											
24	ACD	0-2	-	-	2	1-2	-	-	2	1-2	-	-	1-2
25		0-2	-	-	2	0-2	-	-	1-2	1-2	-	-	2

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

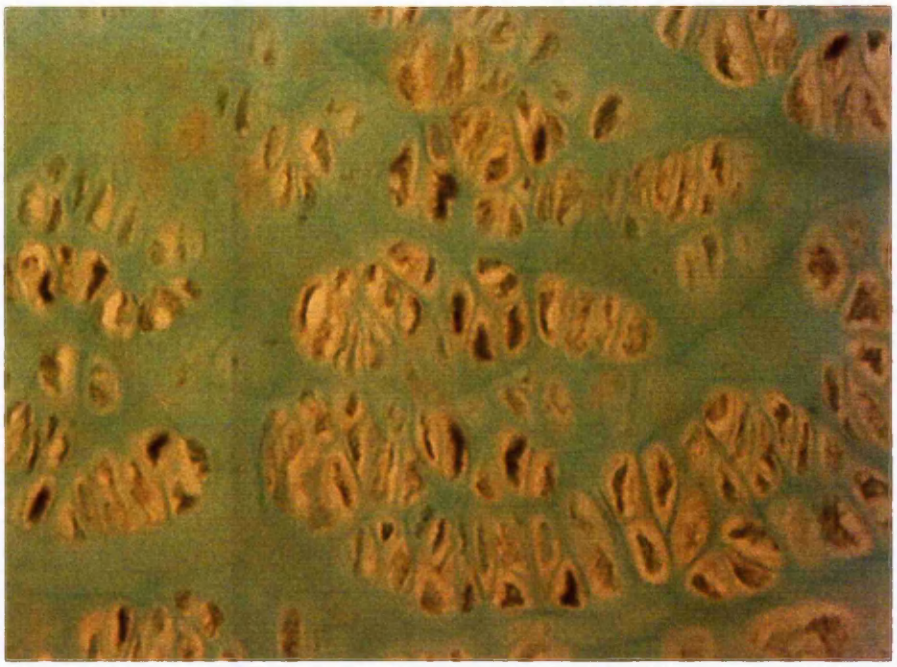


Figure 3.88. E-PHA lectin staining of the normal proliferative zone (X20). Chondrocytes showed a strong reaction but matrix did not.

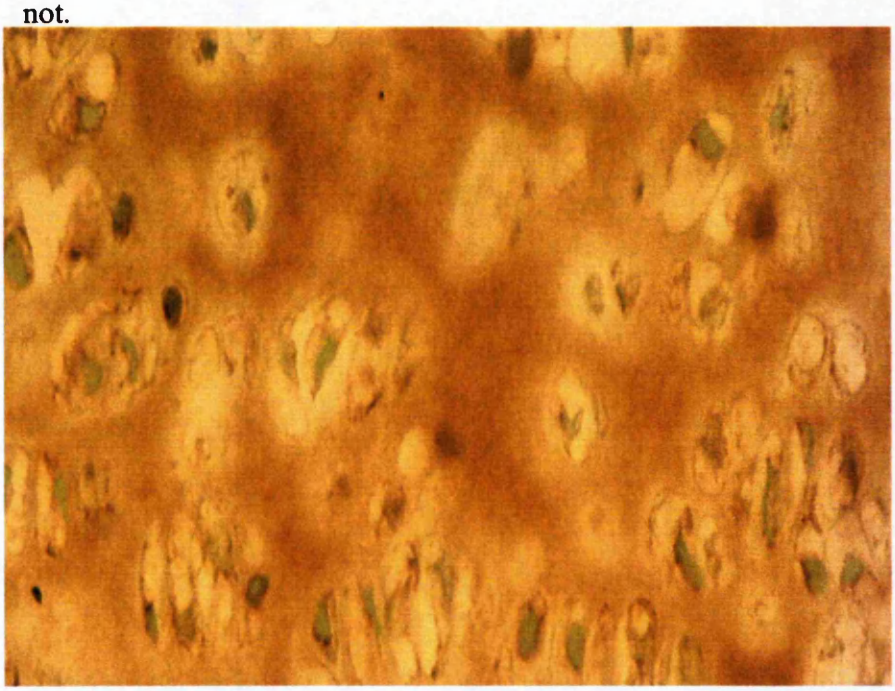


Figure 3.89. E-PHA lectin staining of the thanatophoric proliferative zone (X20). Matrix was stained strongly but chondrocytes were negative.

L-PHA

Matrix: Only the interterritorial matrix of chondrodysplastic growth plate showed a weak reaction with l-PHA, and the matrix of other cases was completely negative.

Chondrocytes: No reactions were seen between the chondrocytes of the growth plate and l-PHA.

After β-elimination:

Matrix and chondrocytes: No changes were found in matrix and chondrocytes of the normal growth plate with l-PHA after β-elimination.

Pre-treatment with hyaluronidase:

Matrix: Interterritorial matrix of the all zones of the normal growth plate showed a weak reaction with l-PHA after pre-treatment with hyaluronidase.

Chondrocytes: No change was seen in the staining of the normal growth plate chondrocytes after pre-treatment with hyaluronidase.

Examples of these features are seen in Figures 3.84 - 3.85.

Table 3.8. Summary of l-PHA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	-	-	-	-	-	-	-	-	-	-	-	-
2		-	-	-	-	-	-	-	-	-	-	-	-
3		-	-	-	-	-	-	-	-	-	-	-	-
4		-	-	-	-	-	-	-	-	-	-	-	-
5		-	-	-	-	-	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	-	-	-	-	-	-	-	-	-	-	-
8		-	-	-	-	-	-	-	-	-	-	-	-
9		-	-	-	-	-	-	-	-	-	-	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	-	-	-	-	-	-	-	-	-	-
13		-	-	-	-	-	-	-	-	-	-	-	-
14	CD	-	-	-	1	0-1	-	-	1-2	-	-	-	0-1
15		-	-	-	0-1	-	-	-	1	-	-	-	0-1
16		-	-	-	1	-	-	-	1	-	-	-	0-1
17		-	-	-	0-1	-	-	-	0-1	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		-	-	-	-	-	-	-	-	-	-	-	-
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		-	-	-	-	-	-	-	-	-	-	-	-
24	ACD	-	-	-	-	-	-	-	-	-	-	-	-
25		-	-	-	-	-	-	-	-	-	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

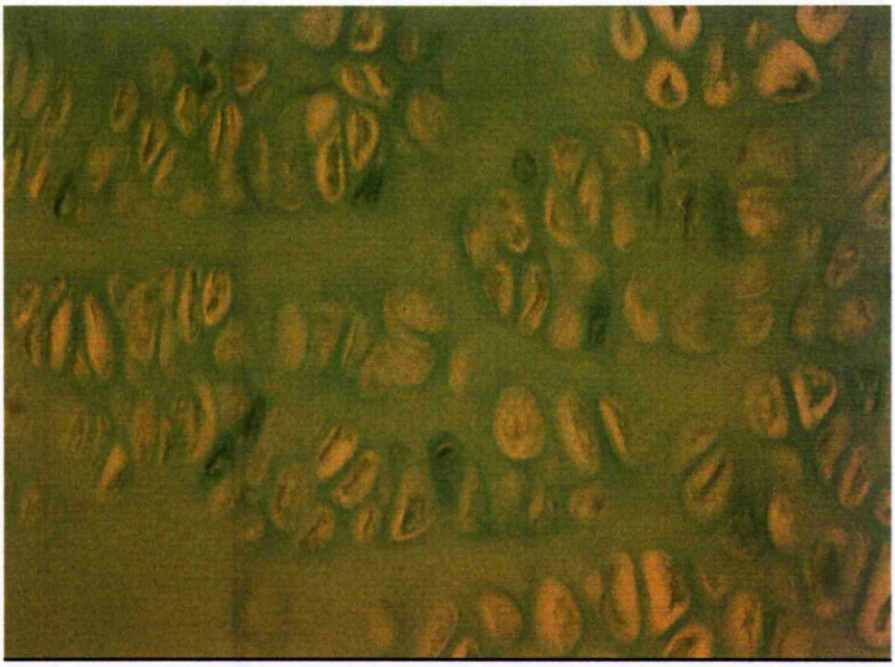


Figure 3.90. L-PHA lectin staining of the normal proliferative zone (X20).
Matrix and chondrocytes were negative.

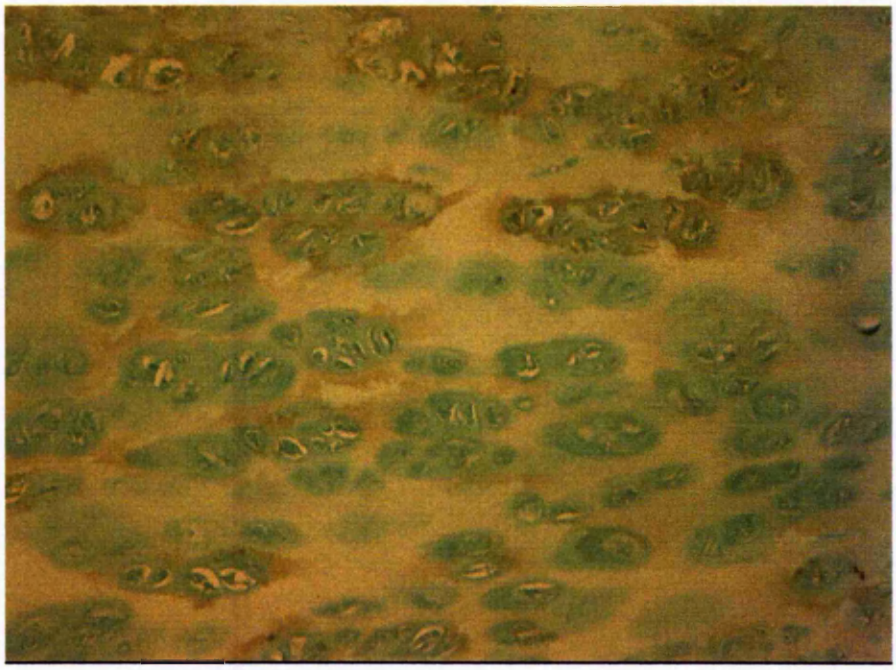


Figure 3.91. L-PHA lectin staining of the achondrogenesis proliferative zone (X10). Matrix only showed a reaction, especially territorial matrix.

LCA

Matrix: Only the interterritorial matrix of thanatophoric dwarfism and short limb dwarfism growth plates showed reactions with LCA.

Chondrocytes: No reactions were seen between the chondrocytes of normal and abnormal growth plate and LCA.

After β-elimination and pre-treatment with hyaluronidase:

Matrix and chondrocytes: These processes did not change any of the staining reactions of the normal growth plate with LCA.

Table 3.9. Summary of LCA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	-	-	-	-	-	-	-	-	-	-	-	-
2		-	-	-	-	-	-	-	-	-	-	-	-
3		0-1	-	-	1	-	-	-	2	-	-	-	2
4		-	-	-	-	0-1	-	-	-	0-1	-	-	-
5		-	-	-	-	-	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	-	-	-	-	-	-	-	-	-	-	-
8		-	-	-	-	-	-	-	-	-	-	-	-
9		-	-	-	-	-	-	-	-	-	-	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	-	-	-	-	-	-	-	-	-	-
13		-	-	-	-	-	-	-	-	-	-	-	-
14	CD	-	-	-	1-2	-	-	-	2	-	-	-	0-1
15		-	-	-	1	-	-	-	0-1	-	-	-	0-1
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		-	-	-	0-1	-	-	-	0-1	-	0-1	-	-
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		-	-	-	-	-	-	-	-	-	-	-	-
24	ACD	-	-	-	-	-	-	-	-	-	-	-	-
25		-	-	-	-	-	-	-	-	-	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis), GD: genetic disorder (19:Campomelic dysplasia, 20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

3.2.3.2. Fucose-binding lectins

UEA-I and AAA

Matrix: No reaction was seen between these lectins and the matrix of the growth plate in any of the cases.

Chondrocytes: The normal and abnormal growth plate chondrocytes showed no reactions with UEA-I and AAA.

After β-elimination:

Matrix: Weak to moderate staining were seen after β-elimination with UEA-1 and AAA in the matrix of the normal growth plate especially in the matrix of the resting zone.

Chondrocytes: Chondrocytes showed a strong reaction with these lectins after β-elimination.

Pre-treatment with hyaluronidase:

Matrix: No change was seen in the staining of the normal matrix after pre-treatment with hyaluronidase.

Chondrocytes: Only chondrocytes of the proliferative zone showed weak reaction with these lectins after pre-treatment with hyaluronidase.

Example of this feature (AAA lectin binding staining) is seen in Figure 3.86.

Table 3.10. Summary of UEA-I and AAA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	-	-	-	-	-	-	-	-	-	-	-	-
2		-	-	-	-	-	-	-	-	-	-	-	-
3		-	-	-	-	-	-	-	-	-	-	-	-
4		-	-	-	-	-	-	-	-	-	-	-	-
5		-	-	-	-	-	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	-	-	-	-	-	-	-	-	-	-	-
8		-	-	-	-	-	-	-	-	-	-	-	-
9		-	-	-	-	-	-	-	-	-	-	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	-	-	-	-	-	-	-	-	-	-
13		-	-	-	-	-	-	-	-	-	-	-	-
14	CD	-	-	-	-	-	-	-	-	-	-	-	-
15		-	-	-	-	-	-	-	-	-	-	-	-
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		-	-	-	-	-	-	-	-	-	-	-	-
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		-	-	-	-	-	-	-	-	-	-	-	-
24	ACD	-	-	-	-	-	-	-	-	-	-	-	-
25		-	-	-	-	-	-	-	-	-	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis), GD: genetic disorder (19:Campomelic dysplasia, 20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

3.2.3.3. β-Galactose-binding lectins

CTA

Matrix: There was no noticeable reaction between the matrix of the normal and abnormal resting zone of the growth plate with CTA. Some of the interterritorial matrix of the normal proliferative and hypertrophic zones showed a weak reaction with CTA.

Chondrocytes: Only the chondrocytes of a few normal cases showed a weak reaction with CTA. Growth-plate chondrocytes in Beckwith's syndrome showed moderate reactions with CTA.

Example of this feature is seen in Figure 3.87.

Table 3.11. Summary of CTA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	0-1	-	-	-	0-1	-	-	1	0-1	-	-	1
2		0-1	1	-	-	1	-	-	-	-	-	-	-
3		2-3	2-3	-	1	3-4	2-3	-	2-3	0-1	2-3	2-3	2-3
4		-	-	-	0-2	0-1	-	-	0-1	0-1	1-2	-	-
5		-	-	-	-	-	-	-	-	-	-	-	1-2
6		-	-	-	-	-	-	-	-	-	-	-	-
7		0-1	0-1	-	-	0-1	-	-	1	-	0-1	-	1
8		0-2	0-2	-	-	2	-	-	1	2	-	-	1
9		-	-	-	-	-	-	-	-	-	-	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	-	-	0-1	0-1	-	1	-	-	-	1
13		-	-	-	1	-	-	-	1	-	1	-	1
14	CD	-	-	-	-	-	-	-	-	-	-	-	-
15		-	-	-	-	-	-	-	-	-	-	-	-
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		0-2	0-1	-	1	0-2	0-2	-	1	0-2	0-2	0-2	1
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		-	-	-	-	-	-	-	-	-	-	-	-
24	ACD	-	-	-	-	-	-	-	-	-	-	-	-
25		-	-	-	-	-	-	-	-	-	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

Figure 3.92. AAA lectin staining of the normal proliferative zone (X40). No reaction was seen.

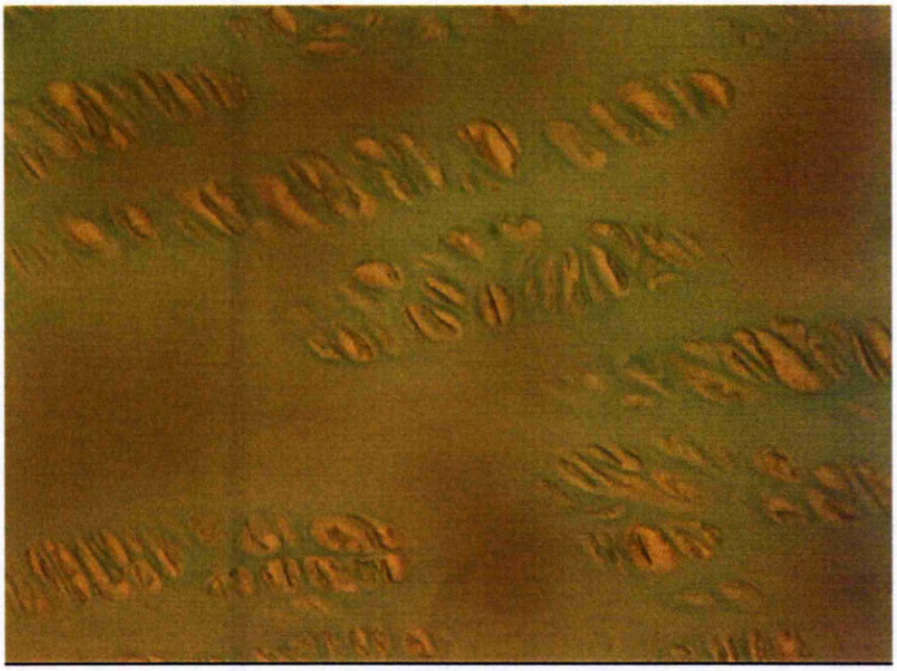


Figure 3.93. CTA lectin staining of the normal proliferative zone (X20). Weak reactions were seen in both chondrocytes and matrix with CTA.

ECA

Matrix: Strong binding reactions were found between the territorial matrices of the normal growth plates with ECA. Interterritorial matrix of these cases showed weak to strong reactions with ECA as well. However, territorial matrix of the normal resting zone showed more affinity for ECA than other zones. Pericellular area of the normal chondrocytes showed strong reaction with ECA. The matrix of achondroplasia, achondrogenesis and campomelic dysplasia cases showed no reaction with ECA. However, territorial and interterritorial matrices of thanatophoric dysplasia, short limb dwarfism and abnormal calcium deposits cases showed strong reactions with ECA. Pericellular areas of chondrocytes of the thanatophoric dysplasia, short limb dwarfism and abnormal calcium deposits cases were stained strongly with ECA.

Chondrocytes: Chondrocytes of the normal growth plates showed strong reactions with ECA. Chondrocytes of thanatophoric dysplasia, short limb dwarfism and abnormal calcium deposits cases were stained strongly with ECA.

After β-elimination

Matrix and chondrocytes: β-Elimination did not change reactions between the matrix and chondrocytes of the normal growth plate and ECA.

Pre-treatment with hyaluronidase

Matrix and chondrocytes: Less staining with ECA was seen in the matrix and chondrocytes after pre-treatment with hyaluronidase.

Examples of these features are seen in Figures 3.88 - 3.89.

Table 3.12. Summary of ECA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	2-3	2	0-2	1	3-4	2-3	2	1	0-3	2	0-2	2
2		2-3	2	0-2	1	0-1	-	1-2	1-2	-	-	-	1
3		4	4	0-3	3	3-4	-	0-3	3	3	3	3	3
4		4	4	0-3	3	4	0-3	0-3	3	4	3	0-3	3
5		-	-	3	-	-	-	2-3	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		2-4	1-2	-	1-2	2-4	1-2	-	1-2	2-4	2	-	1
8		4	4	3	3	4	-	3	2-3	4	3	3	3
9		4	4	0-2	2	4	0-3	0-3	-	3	4	0-2	2
10		4	4	0-1	2	4	3	0-2	2	3	0-3	0-2	2
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	2	1	0-1	-	0-1	2	1	2	-	2
13		0-1	1	2	3	2	2	-	-	2	2	-	0-1
14	CD	1-2	-	3-4	3	1-2	-	-	3-4	3-4	2-3	3	2-3
15		4	0-2	0-3	2	3	-	0-3	3	0-3	0-3	0-3	0-3
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		4	4	0-3	3	4	-	0-3	3	4	0-3	0-3	3
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		3-4	2-3	1	2-3	3-4	2-3	1	2-3	3-4	2-3	0-1	1-2
24	ACD	3-4	3-4	3-4	3-4	3-4	3	3-4	3-4	3	3	3	3
25		4	4	0-3	2	3-4	-	0-2	3	4	4	0-2	3

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

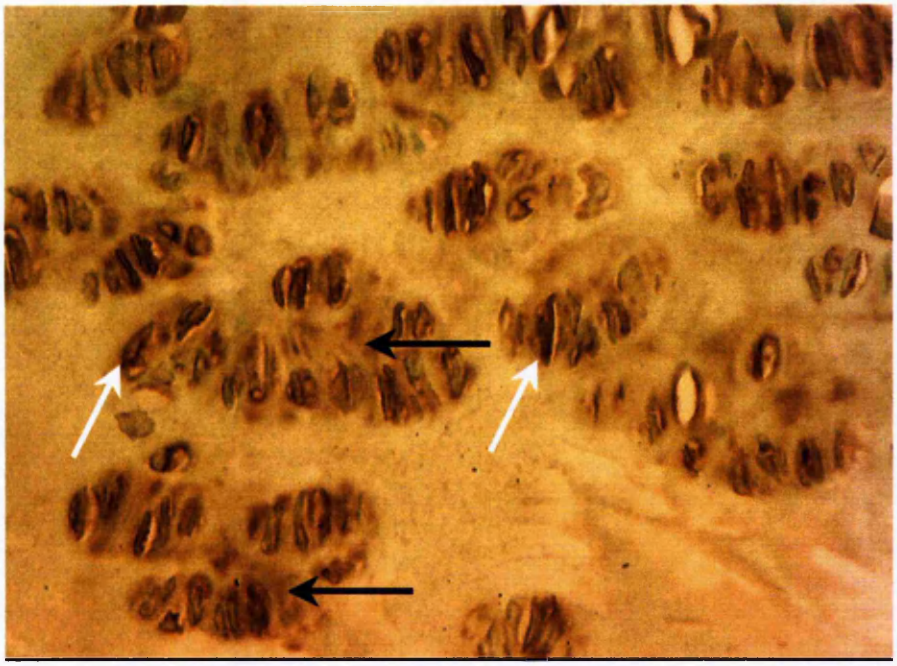


Figure 3.94. ECA lectin staining of the normal proliferative zone (X20). Chondrocytes (white arrows) and territorial matrix (black arrows) were stained strongly.

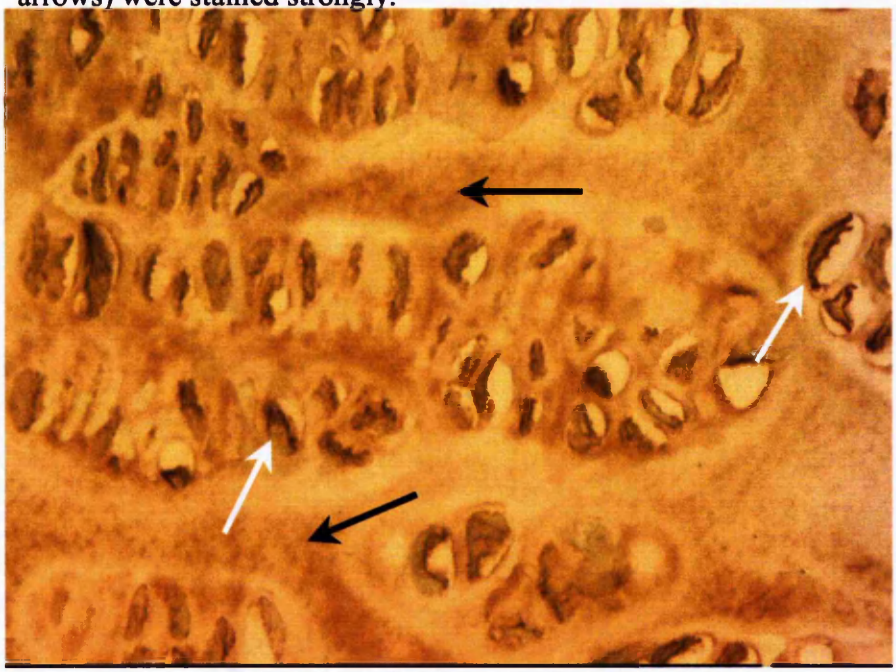


Figure 3.95. ECA lectin staining of the short limb dwarfism proliferative zone (X20). Chondrocytes (white arrows) and interterritorial matrix (black arrows) were stained strongly.

PNA

Matrix: No noticeable reaction was seen between the matrix of normal and abnormal growth plates and PNA.

Chondrocytes: The proliferative and hypertrophic chondrocytes of normal growth plates were stained moderately to strongly with PNA. Chondrocytes of Beckwith's syndrome and abnormal calcium deposition cases showed moderate to strong reactions with PNA.

Table 3.13. Summary of PNA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	-	-	-	-	0-2	-	-	-	1-2	3	-	0-1
2		-	-	-	-	-	-	-	-	-	-	-	-
3		-	-	-	-	-	-	-	-	-	-	-	-
4		-	-	0-1	1-2	0-2	-	2	-	2-3	4	4	0-1
5		0-1	-	-	-	0-1	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	0-1	-	-	0-1	-	-	1	2-3	3	-	-
8		0-1	-	-	-	3-4	3-4	-	-	3	4	-	-
9		-	-	-	-	-	0-3	-	-	-	0-3	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	-	-	0-2	0-2	-	-	1-2	3	-	-
13		-	-	-	-	1-2	2	-	-	2	1-2	-	-
14	CD	-	-	-	-	-	-	-	-	-	-	-	-
15		-	-	-	-	-	-	-	-	-	-	-	-
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		0-3	-	-	-	0-1	0-1	-	-	2-3	4	-	0-1
21	SA	0-1	-	-	-	-	-	-	-	0-1	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		1	-	-	1	1-2	1-2	-	-	3-4	3	-	-
24	ACD	2	-	-	-	3	2	-	-	3	3	-	-
25		2	-	-	-	0-2	0-3	-	-	2	3-4	-	-
26		-	-	-	-	-	-	-	-	-	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

3.2.3.4. N-Acetylgalactosamine-binding lectins

WFA

Matrix: Interterritorial matrix of normal and abnormal cases was stained weakly to strongly with WFA. The territorial matrix of the hypertrophic zone showed strong reaction with WFA, but staining was infrequent in the territorial matrix of the other zones. Matrix of Beckwith's syndrome and rickets showed no reaction with WFA. The strongest reaction was with pericellular areas of normal or abnormal growth plates.

Chondrocytes: Chondrocytes of normal and abnormal cases showed moderate to strong reactions with WFA.

After β-elimination:

Matrix and chondrocytes: Matrix and chondrocytes of the all zones of the normal growth plate showed stronger reactions with WFA after pre-treatment with β-elimination.

Pre-treatment with hyaluronidase:

Matrix and chondrocytes: Matrix and chondrocytes of all the zones of the normal growth plate lost staining reactions with WFA after pre-treatment with hyaluronidase.

Examples of these features are seen in Figures 3.90 –3.91.

Table 3.14. Summary of WFA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	3	3	0-3	1	3-4	3	0-3	1-2	3-4	3	0-3	1-2
2		3	2-3	-	-	3-4	-	1-3	1-2	3-4	2-3	3	3
3		4	4	-	2-3	4	4	-	2-3	3-4	3-4	2-3	2-3
4		1-2	-	-	-	0-1	-	-	-	1-2	-	-	-
5		4	3	-	1	4	-	0-2	2	1-2	3	3	1
6		1-2	0-1	-	1	2-3	-	-	1	1-2	2-3	1-2	1
7		0-3	0-1	-	1-2	0-2	-	-	1	0-2	-	-	-
8		2-4	3-4	-	-	3-4	4	-	-	3-4	2-3	-	-
9		4	4	-	1	4	4	-	-	4	4	-	-
10		4	4	-	2	3	3	-	-	3	3	-	-
11		3-4	-	-	0-1	3-4	-	-	1	2-3	3	-	3
12		1	0-2	0-3	0-1	2-3	2-3	0-3	1-2	1-2	3-4	0-3	1-3
13		1-2	1-2	2	1	3	3	3	1-3	3	3	3	1-3
14	CD	2-4	3-4	-	2	2-3	1-2	-	2-3	2-3	2-4	-	1-2
15		4	4	-	2-3	4	4	-	2	4	4	-	2-3
16		0-1	2-3	-	1-2	2-3	2-3	1	1	4	2-3	-	1
17		2	-	-	3	2	-	-	3	1	-	-	3
18		2-3	-	-	1	3	-	0-1	2	2-3	3-4	-	1
19	GD	3	3	-	-	2-3	3-4	-	1-2	2-3	2-3	-	1-2
20		2-3	-	-	1	2	-	-	1	2	-	-	1
21	SA	2-3	2-3	-	2	4	3-4	-	-	3-4	3-4	-	-
22		1-3	-	0-3	1	3	3	-	-	2-3	-	-	1
23		1-3	1-2	-	1-2	1-3	1-2	-	1-2	1-3	1	1	1-3
24	ACD	1-2	-	-	-	1-2	4	-	-	1-2	-	-	-
25		4	4	-	1	4	4	-	2	3-4	3-4	-	1-2

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

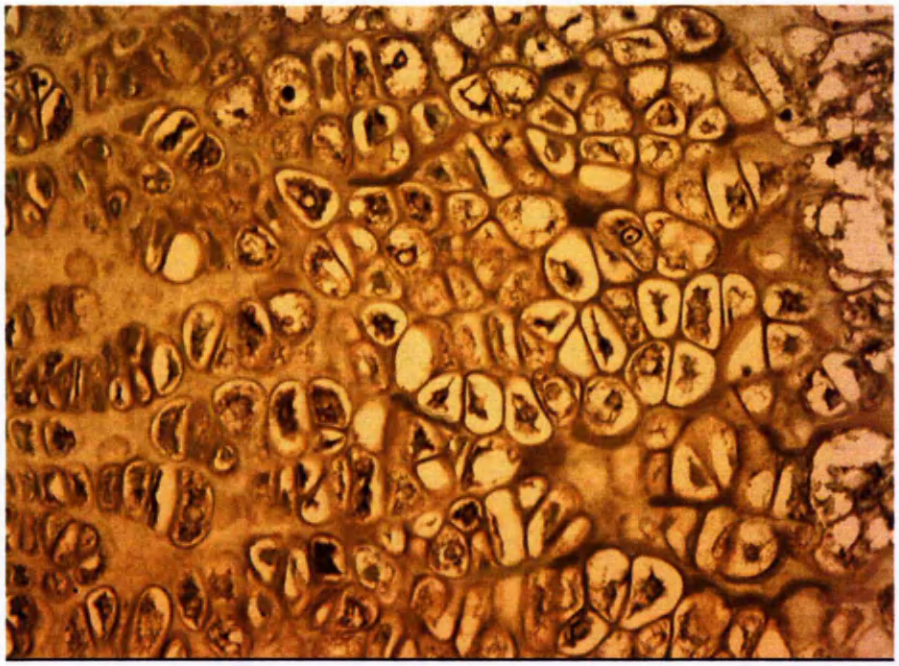


Figure 3.96. WFA lectin staining of normal growth plate (X10).
Matrix and cells showed strong reactions.

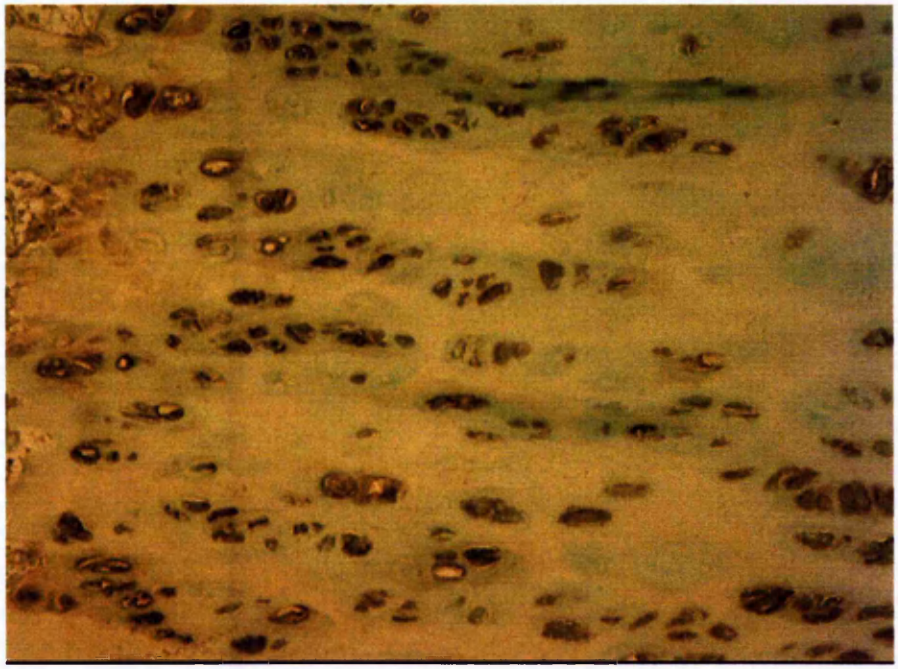


Figure 3.97. WFA lectin staining of the achondroplastic hypertrophic zone (X10).
Chondrocytes showed a strong reaction but the matrix did not.

DBA, VVA and HPA

Matrix: No reaction was seen between these lectins and the matrix of the growth plate in any of the cases.

Chondrocytes: The normal and abnormal growth plate chondrocytes showed no reactions with DBA, VVA and HPA.

After β-elimination:

Matrix: Matrix of the all zones of the normal growth plate showed weak reactions with DBA and VVA, but no changes were seen with HPA.

Chondrocytes: Chondrocytes of the normal growth plate showed moderate to strong reactions with VVA. They showed weak reactions with DBA but no reaction with HPA after β-elimination.

Pre-treatment with hyaluronidase:

Matrix: Interterritorial matrix of the normal resting zone showed weak reactions with DBA, VVA, and HPA. No changes were seen in other parts of the matrix.

Chondrocytes: chondrocytes of the normal growth plate showed a moderate reaction with HPA after pre-treatment with hyaluronidase. Staining of the other chondrocytes did not change with these lectins after pre-treatment with hyaluronidase.

Table 3.15. Summary of DBA, VVA and HPA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	-	-	-	-	-	-	-	-	-	-	-	-
2		-	-	-	-	-	-	-	-	-	-	-	-
3		-	-	-	-	-	-	-	-	-	-	-	-
4		-	-	-	-	-	-	-	-	-	-	-	-
5		-	-	-	-	-	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	-	-	-	-	-	-	-	-	-	-	-
8		-	-	-	-	-	-	-	-	-	-	-	-
9		-	-	-	-	-	-	-	-	-	-	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		-	-	-	-	-	-	-	-	-	-	-	-
12		-	-	-	-	-	-	-	-	-	-	-	-
13		-	-	-	-	-	-	-	-	-	-	-	-
14	CD	-	-	-	-	-	-	-	-	-	-	-	-
15		-	-	-	-	-	-	-	-	-	-	-	-
16		-	-	-	-	-	-	-	-	-	-	-	-
17		-	-	-	-	-	-	-	-	-	-	-	-
18		-	-	-	-	-	-	-	-	-	-	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		-	-	-	-	-	-	-	-	-	-	-	-
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		-	-	-	-	-	-	-	-	-	-	-	-
24	ACD	-	-	-	-	-	-	-	-	-	-	-	-
25		-	-	-	-	-	-	-	-	-	-	-	-
26		-	-	-	-	-	-	-	-	-	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia,), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, TM: territorial matrix, ITM: interterritorial matrix

3.2.3.5. N-Acetyllactosamine-binding lectin

DSA

Matrix: Interterritorial matrix of normal resting zone showed a weak reaction with DSA, but in proliferative and hypertrophic zones of most cases it strongly stained. Territorial matrix was mostly negative. The pericellular areas of the normal chondrocytes were positive.

The matrix of abnormal cases showed no reaction with DSA.

Chondrocytes: Chondrocytes of normal growth plate showed strong reaction with DSA. However, chondrocytes of abnormal growth plates showed no binding sites for DSA, except chondrocytes of abnormal calcium deposit cases.

Examples of these features are seen in Figures 3.92 -3.93.

Table 3.16. Summary of DSA lectin histochemistry

No		Resting Zone				Proliferative Zone				Hypertrophic Zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	N	3D	3	0-3	1	3	3	0-3	1	2-3	2	0-3	0-3
2		0-1	0-1	0-2	1	1-2	-	2-3	2-3	1-2	0-1	-	2-3
3		2-3	2	-	1	2-3	2	-	2	2-3	2	-	2
4		2	-	-	1	0-2	-	-	-	1-3	3	-	1
5		0-2	-	-	-	-	-	-	-	-	-	-	-
6		-	-	-	-	-	-	-	-	-	-	-	-
7		-	-	-	0-2	1-2	1-2	-	0-2	0-1	-	-	0-2
8		0-1	-	-	-	0-2	-	-	-	0-2-	-	-	-
9		0-2	-	-	-	2-3	-	-	-	-	0-1	-	-
10		-	-	-	-	-	-	-	-	-	-	-	-
11		3	2	-	-	4	2	-	1	2	2	-	-
12		1	0-2	0-3	0-1	2	3	3	1	1-2	3	0-3	0-3
13		1	2	0-1	2	3	1-2	0-2	0-2	3	3	0-3	3
14	CD	-	-	0-4	1	2-3	0-1	0-3	3	2	-	-	3
15		-	-	-	-	-	0-1	-	-	0-2	-	-	-
16		-	-	-	-	-	2-3	-	1-2	1	2-3	-	-
17		-	-	-	-	-	1-3	-	-	1			
18		-	-	-	-	-	-	-	-	-	1	-	-
19	GD	-	-	-	-	-	-	-	-	-	-	-	-
20		-	-	-	-	-	-	-	-	-	-	-	-
21	SA	-	-	-	-	-	-	-	-	-	-	-	-
22		-	-	-	-	-	-	-	-	-	-	-	-
23		0-2	0-2	0-2	-	2	0-1	-	0-2	2	-	-	-
24	ACD	0-2	0-1	-	-	2	2	-	-	2	2	-	-
25		0-2	0-1	-	1	0-2	-	-	1	0-2	-	-	-

N: normal, CD: chondrodysplastic (14: Thanatophoric dysplasia, 15: Short limb dwarfism, 16: Achondroplasia, 17: Achondrogenesis, 18:Campomelic dysplasia), GD: genetic disorder (20: Beckwith's syndrome), SA: skeletal disorder, ACD: abnormal calcium deposition (24: Rickets)
CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix

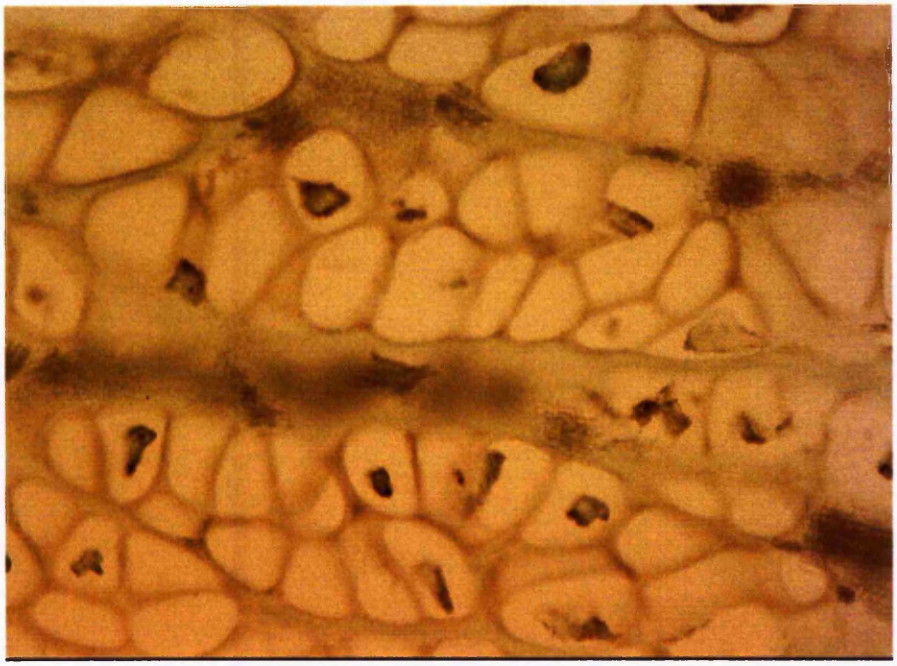


Figure 3.98. DSA lectin staining of normal growth plate (X20).
Matrix and cells showed strong reactions.

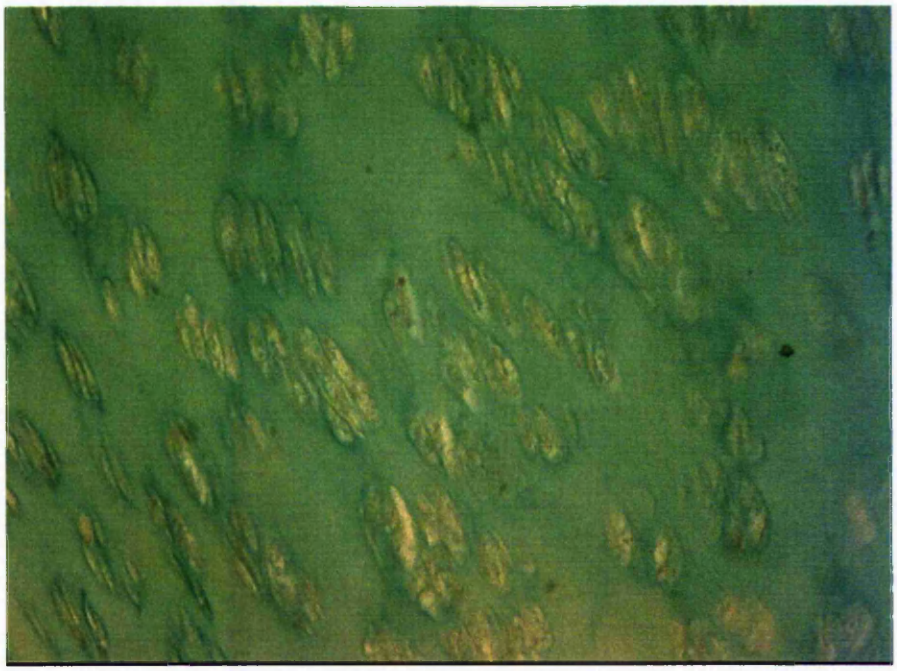


Figure 3.99. DSA lectin staining of achondrogenesis (X10).
No reaction in either chondrocytes and matrix was seen.

Table 3.17. Level of lectin staining after pre-treatment with hyaluronidase on normal GP

No	Lectin	Resting zone				Proliferative zone				Hypertrophic zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	HHA	=	=	=	↑	=	=	=	=	=	=	=	=
2	LCA	=	=	=	=	=	=	=	=	=	=	=	=
3	EPHA	=	=	=	↑	=	=	=	↑	=	=	=	↑
4	LPHA	=	=	=	↑	=	=	=	↑	=	=	=	↑
5	UEA-1	=	=	=	=	↑	=	=	=	=	=	=	=
6	AAA	=	=	=	=	↑	=	=	=	=	=	=	=
7	ECA	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
8	DBA	=	=	=	↑	=	=	=	=	=	=	=	=
9	VVA	=	=	=	↑	=	=	=	=	=	=	=	=
10	HPA	↑	=	=	↑	↑	=	=	=	↑	=	=	=
11	WFA	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix.
↑: staining increased after pre-treatment with hyaluronidase
↓: staining decreased after pre-treatment with hyaluronidase
=: staining not changed after pre-treatment with hyaluronidase

Table 3.18. Level of lectin staining after β-elimination on the normal growth plate

No	Lectin	Resting zone				Proliferative zone				Hypertrophic zone			
		Cell		Matrix		Cell		Matrix		Cell		Matrix	
		CY	PC	T	IT	CY	PC	T	IT	CY	PC	T	IT
1	HHA	↑	↑	↑	↑	↑	=	=	=	↑	=	=	=
2	LCA	=	=	=	=	=	=	=	=	=	=	=	=
3	EPHA	=	=	=	=	=	=	=	=	=	=	=	=
4	LPHA	=	=	=	=	=	=	=	=	=	=	=	=
5	UEA-1	↑	↑	↑	↑	↑	=	↑	↑	↑	=	↑	↑
6	AAA	↑	=	↑	↑	↑	=	↑	↑	↑	=	↑	↑
7	ECA	=	=	=	=	=	=	=	=	=	=	=	=
8	DBA	↑	=	=	↑	↑	=	=	=	↑	=	=	=
9	VVA	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
10	HPA	=	=	=	=	=	=	=	=	=	=	=	=
11	WFA	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑

CY: cytoplasm, PC: pericellular, T: territorial matrix, IT: interterritorial matrix
↑: staining increased after pre-treatment with β-elimination
↓: staining decreased after pre-treatment with β-elimination
=: staining not changed after pre-treatment with β-elimination

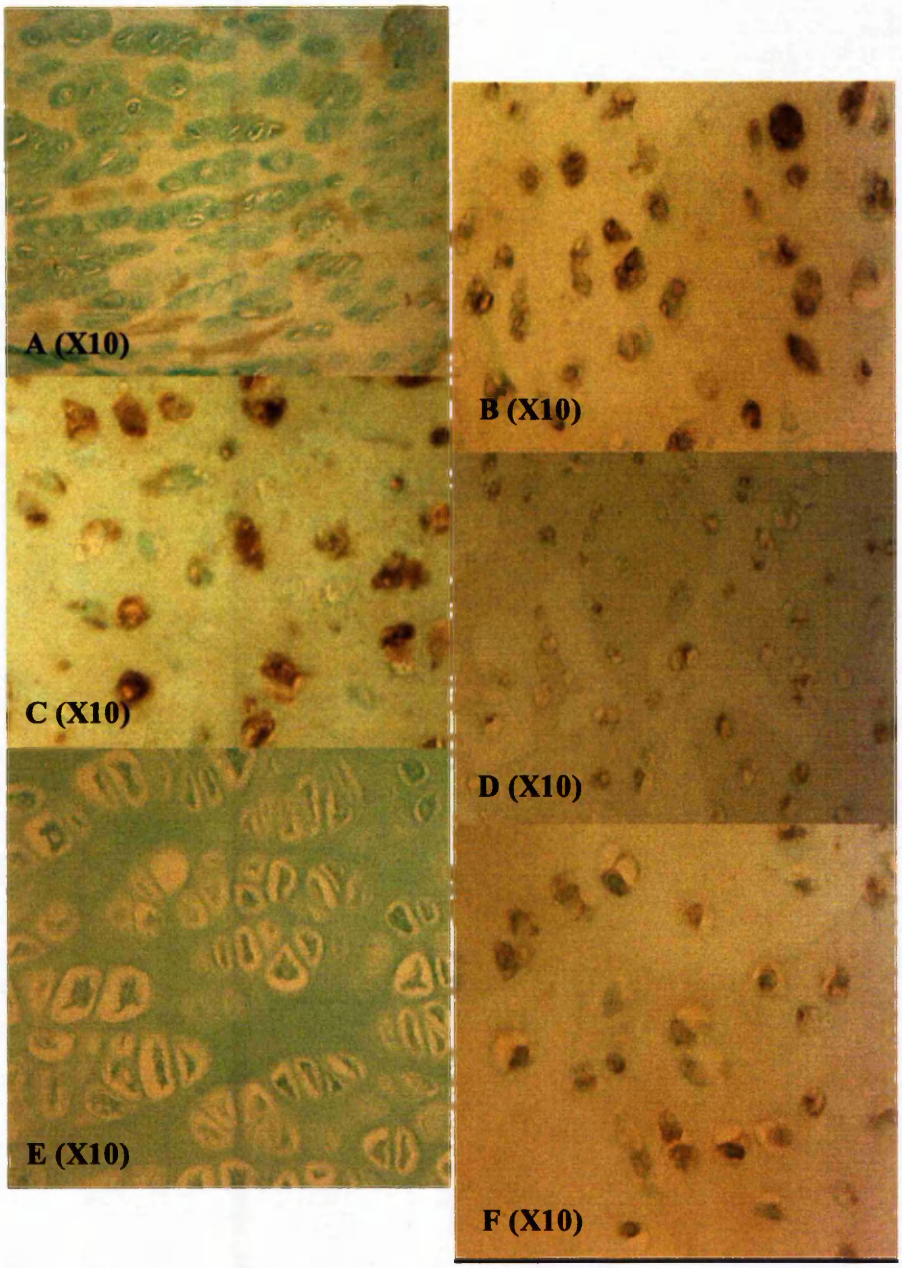


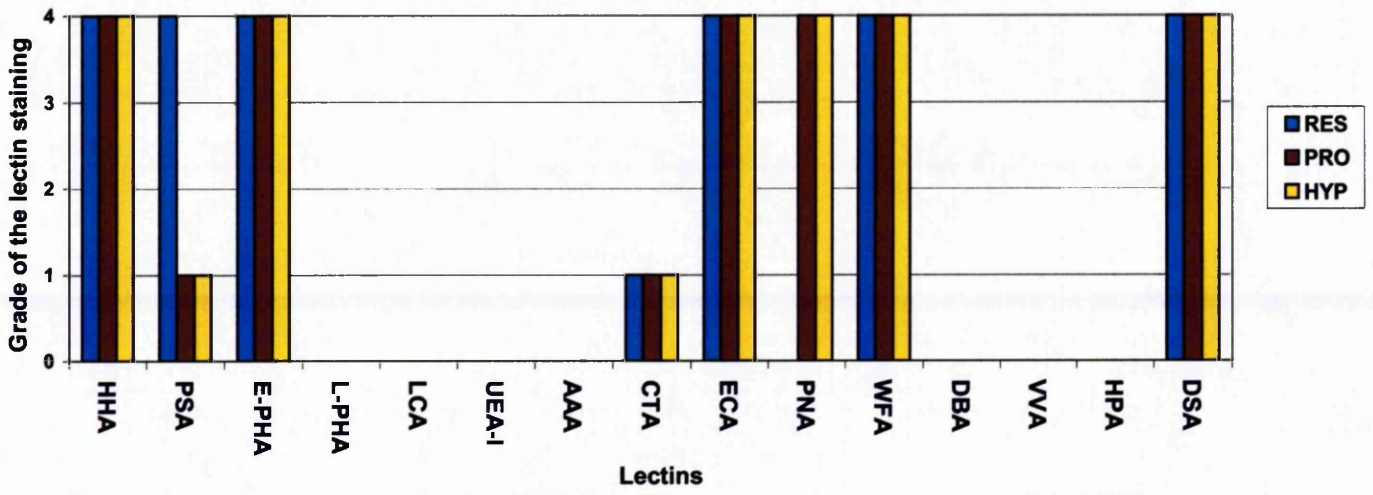
Figure3.100. Lectin staining changes after pre-treatment in normal growth plate.

A:E-PHA lectin staining after using hyaluronidase. B:HHA lectin staining after using beta-elimination. C:UEA-I lectin staining after using beta-elimination. D:HPA lectin staining after using hyaluronidase. E:WFA lectin staining after using hyaluronidase.
F:ECA lectin staining after using hyaluronidase.

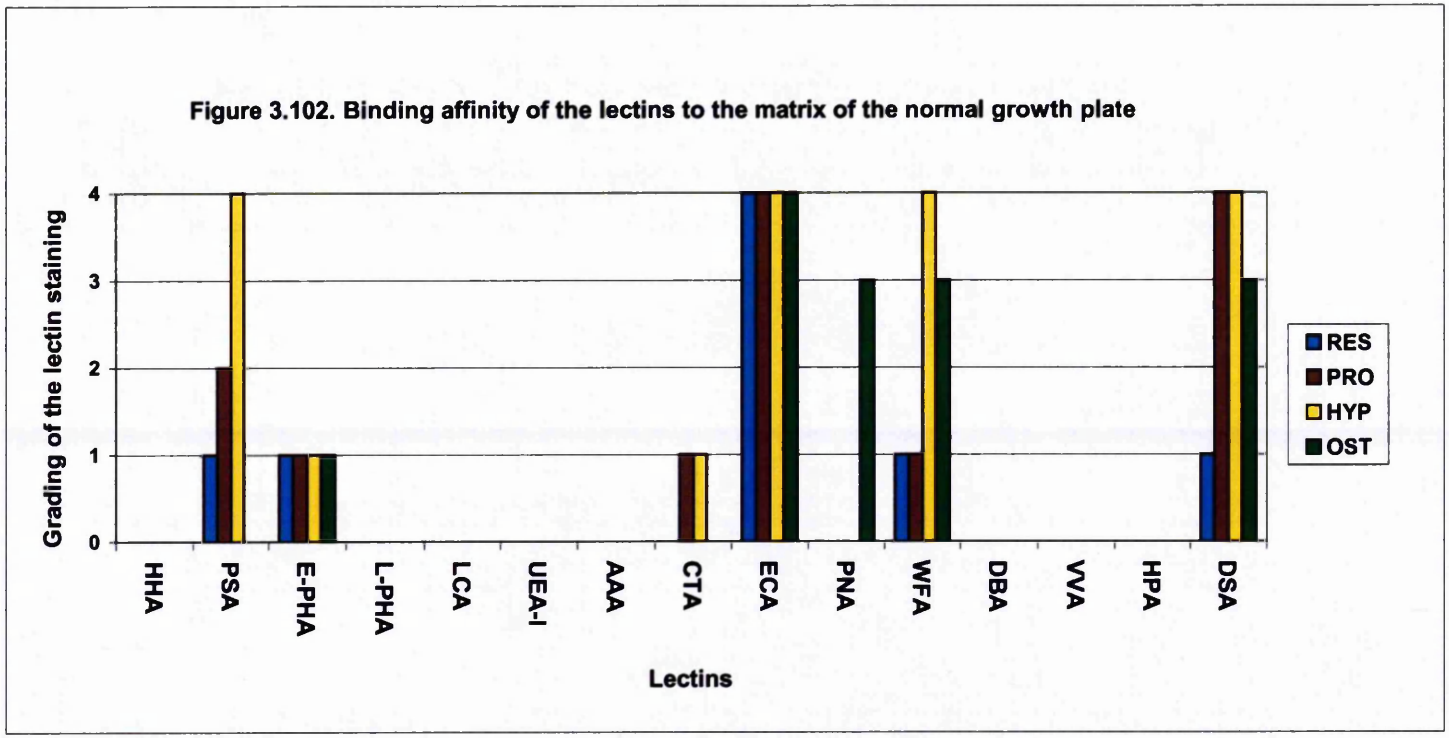
3.2.3.4. Overall summary

For all the cell and matrix staining reactions of normal growth plates the intensities of the lectin staining reactions for each lectin were averaged and recorded in a number of glycoprofiles. These are shown in Figures 3.101 to 3.102. In the growth plate glycoprofiles of resting, proliferative and hypertrophic zone and bone were made. These glycoprofiles are a useful visual method of illustrating the similarities and differences of the lectin reactivities detailed previously.

Figure 3.101. Binding affinity of the lectins to the chondrocytes of the normal growth plate



RES: resting chondrocytes, PRO: proliferative chondrocytes, HYP: hypertrophic chondrocytes.



RES: resting zone , PRO: proliferative zone, HYP: hypertrophic zone, OST: osteoid.

Chapter 4

Discussion

4.1. Introduction

Cartilage is a very important tissue of the body both in adulthood and during development. Normal cartilage protects, gives shape, allows movement and enables development of the skeleton. These important functions are achieved by the cells and their extracellular matrix. The overall importance of cartilage justifies its investigation. This study consisted of a set of investigations on two types of hyaline cartilage (articular cartilage and growth plate) and fibrocartilage. These investigations consisted of histological staining (H&E, toluidine blue, alcian blue, picro sirius red and alkaline Congo red), immunohistochemical staining (anti-amyloid antibodies and anti-CD68), and lectin histochemical staining with a set of 16 lectins. The major findings reported in this thesis are as follows.

1-There were characteristic light microscopical differences between normal and osteoarthritic sternoclavicular joints. Because motion at the SCJ accompanies every movement of the arm, it is one of the most frequently used joints and is involved in osteoarthritic processes even though it is non-weight-bearing. Osteoarthritic changes could be distinguished radiologically, but accurate diagnosis can only be established on the basis of pathological examination.

2-Amyloid deposition was commonly found in the articular cartilage, interarticular discs, synovium and capsule of sternoclavicular joints. The amyloid in these deposits was chemically more complicated than had been previously documented. This study suggests that mixed amyloid deposits were present in different parts of the joints, since monoclonal antibodies confirmed the existence of β2 microglobulin, κ and λ immunoglobulin light chains and P-component. It is noteworthy that there was no record of dialysis, renal failure, myeloma or systemic amyloidosis in any of the cases.

3-Lectin histochemistry of sections of normal and osteoarthritic sternoclavicular cartilage, using a panel of 14 lectins from five specificity groups, showed a very varied pattern of glycan expression. In extracellular matrix, the patterns of glycan expression were different between normal and osteoarthritic cartilage. Mild to moderately osteoarthritic cartilage showed a more abundant expression of several glycans than did normal cartilage or severely osteoarthritic cartilage. Chondrocytes

of both the normal and osteoarthritic cartilages showed a diversity of glycan expression. Both the matrices and chondrocytes of the different zones in articular cartilage, normal and osteoarthritic cases, showed variety in glycan expression.

4-There were characteristic light microscopic findings in normal and abnormal growth plates at the costochondral junctions. Some growth plates from abnormal cases showed changes in glycoprofiles despite having a normal histological appearance.

5-Lectin histochemistry of the normal and abnormal growth plate sections, from the costochondral junctions, using a panel of 15 lectins from five specificity groups, showed that there was variation in the glycan expression between normal and abnormal cases and between abnormal cases.

6-The matrix of the zones of the growth plates showed between-zone variation in glycan expression i.e. the hypertrophic and proliferative zones in most cases were different from the resting zone. In addition the glycoprofiles of the chondrocytes of the different zones varied.

4.2. Osteoarthritic features in sternoclavicular joints

Osteoarthritis (OA) is the most common of all joint diseases. The exact definition of OA is still debated (Fiske and Edwards 1995), in large part because of the disorder's heterogeneity. Due to the absence of a single diagnostic test, sets of criteria are relied upon to enhance the probability of making the correct diagnosis.

The joints usually involved in OA include those of the hands, hips and knees, metatarsophalangeal joints and the cervical and lumbar intervertebral joints. Sternoclavicular joints did not feature on this list, but OA of the SCJ is not unusual in autopsy material (Yood and Goldenberg 1980) and in diagnostic biopsy material (Le Loet and Vittecoq 2002). In the present study osteoarthritic changes were seen in 85% of sternoclavicular joints. The patients from whom the specimens were obtained did not have any evidence of joint disease in their medical records.

The accurate diagnosis of OA depends on pathological evaluation (Aigner and McKenna 2002). Studies of the whole joints were very important in diagnosis and grading of OA, because the location and degree of severity may be focal (Lafeber et al 1993). In the present study, OA changes were seen in 85% of the sternoclavicular joints. This is the same incidence as found by Kopp et al (1976). There is no record of how

often osteoarthritic changes of the SCJ are symptomatic. In the present study, the osteoarthritic changes were severe in 36.6% of cases and mild to moderate in 48.4%. Le Loet and Vittecoq (2002) reported a high frequency of degenerative changes in SCJ. De palma (1963) stated that degenerative changes in SCJ was not observed until the third decade and these became progressively more severe and frequent after the fifth decade. Similar results have been reported by Kier et al (1986).

The present study showed that osteoarthritic changes were more severe in the right SCJ than in the left. Since the majority of people are right-handed this suggests that the greater use of the dominant limb may play an important role in localising the degenerative processes. Lane et al (1989) believed that OA was not more prevalent in the dominant hand, but they found more severe osteoarthritic changes in the dominant hand. As every motion of the hand is accompanied by some motion in the SCJ, it suggests that the same may happen in the SCJ. There are no published reports comparing OA in the right and left SCJs.

The current study also showed that the inferior parts of the joints were more affected than the superior part. These findings confirm a previous study (Constant 1989). In other joints, such as hip and knee, OA affects some parts more severely than others (Appleyard et al 1999). In the present study subchondral bone changes were more marked on the right side than on the left.

The SCJs, like the temporomandibular joints, are divided into two compartments by intraarticular discs, which probably influence the development of degenerative changes (Kopp et al 1976). Osteoarthritic changes were more severe in clavicular than in sternal articular cartilage. The fibrocartilaginous disc is closer to the sternal cartilage and, hence, it could be protecting the sternal cartilage from external forces, so limiting the damage caused (Sutro 1974). Berteretche et al (2001) believed that displacement of the disc in temporomandibular joints always resulted in degenerative changes in the cartilage. In addition, malposition of the disc causes degenerative changes (Luder 2002). The costoclavicular and interclavicular ligament attachments could also help to stabilise the joint (Sutro 1974). However, De palma (1959) claimed that the disc was able to protect both clavicular and sternal articular surfaces from osteoarthritic changes before the seventh decade of life. Berteretche et al (2001) believed that condylar fibrocartilage absorbs considerable stress in joints. In addition, meniscectomy in knee joints induced OA in both articular surfaces (Appleyard et al 1999). None of these studies discussed the severity of degenerative changes in the articular surfaces of the SCJ.

Osteoarthritic changes in the SCJ consisted of fibrillation, clefts and disorganisation of the clavicular and sternal articular cartilage and fibrocartilage, hyperplasia and cyst formation in subchondral bone, and osteophytosis. These changes have been reported in previous investigations in the SCJ (De palma 1963, Yood and Goldenberg 1980, Constant 1989) and in other joints (Cushnaghan and Dieppe 1991, Huch 2001). Minimal changes were noted in some joints by the third decade and were found in almost all subjects by the fifth decade (Yood and Goldenberg 1980). According both to microanatomical and histochemical criteria, various degenerative changes were seen in the present study. In OA cartilage, the loss of GAGs has been demonstrated by a reduction of the area stained with cationic dye (Shimizu et al 1997). Matrix metalloproteinase cleave aggrecan within the interglobular domain, which is the keratan sulphate attachment site (Fosang et al 1992). Sandy et al (1992) found aggrecan fragments, such as a short amino-terminal stretch of the interglobular domain, the G2 domain, the keratan sulphate domain and variable lengths of the chondroitin sulphate domain, in synovial fluid of the osteoarthritic cartilage. However, the rate of PG synthesis is increased in mild to moderate OA (Venn et al 1995). In the present study, fibrillated areas of the matrix showed loss of staining for GAGs using toluidine blue and alcian blue stains, especially in severe OA. This result supports the results of Appleyard et al (1999). The superficial matrix in fibrillated areas appeared striped, like a "tiger tail", because of degeneration of collagen fibrils. Collagenases (matrix metalloproteinase 1 and 13) cleave and denature type II collagen in osteoarthritic cartilage, especially in the superficial zone (Wu et al 2002). Thinning and cracking are other reactions, which change the matrix components. Fibrillation and thinning of the superficial zone were usually present without subchondral changes. However, in a few cases, subchondral bone changes (hyperplasia, cysts or osteophytosis) happened before fibrillation. This suggests that changes in the subchondral bone might antedate cartilage changes or both might occur over a fairly narrow period of time, rather than sequentially, as had been suggested by Pritzker (1994). Bone hyperplasia was seen in most cases with high-grade osteoarthritic changes in articular cartilage and fibrocartilage.

The risk of degenerative changes increases with age (Hamerman 1993). The results of this present study agree with the findings of Kopp et al (1976) that there was not an exact relationship between age and osteoarthritic changes. These investigations showed that osteoarthritic changes probably are due mostly to factors within the individual joints, but the influence of age cannot be excluded.

Sutro (1974) found various stages of inflammation in the synovial tissue in instances of OA. Haynes et al (2002) believed that synovial inflammation plays a significant role in OA. Mitrovic and Riera (1992) found that synovial hyperplasia showed a strong correlation with the occurrence of osteocartilagenous debris in OA. In the present study synovial hypertrophy was found in 53% of SCJ and macrophages were found in 37% of synovial tissues.

4.3. Amyloid deposits in sternoclavicular joints

Amyloid deposits can involve articular and periarticular tissues (Goffin 1981, Egan et al 1982, Ladefoged 1982, Moe and Chen 2001). SCJ is a very common site for amyloid deposits after long-term haemodialysis (Zingraff et al 1989, 1990, Jadoul et al 1997, Garbar et al 1999), although Cameron et al (1997) believed that haemodialysis-related amyloidosis rarely affected the SCJ. This present study has found mixed amyloid deposits in 85% of SCJs, without any evidence of kidney failure or any type of dialysis treatment. Therefore, amyloid can be deposited in the SCJ *without* haemodialysis. In addition, the pattern and types of amyloid deposits in the SCJ, found in this study, were different from the amyloid deposits associated with after haemodialysis (Garbar et al 1999). No other studies have reported such a prevalence of amyloid deposition in SCJs in cases without haemodialysis.

In this study amyloid deposits were found more commonly in clavicular cartilage and disc than in sternal cartilage. There were localised bilaterally and no differences were found between the amounts in the left and the right joints. Osteoarthritic changes were seen in clavicular cartilage more than in the other parts of the joints, but they were more severe in the right joints than in their left counterparts. The results of this study did not support a spatial correlation between OA and the occurrence of amyloid, as was reported by Goffin et al (1981) and Egan et al (1982). In the present study, a relationship between severity of OA and the amount of amyloid deposited cannot be excluded, as the heaviest deposits were found in the older subjects with severe OA, as was reported by Egan et al (1982). This could be because both OA and amyloid deposition are age dependent and both happen in advanced age, but without any casual relationship between them. However, β2-M amyloid deposition and serum amyloid A both induce synovial tissue to increase production of matrix metalloproteinase (Moe and Chen 2001, Ohashi 2001, Vallon et al 2001), which can cause cartilage degradation (Ishiguro et al 1999, Chambers et al 2001, Ray et al 2003). These findings are against the hypothesis of Athanasou et al (1995), who considered that amyloid deposition in cartilage is associated with GAGs, since in severe OA cartilage much of the GAG content was lost, especially in the

superficial area where the amyloid deposition commonly was found. A relationship was found between the occurrence of amyloid deposits and advanced age, supporting the findings of Goffin et al (1981), Ladefoged (1982) and Athanasou and Sallie (1992).

A large number of amyloid deposits in cartilage were found to contain β2 microglobulin (β2-M) mixed with κ and λ immunoglobulin light chains and the universal P-component. β2-M is a feature of dialysis amyloid arthropathy (Ohashi et al 1992, Ogawa et al 1995, Zingraff and Drueke 1998, Garbar et al 1999), and it was not reported in non-dialysis autopsy material. This present study found β2-M deposits in articular cartilage and fibrocartilage, without a history of any type of dialysis or kidney failure. The results showed two patterns of β2-M deposition in autopsy materials. In some cases it was present only in the cartilage and in others it was in cartilage, synovium and tendons. This suggests that β2-M was deposited primarily in the cartilage and subsequently extended to the synovium and tendons when there were extensive deposits in the cartilage. Macrophages were not seen in articular cartilage or fibrocartilage, even in the most abundant deposits. When the amyloid deposits were very extensive, extending to the synovial tissue, then macrophages were seen around the deposits within the synovium and tendon. Therefore macrophages are not needed for β2-M deposition but are reactive to it (Garbar et al 1999). It has been claimed β2-M deposits may elicit a local inflammatory response (Hou et al 2001).

The protein component of the localised amyloid deposits in the joint tissues was not amyloid AA, which is identical with the results reported by Mohr et al (1991). However, Garbar et al (1999) found AA amyloid deposits in joint tissues, but after haemodialysis. So the cause of amyloid deposition in the present cases may be not the same as the amyloid deposition in haemodialysis cases.

Light chain amyloidosis usually shows a clonal restriction related to an underlying plasma cell disorder (Husby and Sletten 1986). The polyclonality and mixed chemical nature of the amyloidosis reported in this present study is unusual.

Amyloid deposits were found in articular cartilage, fibrocartilage, capsule and synovium, which is similar to the results found by Ladefoged et al (1989). These deposits were mostly seen in superficial areas and along the fissures or cracks of fibrillated cartilage. In capsule and synovium, they were found mostly around blood vessels. These findings were also supported by those of Ladefoged (1986). The use of whole joints in the current study showed the topographical distribution of depositions, enabling the construction of a map of amyloid deposition within articular cartilage and fibrocartilage.

This is an important aid in predicting how the amyloid precursor might enter the cartilage (Rumplet et al 1996). Possibly the amyloid fibril or precursor might enter cartilage from the synovial fluid, via the synovial membrane (Cary 1985, Moe and Chen 2001). The finding of amyloid deposits in synovium, but not in subchondral bone of any of the present cases, would support this hypothesis. The localisation of amyloid in the surface of the cartilage and adjacent to tissue and clefts in osteoarthritic cartilage are also consistent with this hypothesis. However, amyloid deposits can be present in the cartilage and not in the synovium as Garbar et al (1999) have reported. Normal cartilage surfaces have a low permeability especially for large molecules, such as amyloid precursor protein (about 120 kD), and specific situations, such as a damaged osteoarthritic surface, might be necessary for the penetration of amyloid fibrils or their precursors from synovial fluid. However, in the present study, amyloid deposits were found in the deep zone of intact cartilage *without osteoarthritic changes*. In addition, β2-M deposits were mostly found within, or adjacent to, chondrocytes in *all* positive cases, as has also been reported by Argiles and Mourad (2000). Thus, there is evidence to suggest that β2-M in the deposits might have been secreted by the chondrocytes. The further processes involved in transformation of the various proteins and precursors into amyloid fibrils and their deposition in cartilage are still not clear (Zingraff and Drueke 1998).

Overall none of these post-mortem materials were obtained from persons with a history of any type of dialysis, kidney failure, generalised amyloidosis or plasma cell disorders. These depositions only showed a relationship with age. The types of amyloid deposit found were different from those found in various cartilages in other studies (Cary 1985, Mohr 1991) and in senile amyloidosis (Gorevic et al 1985).

4.4. Lectin histochemical staining of the sternoclavicular joints

The integrity of cartilage matrix depends on the homeostasis of synthetic and degradative processes. Any disturbance of either the rate of synthesis or the rate of catabolism may alter the amounts of matrix components and the functional properties of the cartilage. Lectin binding was used, in this study, to visualise the localisation and distribution of some monosaccharide or oligosaccharide components of the matrix and cells in normal and osteoarthritic sternoclavicular joint tissue. The main advantages of lectins as histochemical probes, in comparison to the other staining methods for carbohydrate content, is the high specificity of their reactions towards certain glycans (Lis and Sharon 1998). The entire size of most glycans is too great for them to be

accommodated by one lectin-binding site, so that only limited parts of their structure can be identified by any one lectin. By using a panel of lectins with overlapping binding requirements larger glycans can be analysed. The main glycans in cartilage are GAGs - chondroitin sulphate, keratan sulphate and hyaluronan and glycoprotein. Keratan sulphate and N and O-linked oligosaccharides showed binding site for lectins. However, Mallinger et al (1986) determined that the lectin-binding capacity of cartilage was minimal, since lectins with specificities for hyaluronan and chondroitin sulphate have not been found apart from a few lectins, mostly from snake venoms, which are not stable enough for use *in vitro*. The advantage of this study is the use of the whole depth of the cartilage tissue, which is a reliable way to assess carbohydrate content, synthesis and release in normal and OA cartilage.

4.4.1. Lectin binding with articular cartilage

4.4.1.1.Matrix

Introduction: Most lectins showed a mild to moderate reaction with the surface of the articular cartilage, suggesting a greater expression of glycan residues in this zone. Superficial staining was affected slightly by osteoarthritis, which could be reflecting changes in glycan expression produced by osteoarthritic chondrocytes or could reflect the degradation of glycans. The presence of fragments of aggrecan in synovial fluid from OA joints suggests that there is degradation of aggrecan in OA (Lohmander et al 1993). A few lectins showed lesser affinity for osteoarthritic than normal surfaces, but most showed stronger reaction with the osteoarthritic surface than the normal. Degradation of the PGs in osteoarthritis could cause lower concentrations of certain glycans, with other glycans being made more accessible. Takagi et al (1988) and Della et al (1995) used degradative enzymes for pre-treatment of cartilage and found increased subsequent reaction between the matrix and the lectins. The same results could be found after enzyme treatment in vivo. Lyons (2000) mentioned that surface staining could be an artefactual edge effect, but it did not happen with all lectins or in all cases and was not present in other immunohistochemical staining, arguing that it is a real effect. In addition, the lectins of small hydrodynamic size, such as UEA-I (49 kD) did not show any reaction with the superficial part of the cartilage while the larger lectins, such as PNA (110 kD) showed strong reaction. Simple penetration should be easier for small lectins than big ones and so these results support the argument that the staining reactions of the surface cartilage are real.

Group 1 (HHA, LCA, PSA, e-PHA and l-PHA)

These lectins showed moderate to strong reactions with the matrix of different zones of the normal and osteoarthritic articular cartilage. All of these lectins bind to the Asn-GlcNAc4,1βGlcNAc4,1βMan core with a variety of sugars linked to it. Articular cartilage contains various types of N-linked oligosaccharides, which can bind to these lectins. However, it was not positive for all of them. The activities of mannosyl transferase (Richard et al 1990) and matrix metalloproteinase (Fosang et al 1992) are increased in OA. MMPs open the matrix by degradation of hindering molecules making more N-glycan accessible, and binding sites for these lectins are increased by mannosylation. Therefore the binding sites for these lectins are likely to be increased in osteoarthritic cartilage. HHA binds to N-linked oligosaccharides with non-reducing αD-mannosyl termini. It showed a weak to moderate reaction with normal matrix, but a strong reaction with the matrix of mild to moderately osteoarthritic joints. This might be because, in mild to moderate OA, the synthesis of cartilage matrix is stimulated (Kiviranta et al 1987). In addition, degradative enzymes in low concentrations would give only partial digestion of high molecular weight proteoglycans. Therefore accessibility of glycan ligands to lectin would be higher in mild to moderate OA than in normal cartilage (Della et al 1995). The matrix of the severe osteoarthritic cartilage showed a weak reaction with HHA, suggesting that in such specimens, the cartilage has lost matrix macromolecules due to higher concentrations of the degradative enzymes, and consequently more complete depolymerisation and loss of proteoglycans (Della et al 1995). Farnum and Wilsman (1986) found a lack of lectin 'stainability' in cartilage because they used a higher concentration of enzyme in pre-treatment than did Della et al (1995). In OA, matrix metalloproteinases (MMP7 and MMP9) are activated and cleave aggrecan core protein within the proteinase sensitive interglobular domain between G1 and G2. Two specific cleavage sites were found, one at Asn341-Phe342 and another Asp441-Leu442 (Fosang et al 1992, Fosang et al 1993). There was approximately 30-40 kD of keratan sulphate attached to this part, probably derived from matrix. The specific pattern of HHA binding sites in the matrices, suggests that the accessibility or the quantity of carbohydrate residues in matrix varies between normal and degenerating cartilage.

PSA binds to complex N-linked oligosaccharides and preferentially to those with terminal mannose, and the result of the staining with it was similar to that with HHA. LCA showed similar staining reactions to HHA, but it bound to osteoarthritic cartilage more strongly than did the latter lectin. LCA binds to non-bisected bi/tri-antennary N-glycan residues with highest affinity where α1,6 fucosyl residues are attached to asparagine linked N-acetyl lactosamine (Kornfeld et al 1981). It interacts with 2-O-

Nacetylglucosaminyl-mannose, which provides evidence that it binds to internal 2-O-substituted mannose residues as well. In addition, αD-linked glucose disaccharides interact with LCA (Goldstein and Portez 1986) but it generally binds very weakly with glycogen. These residues are increased in osteoarthritic cartilage because of the over activity of the glucosyl transferase and N-acetylglucosamine transferase (Richard et al 1990). Bond et al (1997) found a parallel increase in the expression of both galactose and N-acetyl glucosamine in osteoarthritis. In addition, LCA bound to osteoarthritic cartilage more strongly, perhaps because of exposure of terminal N-acetylglucosamine after degradation of the GAGs in the osteoarthritic process.

E-PHA bound moderately to strongly to the superficial and medial zones of normal and osteoarthritic matrix. L-PHA showed reaction only with the superficial part of the osteoarthritic matrix. E-PHA has high affinity for bi/tri-antennary complex oligosaccharides with a bisecting N-acetylglucosamine residue (Yamashita et al 1983), which can be released from glycoproteins by cleavage of the GlcNAc-Asn bond with N-glycanase (Green and Baenziger 1987). L-PHA binds to non-bisected tri/tetra-antennary sequences with outer galactosyl residues and α-mannose substituted at C-2 and C-6 (Yamashita et al 1983). These results show that the matrix expresses complex N-linked oligosaccharides, including bisected bi/tri antennary and non-bisected bi/tri/tetra-antennary N-glycans. L-PHA binds to the pentasaccharide Galβ1,4GlcNAcβ1,2 (Galβ1,4GlcNAcβ1,6) in tri/tetra-antennary oligosaccharides, but it cannot bind pentasaccharide Galβ1,4GlcNAcβ1,4 (Galβ1,4GlcNAcβ1,6) in tri/tetra-antennary sequences, therefore small changes in the structures of glycans may change their affinity for lectins (Green and Baenziger 1987). Another possibility is that, in normal matrix, tetra-antennary non-bisected complex N-glycans may be not accessible and the binding site is disclosed after osteoarthritic degradation.

The matrix of chondrocytes clones showed various results. HHA and LCA showed strong reactions with some of the clonal matrices, while other clones were negative. This may be because some clones contain intensively proliferating chondrocytes with high metabolic activity, while others do not show any synthetic metabolic activity as suggested by Schunke et al (1985). PSA and e-PHA stained most of the clonal matrices moderately to strongly, and l-PHA detected no ligands in them. So glycan expression in the new matrices was not exactly the same as in the old matrices.

Group 2 (UEA-I)

In all zones, normal matrix showed a weak reaction with UEA-I. UEA-I binds specifically to L-fucosyl termini of N or O-linked oligosaccharides. Terminal fucosyl

residues are only found infrequently in normal articular cartilage matrix, but more frequently in mild to moderately osteoarthritic matrix (Hoedt-Schmidt 1989). Surfaces of mild to moderately osteoarthritic cartilage showed strong reactions with UEA-I, probably because the expression of some glycans is activated in chondrocytes of osteoarthritic cartilage, as suggested by Pfander et al (2001). Osteoarthritic changes induce increased activity of fucosyl transferase (Richard et al 1990), which changes Galβ1,4GlcNAc, an extensive part of aggrecan, to Fucα1,2Galβ1,4GlcNAc. This residue is bound strongly by UEA-I. UEA-I showed a weak reaction with the matrix in severe OA, because there is a measurable loss of matrix components in the late stages of OA as suggested by Ishiguro et al (1999), which would probably includes loss of some glycoprotein glycans. The present result is consistent with the result of Schunke et al (1985).

The matrix of the clones showed no reaction with UEA-I, suggesting that newly formed matrix does not contain L-fucosyl terminal residues like old normal matrix.

Group 3 (MAA)

Normal cartilage matrix showed a reaction with MAA. The superficial zone was stained moderately and medial and deep zones were stained weakly. Superficial parts of the osteoarthritic cartilage showed moderate to strong reaction with MAA. MAA labels, specifically, N-acetylneuraminic acid residues α 2,3 linked to β galactose (Chapman et al 1994). Generally, most sequences of disaccharides in normal articular cartilage are the bridging agent between collagen and proteoglycans; therefore they are masked from detection by lectin. In OA, the superficial collagen fibril network deteriorates (Panula et al 1998); therefore the underlying proteoglycans are more accessible (Schunke et al 1985). In addition, OA induces increased synthetic activity to produce terminal sialyl residues (Richard et al 1990).

Group 4 (PNA and ECA)

PNA showed a weak reaction with the normal and severe osteoarthritic matrix. There was strong reaction between mild to moderate osteoarthritic matrix and PNA; especially, this was shown in the interterritorial matrix. Takagi et al (1988) found a strong reaction between PNA and cartilage matrix after enzymatic digestion with chondroitinase ABC. This could explain how the matrix comes to show more reaction with PNA after OA. PNA has a dominant specificity for the terminal disaccharide β-Gal-1,3-NAcGal, which is found largely in O-linked oligosaccharides and keratan sulphate (Mallinger et al 1986). The possible binding site is located close to the core protein of aggrecan, therefore it is masked by GAG side chains and their degradation exposes these ligands to PNA (Schunke et al 1985).

ECA showed weak to moderate reaction with the matrix of the normal articular cartilage. Mild to moderate osteoarthritic cartilage was stained more strongly, but, in severe OA, only weakly. ECA binds strongly to subsets of glycans containing N-acetyllactosamine and also to the Galα1,3Galβ1,4GlcNac linked analogue of the disaccharide (Iglesias et al 1982). Strong reaction in mild to moderate OA might be caused by revealing β-galactosyl termini after degradation starts, but when the degradation continues, those terminal residues might be mostly destroyed.

Group 5 (DBA, VVA, MPA, HPA and WFA)

There was very little reaction between this group of lectins and the matrix of either normal or osteoarthritic articular cartilage. Only the interterritorial matrix of the normal cartilage showed moderate reaction with DBA. This group requires N- or O-linked oligosaccharides with α-2-deoxy,2-acetamidogalactosyl residues in various linkages. These results show that there are few of these glycans in cartilage matrix. Takagi et al (1988) studied other lectins of this group and they found similar results. McClure et al (1997) studied the binding of HPA, MPA and DBA to chick cartilage and found the same results, but when they used WFA a strong reaction was seen with the cartilage matrix. This difference could be the result of species differences (Malinger et al 1986) or may reflect the developmental stages of the cartilage (Miosge et al 1998). WFA has a high affinity for GalNAcα1,6Galβ1 and it can bind GalNAcα1,3Galβ1 weakly (Baker et al 1983).

Summary:

According to the findings above, the expression of carbohydrates in the extracellular matrix is very complex. The lectins bind to various N- and O-linked oligosaccharides with a range of structural units and termini. Both terminal and core sequences were expressed in the superficial zone and there were alterations during osteoarthritic changes. These changes were seen sometimes in other zones and indicate that all zones of the cartilage are involved in the pathological process, but to a varying extent depending on the severity of the pathogenesis. Aggrecan contains about 100chains of chondroitin sulphate and 40 of keratan sulphate (Sandy et al 1991). Keratan sulphate can be attached via either N and or O-linked oligosaccharides, while chondroitin sulphate has attached only via O-linked oligosaccharide (Hardingham et al 1991). Other small proteoglycans of the cartilage including fibromodulin (Svensson et al 2000), biglycan and decorin (Kresse et al 1993) contain N-linked oligosaccharides. Link protein and other matrix glycoproteins also have saccharide side chains (Neame and Barry 1993). Cartilage collagens have N or O-linked oligosaccharides as well (Grynpas et al 1980).

Carbohydrate groups appear to influence the orientation of molecules involved in matrix-matrix, matrix-cell and cell-cell interactions. Alterations in the lectin staining patterns show changes in the carbohydrate groups and add to the understanding of the pathological processes that are involved in articular cartilage and its changed biomechanical properties. The expression of carbohydrate mostly was increased in mild to moderate OA and decreased in severe OA. Territorial matrix and interterritorial matrix tended to be stained with about the same affinity. These results make it evident that lectins can demonstrate small differences between normal and arthritic cartilage, therefore they are sensitive and specific tools for the study of degenerative joint disease.

4.4.1.2. Chondrocytes

Introduction: Chondrocytes were stained with various lectins, implying the presence of cellular glycoproteins. These results suggest that chondrocytes from different anatomical sites might be differentially metabolically active and express different carbohydrate residues.

Group 1 (HHA, LCA, PSA, e-PHA and l-PHA)

HHA, PSA and e-PHA showed strong reactions with most chondrocytes in the superficial zone of the articular cartilage, but l-PHA and LCA showed no reactions. These findings suggest that chondrocytes express high-mannose glycans, the specific ligands for HHA, and bi-antennary complex N-linked glycans, the specific ligands for PSA and e-PHA (Kornfeld et al 1981), in their cytoplasm. The absence of the staining in some cells in the same zones showed that they were not all in the same phase of the activity, and that a few of them might complete production of the outer chains of the glycans while others might not. Another possibility is that these differences could be because of the exact part of the cell that is exposed when the cells are sectioned. L-PHA stains non-bisected tri/tetra-antennary complex N-glycans; they were detected in the matrix, but not inside the chondrocytes, suggesting they leave the cells quickly and do not develop intracellular 'pools', or that they have another origin.

Group 2 (UEA-I)

UEA-I showed no reaction with chondrocytes and Schunke et al (1985) found the same result. This shows that L-fucosyl termini are not present or are not accessible in chondrocytes.

Group 3 (MAA)

Most of the chondrocytes of the surface of the normal articular cartilage were stained moderately showing that chondrocytes of the normal articular cartilage can produce N-acetylneuraminic acid. Osteoarthritic chondrocytes of the superficial zone mostly showed

a weak reaction, but it was strong in the cells of the medial and deep zones, suggesting that a lower amount of carbohydrate in the matrix is a trigger for the increased intercellular production of them (Bock et al 2001). These results show that N-acetylneuraminyl residues are expressed in these areas at different concentrations. Superficial chondrocytes in OA do not appear to be triggered to produce N-acetylneuraminic acid because the concentration of this residue is stable in comparison to other residues.

Chondrocytes of the clones were stained strongly, suggesting they are active for repair, and operate like normal chondrocytes.

Group 4 (ECA and PNA)

Chondrocytes of normal and osteoarthritic cartilage showed moderate to strong reactions with ECA. This shows that they are active in expressing Galβ1,4GlcNAcβ1, residues. Chondrocytes of severe OA showed strong reactions with ECA, but the matrix showed no ligands for it. This suggests a possible restriction of secretion of ECA-positive material from the chondrocytes to the matrix, or that this material is a cellular component.

PNA showed no reaction with chondrocytes of normal and osteoarthritic cartilage. Hoedt-Schmidt (1989) found no reaction between PNA and chondrocytes of the cartilage before any pre-treatment, suggesting there are Galβ1,3GalNAcα1 and Galβ1,4GlcNAcβ1 present, but they are accessible only after pre-treatment.

Group 5 (DBA, VVA, MPA, HPA and WFA)

No reaction was seen between these lectins and chondrocytes of the normal or osteoarthritic articular cartilage. This shows they might be not capable of producing N-acetylgalactosamine residues with any linkage on N- or O-linked oligosaccharides or they might not be accessible.

Summary

There were various patterns of staining with lectins indicating that a wide range of carbohydrate sequences were expressed in the chondrocytes. Variable staining of these cells suggests that they were in various phases of metabolic activity. Superficial and middle zone chondrocytes appeared to be more metabolically active than deep zone chondrocytes and their glycoprotein glycan products were mostly N-linked oligosaccharides. Carbohydrate expression of the chondrocytes of the clones showed that their metabolic functions differed from normal chondrocytes. In addition, the chondrocytes of the clones showed variable carbohydrate expression or variable amounts of glycan production.

4.4.2. Lectin binding with fibrocartilage

Introduction: There are several types of cartilage in sternoclavicular joints, which have some differences in carbohydrate expression in both matrix and chondrocytes. The expression of the carbohydrates in fibrocartilage was homogeneous and the distance from the surface did not have an affect. This supports the conclusion that the superficial staining found in articular cartilage was not an artefactual edge effect.

4.4.2.1. Matrix

The matrix of the normal and osteoarthritic fibrocartilage showed a weak to moderate reaction with HHA, PSA, LCA, e-PHA, l-PHA. These results show that the matrix of the fibrocartilage contains moderate amounts of high-mannose N-glycans and bisected and non-bisected bi/tri/tetra-antennary complex N-glycans. Osteoarthritic changes do not alter any of the binding sites for these lectins.

UEA-I moderately stained the matrix of the normal and the mild to moderate osteoarthritic fibrocartilage. It stained the severely osteoarthritic fibrocartilage only weakly. These results show that fibrocartilage has N or O-linked oligosaccharides with L-fucosyl termini and that in severe OA this carbohydrate residue is either degraded or the cells do not produce it.

The matrix of the normal fibrocartilage showed a weak reaction with MAA, and osteoarthritic fibrocartilage showed a moderate reaction because osteoarthritic changes disclosed terminal carbohydrate residues (Schunke et al 1985). The matrix of the osteoarthritic fibrocartilage showed more concentration of N-acetylneuraminyl residues, arising either because of excess production or increased accessibility of the ligands after degradation of the matrix.

PNA and ECA showed only a weak reaction with the matrix of the normal and osteoarthritic fibrocartilage, suggesting that only a little N or O-linked glycan with β-galactosyl termini, was present in the matrix of the fibrocartilage.

The matrix of the normal and osteoarthritic fibrocartilage showed no reaction with DBA, VVA, MPA, HPA and WFA. This suggests there is no N or O-linked glycan with α-2-deoxy2-acetamidogalactosyl terminal residues in fibrocartilage.

4.4.2.2. Chondrocytes

Various types of carbohydrate residues were found in chondrocytes of the fibrocartilage. The expression of glycans in chondrocytes of the fibrocartilage was different from that of the chondrocytes of the articular cartilage, perhaps because of their different functions and anatomical origins.

Chondrocytes of the normal and osteoarthritic fibrocartilage generally showed weak to moderate reactions with HHA, PSA, LCA, e-PHA and l-PHA. This suggests that they produce moderate amounts of complex bi/tri/tetra-antennary and high mannose N-glycans.

Chondrocytes of fibrocartilage showed no binding sites for UEA-I, suggesting that there was little or no production of N or O-linked oligosaccharides with L-fucosyl termini and/or they are exported quickly into the matrix.

Chondrocytes of the normal fibrocartilage mostly showed weak reactions with MAA, whereas chondrocytes of the osteoarthritic fibrocartilage generally gave moderate reactions, probably because they tend to be compensating for degradation (Hoedt-Schmidt 1989).

PNA showed no reaction with chondrocytes of the fibrocartilage as with articular cartilage. This shows that chondrocytes do not have N or O-linked glycans with β-galactosyl termini available to bind to PNA.

ECA showed weak reactions with normal chondrocytes of the fibrocartilage and moderate staining of chondrocytes in mild to moderate OA. Chondrocytes of severe OA showed no reaction with ECA. This may be because osteoarthritic changes in cartilage first stimulate metabolic activity in cells, but in severe OA the chondrocytes fail in essential biosynthetic activity. These reactions showed there were N or O-linked glycans with β-galactosyl termini available to ECA in fibrocartilagenous chondrocytes.

DBA, VVA, MPA, HPA and WFA showed no reaction with chondrocytes of any fibrocartilage, therefore fibrocartilagenous chondrocytes do not produce N or O-linked oligosaccharides with α-2-deoxy2-acetamidogalactosyl termini and are similar in this respect to chondrocytes of the articular cartilage.

Summary

The expression of the carbohydrate residues in sternoclavicular fibrocartilage had not been studied previously using lectin histochemistry. These results show that different types of N and O-linked oligosaccharides, with various carbohydrate termini, were found in fibrocartilage, and that those were differences from the glycans in articular cartilage. There was no strong reaction between any of the lectins and fibrocartilage, suggesting that this cartilage either contains less glycoprotein glycan than articular cartilage, as Malinger et al (1986) have determined by lectin and alcian blue staining, or that less of it is available for lectin binding.

4.4.3. Lectin binding with the first rib cartilage

The expression of glycans in the hyaline cartilage of the first rib was compared to that in the articular hyaline cartilage. No reaction was seen between three groups of the lectins (HHA, PSA, LCA, e-PHA and l-PHA; PNA and ECA; DBA, VVA, MPA, HPA and WFA) and the matrix and chondrocytes of the first rib. These results show that complex N-linked glycans and N or O-linked glycans with α-2deoxy,2-acetamidogalactosyl termini were not identified in the first rib. It is probable that the ligands for most of these lectins were masked by steric effects caused by the densely packed proteoglycan aggregates in the matrix (Hoedt-Schmidt 1989) rather than being truly absent. Therefore, expression of glycans in the first rib, or the density of the matrix materials, differed from that in articular cartilage, reflecting the specific structures and functions of these matrices.

The first rib was not present in all the slides used for lectin histochemistry, because of technical problems with detachment of the delicate structure of the rib from the slides during the processes of staining.

4.4.4. Lectin binding with subchondral bone

Subchondral bone trabeculae showed moderate to strong reactions with e-PHA, PSA and MAA. There was a weak reaction between the bone trabeculae and other lectins (HHA, LCA, l-PHA, UEA-I, ECA, PNA, DBA, MPA and WFA). No reaction was seen with and VVA or HPA. These results show that subchondral bone trabeculae contain an abundance of the complex N-linked glycans and N or O-linked glycans with N-acetylneuraminyl termini, and small amounts of N- or O-linked glycans with β-galactosyl and α-2deoxy 2-acetamidogalactosyl termini.

Osteocytes showed a strong reaction only with MAA indicating the intracellular presence of structures with N-acetylneuraminyl termini, but not the other terminal or subterminal glycans which this study investigated.

4.4.5. Lectin binding with synovium and capsule

Synovium and capsule showed moderate to strong reactions with PSA, UEA-I, MAA and ECA. Capsule were stained moderately with WFA, but synovium showed no reaction. L-PHA showed moderate reaction with synovium, but no reaction with capsule. Neither structure reacted with the other lectins used in this study. These results showed that expression of glycans in synovium and capsule had some differences from other joint tissues and that glycan expression in synovium and capsule were not completely the same.

4.5. Histological features of the growth plate

4.5.1. Introduction

The growth plate is a complex structure composed of cartilage, bone and fibrous tissue, which combines chondrogenesis with osteogenesis at the same time and at the same rate, in response to local and systemic factors. Parathyroid hormone-related peptide (PThrP), growth hormone (GH) and Indian hedgehog (Ihh) are essential for normal skeletal development (Medill et al 2001, Richardson et al 2003). The growth plate is an active structure, in which chondrocytes proceed through proliferative and hypertrophic stages, as well as an additional process that causes their death. Finally the cartilage is replaced by newly formed bone in the process of endochondral ossification. Terminal differentiation of the chondrocytes, cell hypertrophy and apoptosis, should happen before the conversion of the calcified cartilage to bone (Jikko et al 1999). The rate of bone formation is determined by the rate of chondrocyte proliferation, matrix production and increase in chondrocyte size, in the direction of growth, during cellular enlargement (Breur et al 1991). Chondrocytes produce macromolecules, PGs and collagens, and export them out to extracellular matrix. In the ECM these are assembled into a large network, which is involved in cell-cell, cell-matrix and matrix-matrix interactions (Muramatsu 1994). Therefore, glycans, as a notable part of these macromolecules, should be investigated in abnormal embryonic and fetal development (Miosge et al 1998). Modifications of carbohydrate residues may possibly play a functional role in abnormal development in humans and it is important to determine carbohydrate patterns elaborated in human malformations. In this study features of the chondrocytes and their production of the extracellular matrix, were investigated in normal growth plate and compared with the equivalent features in various abnormalities of the growth plate.

4.5.2. Microanatomy of the growth plate

In this present study examination of the growth plate, using light microscopy, revealed that the cartilaginous part of the plate is organised into three, different zones. In longitudinal sections, cell profiles and the orientations of cells differed considerably between zones, but in transverse sections, cell profiles did not change among zones as Buckwalter et al (1985) had found. The reserve zone contained spherical chondrocytes, in which single or paired chondrocytes were separated by extracellular matrix. The function of this zone is not well understood (Abad et al 2002) and primary dysfunction does not take place in the reserve zone (Brighton 1987). However, manipulation of the resting zone showed that it could regenerate the other zones (Abad et al 2002). The

number and size of the resting chondrocytes in the reserve zone is less than in other zones.

In the proliferative zone, flattened chondrocytes form longitudinal columns perpendicular to the long axis of the bone. The average number of the cells in each column was between five and ten. Chondrocytes within a column are more synchronised with each other than are chondrocytes in different columns, because of different original stem cells (Farnum and Wilsman 1993). Endochondral ossification includes a well-organized evolution from cell division through hypertrophy and differentiation to cell death (Gibson 1998). At the end of the proliferative zone, the hypertrophic zone began with one or two cells at the end of each column. A constant distance was maintained between proliferating cells and the underlying bony metaphysis, because of coordination between cell production in the proliferative zone and cell death in the hypertrophic zone (Farnum and Wilsman 1989). Hypertrophic chondrocytes occupied the epiphyseal cartilage within regular cell columns (Morini et al 1999). They became more rounded and the degree of cell orientation was decreased between the proliferative zone and the hypertrophic zone (Buckwalter et al 1985). The average size of the hypertrophic chondrocytes at the bottom of the zone was many times bigger than their size in the proliferative zone (Noonan et al 1998). This increase in size happened not only in cell volume, but also in the amount of extracellular matrix of the hypertrophic zone (Noonan et al 1998) and the largest increase in matrix volume occurred in the hypertrophic zone (Vanky et al 1998). In hypertrophic cells, increases in cell volume were associated with an increase in number of internal organelles including endoplasmic reticulum, Golgi apparatus and mitochondria, but the volume increment in cytoplasm was clearly greater than that attributable to increased occupancy by organelles (Buckwalter et al 1986). The chondrocyte enlargement started immediately following cell division in the proliferative zone (Breur et al 1994). In the very bottom of the hypertrophic zone, the cells were dead and the lacunae were empty. However, hypertrophic chondrocytes were metabolically active and they were capable of proliferation (Alberty and Peltonen 1993). Both morphological and biochemical evidence was found for apoptosis of the terminal hypertrophic chondrocytes (Zenmyo et al 1996). Apoptosis of the terminal hypertrophic chondrocytes is thought to play an important role in the control of normal endochondral ossification (Horton et al 1998). Death of the hypertrophic chondrocytes occurs prior to invasion by vascular endothelial cells and endothelial cells or blood vascular cells then fill the space of the empty lacunae (Farnum and Wilsman 1989, Gerber and Ferrara 2000). Cartilage canals are penetrations of blood vessels and connective tissue that were found

within the growth plate of the costochondral junction (Hirano et al 1994). Cartilage canals are believed to participate in the development of secondary ossification centres of the epiphysis (Roach et al 1998). Cells resembling fibroblasts were present adjacent to the canals (Cole and Wezeman 1985).

In this study, growth plate from the abnormal samples showed different features from normal, but some of their features looked normal. The skeletal dysplasias are very rare disorders and it takes a long time to find numbers of these disorders to study. Four cases of fetal skeletal dysplasia were studied here, thanatophoric dysplasia, achondrogenesis and achondroplasia, which are three of the most common types of skeletal dysplasia, and Campomelic dysplasia, a rare type of skeletal dysplasia (Lee et al 2002). In thanatophoric dysplasia, the reserve zone was hypercellular with less extracellular matrix than normal. Some of the chondrocytes were of a discoid shape. These findings confirmed those of Rimoin (1974), Hwang et al (1979) and Sillence et al (1979). Diminution in number of the proliferative cells was seen and they made columns only infrequently, as was found by Kolble et al (2002). In the hypertrophic zone, not all cells underwent hypertrophy and small cells were seen between the hypertrophic cells. Kolble et al (2002) found fibrous tissue and irregular ossification in some parts of the zones. Individuals affected by thanatophoric dysplasia usually die of pulmonary hypoplasia perhaps because their ribs are shorter than normal, like their limbs (Lee et al 2002). Growth abnormality is more severe at a late stage of development (Fieni et al 2002).

In the case of achondrogenesis type I studied here, some of the normal chondrocytes were replaced by vacuolated chondrocytes with a thin layer of matrix. Borochowitz et al (1988) support this finding. In the present study no columns were formed in the proliferative zone and not all of the cells in the hypertrophic zone underwent hypertrophy. Rimoin (1974) and Hwang et al (1979) described similar observations. In achondrogenesis, total collagen, essentially type II collagen, was shown to be decreased when compared to an age-related control, but it was unlikely to be the primary defect (Freisinger et al 1994). In addition, interterritorial matrix was missing, cartilage chondrons were separated by thin spaces and mineralised cartilaginous septa were found to be absent (Corsi et al 2001).

In this present study, resting chondrocytes looked normal in achondroplasia, but proliferative cells were not flattened and infrequently made a column. Wang et al (1999) found a longer growth plate, as compared with normal, in homozygous achondroplasia because of an increase in proliferating chondrocytes. Here the variable sizes of the chondrocytes, hypertrophic and non-hypertrophic, were admixed. However, Rimoin

(1974) stated that achondroplasia is characterised by well-organised, regular, endochondral ossification. Wang et al (1999) and Lemyre et al (1999) believed that the homozygous achondroplasia is less well organised than heterozygous achondroplasia. Thanatophoric dysplasia resembles a degree of abnormality near to homozygous achondroplasia. Therefore the specimens used here could be from homozygous achondroplasia. Mutations in the fibroblast growth factor 3 (FGF3) have been shown to be responsible for achondroplasia (Roussau et al 1994). It is caused by disorganisation in the proliferation and hypertrophy of chondrocytes of the growth plate (Wang et al 1999), which leads to the characteristic short, flat bones. These aberrant features are characteristically associated with the abnormal statures of individuals with these chondrodysplastic conditions. However, it is not clear how these abnormal features affect growth (Rimoin et al 1976).

In the present study, the resting zone of the rachitic growth plate was normal, but irregular proliferative cell columns, and expansion of the hypertrophic zone, were obvious. Liu (1996) found deficient mineralisation, irregular proliferative cell columns, extending to other zones, and dilated vascular channels were seen in rachitic growth plate (Liu 1996). The expansion of the hypertrophic zone was present, because extracellular calcium plays an important role in cellular maturation (Donohue and Demay 2002).

In some types of abnormal skeletal development, the growth plate could be the site of secondary malformation associated with the primary defect. Therefore, it was expected to find normal histology with an altered carbohydrate pattern (Miosge et al 1998). The malformation could be because of abnormal chondrocytes behaviour (Horton 1988).

4.5.3. Histochemistry of the growth plate

The normal extracellular matrix of the growth plate was stained intensely by TB, which showed that there was abundant GAG, presumably as aggrecan, present in the ECM of the growth plate (Plaas and Sandy 1993). However, the resting zone was stained less than other zones indicating fewer GAG components, and Farquharson et al (1994) found that chondroitin sulphate and keratan sulphate were lower in the hypertrophic zone than in the proliferative zone. Della et al (1995) found that cationic dye stains the territorial matrix intensely, whereas pre-treatment with hyaluronidase induces intense staining with cationic dye in interterritorial matrix. Chondrocytes of the proliferative and hypertrophic zones show a functional heterogeneity (Simon and Cooke 1988, Farnum and Wilsman 1993). Cell and matrix volume increase from the proliferative zone to the hypertrophic zone. However, cell density decreases from the proliferative zone to the hypertrophic zone (Noonan et al 1998). The structure of the aggrecan is similar in the

matrix of the resting and proliferating zones, but in the lower hypertrophic zone the hydrodynamic size of the aggrecan monomer is increased because the sizes of the GAG side chains and the core protein increase (Plaas and Sandy 1993). In the hypertrophic zone, the filament length of hyaluronan and the number of hyaluronan monomers per aggrecan molecules decreases. Proteoglycan aggregate size and the proportion of aggregate monomers also decrease (Buckwalter et al 1987). Histochemical staining in the present study showed heterogeneity of the matrix of the growth plate between the zones and with distance from the chondrocytes.

The extracellular matrix of abnormal growth plates showed different toluidine blue staining from normal across the zones and a homogeneous reaction was seen between various parts of the ECM of the zones. In abnormal growth plates, chondrocyte maturation is arrested, therefore few changes occur in matrix components, as was found by Farquharson et al (1994). The structure of the GAGs may, however, not be completely normal (Brighton 1987), therefore TB staining can be abnormal.

PSR stained the extracellular matrix of the normal growth plate intensely, which shows the presence of large amounts of fibrous collagen in the matrix of the growth plate as Noonan et al (1998) concluded. Alini et al (1992) found that the concentration of collagen is highest in the proliferative and upper hypertrophic zones and Fujii et al (2000) suggested that the collagen fibres are more randomly aligned in the resting zone than in the other zones. When hypertrophic chondrocytes enlarge, the collagen content of the territorial matrix decreases and the collagen content of the interterritorial matrix increases, from the proliferative zone (Buckwalter et al 1986). Abnormal growth plates showed irregular and lessened staining with PSR. This suggests that the amounts of collagen in the abnormal growth plate matrices, or that the structure of collagen is different from the normal.

4.6. Lectin histochemistry of the growth plate

Considerable attention should be focused on the role of growth factors and their interactions as control mechanisms, regulating morphogenesis and development. Carbohydrates can serve as important information-storing molecules and information about glycoconjugate-receptor interaction is important (Zanetta et al 1994). Application of a set of lectins, characterised by the same monosaccharide affinity, but with different fine specificities, may be used to analyse the chemical structure of complex carbohydrates in histochemistry. Little information is available with respect to the analysis of alterations of N or O-linked carbohydrate residues during growth plate maturation. Therefore, in the present study the distribution, concentration and organisation of matrix

carbohydrates, in the different zones of the growth plate, have been studied through the use of lectin histochemistry with pre-treatment by hyaluronidase and β-elimination as shown in Figure 4.1. Hyaluronidase depolymerises hyaluronan, so making some glycans of aggrecan accessible, which had previously been hidden. Also, some glycans could be lost if they were attached near to the hyaluronan and link protein, or if they were trapped as clathrates in the matrix. β-Elimination releases the O-glycans, but not the N-glycans, and so enables identification of this latter general class of glycans.

A difference in the lectin binding patterns of normal growth plate and diseased tissue has been established (Horton 1982, Stanescu et al 1994, Miosge et al 1998). Abnormal tissue showed alteration in carbohydrate residues compared to normals, even in those cases with the same histology as the normal growth plate. Lectins have been used in a variety of tissues to examine differentiation, maturation and neoplastic transformation involving the sugar moieties of both cellular and matrix glycoconjugates, and this kind of information is not available in such detail from other histochemical techniques such as immunocytochemistry.

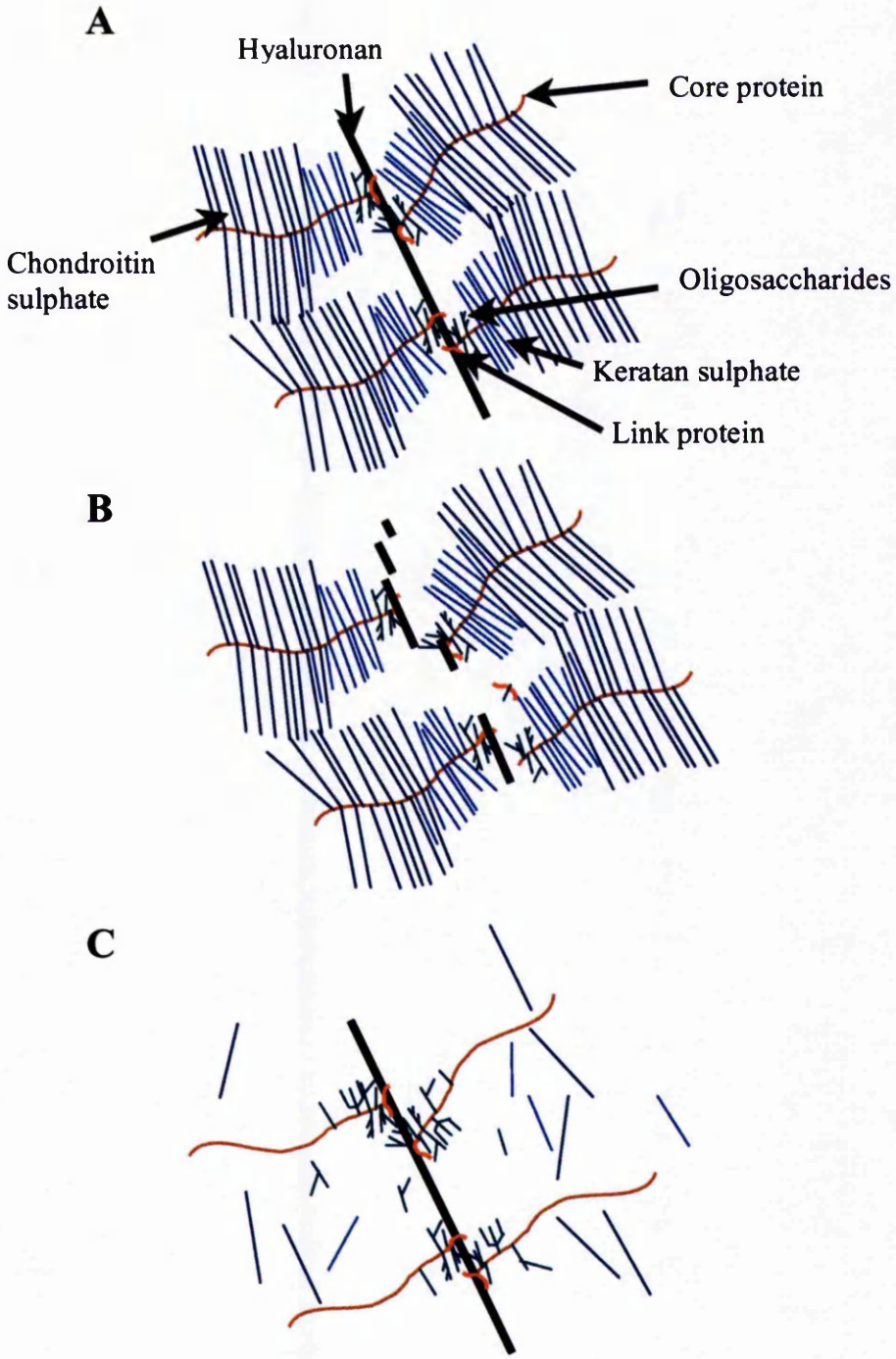


Figure 4.1. Diagram showing part of the aggrecan molecule and after pre-treatment. (A), after hyaluronidase (B) and after β-elimination (C).

4.6.1. Matrix

Group 1 (HHA, PSA, LCA, e-PHA and l-PHA)

Different reactions with the matrix of the normal and abnormal growth plates were seen among these lectins. HHA showed no reaction with the matrix of either the normal or the abnormal growth plate. This suggests that either there are no non-reducing terminal αD-mannosyl residues in the matrix of the growth plate or, perhaps, other factors prevent lectin binding even in presence of these residues (Farnum 1985). Both pre-treatment with hyaluronidase and β-elimination caused a mild to moderate reaction between HHA and the matrix of the normal growth plate, indicating that the matrix did contain non-reducing αD-mannose residues (Zschabitz et al 1995) and that they were not accessible because of long and compact branches of chondroitin and keratan sulphates (Zschabitz et al 1995). Pre-treatments, which remove GAG chains from PGs (using β-elimination) or cause depolymerisation of hyaluronan (using hyaluronidase) may result either in an increase of lectin penetration or unmasking of lectin reactive sites (Takagi et al 1988).

The interterritorial matrix of normal growth plate showed a weak reaction with PSA, as did the territorial matrix of the normal hypertrophic zone. These results suggest that non-bisected bi-antennary N-glycans, probably with fucose residues in the α1,6 linkage (Kornfeld et al 1981), are produced by normal growth plate chondrocytes and especially by normal hypertrophic cells. Zschabitz et al (1995) found a similar result, but not with the hypertrophic zone probably because of the different anatomical position of the specimens. Proliferative and hypertrophic interterritorial matrices of the thanatophoric and short limb dysplasias showed a strong reaction with PSA. This was also seen in their binding of LCA. Sillence et al (1979) suggested that the concentration of the carbohydrate residues is higher in their matrices than normal. These could cause a problem in binding of growth factors to these matrices.

The interterritorial matrix of abnormal growth plate was stained strongly with e-PHA, but normal matrices showed no reaction. A similar result was found by Zschabitz et al (1995) in the normal growth plate. Strong staining in interterritorial matrix of abnormal growth plates could be because of degenerative changes in abnormal matrices. L-PHA did not show any reaction with the normal or abnormal matrices. High affinity binding to e-PHA occurs with bi/tri-antennary glycoprotein glycans containing two outer chain galactosyl residues and a 'bisecting' residue of N-acetylglucosamine linked β1,4 to the β-linked mannosyl residue in the core. This specificity is not the same with l-PHA binding

affinity. In contrast l-PHA binds to tri/tetra-antennary complex N-glycans containing outer galactosyl residues and an α-linked mannosyl residue alternative at position C-2 and C-6 (Cummings and Kornfeld 1982). The difference between the binding requirement of e-PHA and l-PHA was confirmed by the present study, which indicates that e-PHA and l-PHA interact with oligosaccharides differing in their requirement for antennation and bisection. β-Elimination did not change any reactions between e-PHA or l-PHA and matrix. However, a mild reaction was seen between l-PHA with normal interterritorial matrix after pre-treatment with hyaluronidase. This showed that a few tri/tetra-antennary, non-bisected, complex N-glycans were near to the hyaluronan, which were disclosed after pre-treatment with hyaluronidase. Zschabitz et al (1995) found a reaction between l-PHA and the matrix of the normal growth plate. This showed the presence of tri/tetra-antennary complex N-glycans in the matrix, but it is not clear why it was negative before pre-treatment in the present study. It could be caused by the different technique or concentrations of materials used in this study.

Group 2 (UEA-I and AAA)

The matrix of normal and abnormal growth plates showed no reaction with these lectins. The lack of binding with UEA-I and AAA indicated that terminal α-L-fucosyl residues in α1-2 or 1-3 linkages were apparently not expressed in glycoconjugates of growth plate (Zschabitz et al 1995) or they were not accessible. The latter conclusion is more acceptable because after β-elimination both lectins showed reactions with the matrix of normal growth plate. UEA-I showed a weak reaction with resting zone matrix and AAA showed a weak to moderate reaction with matrices of all zones. Zschabitz et al (1995) found a reaction between UEA-I and matrix of the normal growth plate, but it is not clear why it was not positive before pre-treatment in the present study. It could be because of the different developmental stage of the specimens. Miosge et al (1998) found exposure of free ligands due to diminution of other ligands in different ages. These results show the presence of α-L-fucosyl termini in normal matrix. Mallinger et al (1986) suggested that keratan sulphate has fucosyl terminal residues.

Group 3 (CTA, ECA and PNA)

CTA and PNA showed no reaction with the matrices of normal or abnormal growth plate. PNA binds to β-galactosyl termini and lack of PNA binding sites confirmed that this residue was not there or not accessible. The present results support the result of Zschabitz et al (1995) and Miosge et al (1998). Takagi et al (1988) used chondroitinase ABC on normal growth plate and PNA showed strong reaction after this pre-treatment.

This shows matrix of normal growth plate contains galactosyl-rich substances, but they are not accessible.

ECA bound strongly with normal matrix, especially with the territorial matrix of the normal resting zone, supporting suggestions that the components of the zones vary from each other (Brighton 1987, Zschabitz et al 1995). In addition, matrices of the growth plate of the thanatophoric dysplasia, short limb dwarfism, abnormal calcium deposition and Beckwith's syndrome showed strong reactions with ECA. This could result from the presence of Galβ1,4GlcNAcβ1, like normal growth plate, or from fewer components, but with more lectin penetration. This lectin is mostly reactive with N-acetyllactosamine residues (Iglesias et al 1982). β-Elimination did not alter either the sites of reaction with ECA in matrix or the intensity of staining, but less reaction was seen between ECA and the normal matrix after pre-treatment with hyaluronidase. This showed either that some ECA ligands were trapped in the matrix the loosing the matrix washed them out, or they are on small proteins, such as link protein, which are released when hyaluronan is broken.

Group 4 (DBA, VVA, HPA and WFA)

In this group only WFA showed reaction with interterritorial matrix of all the cases, with the exception of Beckwith's syndrome. This is a rare genetic disorder, which is characterised by pre-and postnatal overgrowth, macroglossia and anterior abdominal wall defects. Additional, but variable complications include organomegaly, hypoglycaemia, hemihypertrophy, genitourinary abnormalities and in about 5% of children embryonal tumours. WFA has a high affinity for GalNAcα1,6Galβ1 and it can bind GalNAcα1,3Galβ1 weakly. VVA binds the latter strongly and DBA needs terminal fucosyl residues. In Beckwith's syndrome most glycan production is probably greater than normal, but a few are less, such as the WFA-specific binding site, GalNAcα1,6Galβ1. Another possibility is that WFA may not be able to access the specific binding sites in the packed matrix. Lack of reaction with VVA could result from the deletion of GalNAcα1,3Galβ1 residue in growth processes or they were not accessible. HPA binds to similar ligands as WFA but with less affinity. Farnum and Wilsman (1986) found similar results. DBA showed no reaction with any of the matrices. However, Nakamura and Ozawa (1996) found that DBA bound to the matrix of the normal growth plate. In addition, in the present study, DBA applied after β-elimination showed a weak reaction with matrix, suggesting that the DBA ligands were disclosed after pre-treatment because β-elimination would have removed the long GAG molecules and so small glycans were available to the lectins. Nakamura et al (1989) found the same

result with other lectins of this group. Takagi et al (1988) used chondroitinase ABC on the normal growth plate and found binding between SBA and the matrix of the normal growth plate. These results show the presence of α-2-deoxy,2-acetamidogalactosyl termini in the matrix of the normal growth plate, but they are not accessible without pre-treatment.

Various reactions are seen with these lectins as they have hydrogen bonding interactions, making stable binding between some of the lectins and carbohydrate (Baker et al 1983). WFA showed no reaction with the matrix of normal growth plate after pre-treatment with hyaluronidase. WFA probably bound to glycans that were trapped in the matrix and opening the matrix washed them out, or they were near to hyaluronan, for example on link protein, and they were released after the hyaluronan was broken. Therefore, the matrix loses these residues after pre-treatment as Picton et al (2000) suggested. In addition, Della et al (1995) concluded that lectin staining is sensitive to the concentration of the enzyme used for pre-treatment, as Farnum and Wilsman (1986) observed lack of lectin staining after hyaluronidase pre-treatment probably due to a higher enzyme concentration. More reaction was seen in the matrix with these lectins after β-elimination, because the release of GAGs and O-glycans disclosed other N-glycans to lectins. HPA showed more reaction with matrix after pre-treatment with hyaluronidase, which probably allowed full penetration of the lectin, because it altered the relationship between PG and other matrix and plasma membrane macromolecules (Poole et al 1982).

Group 5 (DSA)

Interterritorial matrix of normal proliferative and hypertrophic zones showed strong reactions with DSA. This showed the presence of N-acetyllactosamine and/or N-acetylglucosamine oligomers (Crowley et al 1984), but the normal resting matrix and the abnormal matrices either did not contain them or the lectin had no access to them.

Summary

These results show dissimilarity between the matrices of the normal and abnormal growth plate as has been reported by Stanescu et al (1994). The altered expression of carbohydrate in abnormal growth plate, as compared to the normal, could risk lack of binding to important growth factors. This alteration was seen also between various zones in growth plate (Farquharson et al 1994). It was mostly found between the zones of the normal growth plate. The variety of the carbohydrate expression could be explained by the main functions of the zones (Brighton 1987). So the zones probably contain the glycans that are needed for the particular growth processes taking place in them. Interleukin 1 (IL-1), which stimulates chondrocytes to produce collagenase and

proteoglycanase, has been detected in the normal hypertrophic chondrocytes (Yamashita et al 1989). In addition, growth factors were found only a little in the normal resting chondrocytes (Jingushi et al 1995). These factors, IL-1 and growth factor, could cause a variation in the expression of macromolecules between the zones. However, according to the results of this present study some similarity was found between normal and abnormal growth plates. Using pre-treatments that disrupt the matrices, it was found that many glycans were present in the growth plate and access to some of them was not possible before the use of β-elimination or hyaluronidase, which rendered them accessible to the lectins. Thus, negative results do not imply the absence of lectin binding sites (Farnum 1985). Sometimes, lectins cannot reach the carbohydrates because they are embedded in tertiary protein structures or they are masked by other molecules (Della et al 1995).

4.6.2. Chondrocytes

Chondrocytes of the normal growth plate are metabolically very active. They showed strong cytoplasmic and surface reactions with most of the lectins, meaning that they expressed a wide range of carbohydrates. Growth plate chondrocytes are known to change their phenotypic expression with their stage of cellular maturation (Poole et al 1984) so staining of the chondrocytes of the zones could vary. Lectins may be used as cytochemical tools in order to determine carbohydrate residues that are inserted in the growing oligosaccharide chains in chondrocytes (Tartakoff and Vassalli 1983). The lectins used showed that the chondrocytes of the various zones had different staining patterns and, hence, different glycans which suggests diverse functions of the chondrocytes in the zones, and also that there were differences between chondrocytes of the normal and the abnormal growth plates.

Group 1 (HHA, LCA, PSA, e-PHA and l-PHA)

The chondrocytes of the growth plate showed reactions with HHA, PSA and e-PHA. Resting chondrocytes showed more reaction with these lectins than other chondrocytes. It is consistent with the proposition that the chondrocytes of different zones have different functions (Farnum and Wilsman 1989). Chondrocytes produced bisected and non-bisected bi-antennary complex N-glycans. Abnormal proliferative and hypertrophic chondrocytes showed stronger reactions with PSA than the resting chondrocytes. They expressed more carbohydrate, because chondrocyte maturation was arrested in abnormal growth plate (Farquharson et al 1994). E-PHA showed no strong reaction with chondrocytes of the abnormal growth plate. HHA stained most of the normal chondrocytes, and the chondrocytes of the thanatophoric dysplasia, short limb dwarfism, Beckwith's syndrome and abnormal calcium deposits were stained. This suggests that

these chondrocytes expressed complex N-glycans with non-reducing terminal αD-mannose. LCA and l-PHA did not bind to chondrocytes of normal and abnormal growth plate. This suggests the chondrocytes do not produce any tri/tetra-antennary N-glycan complexes with N-acetylglucosaminyl termini.

Group 2 (UEA-I and AAA)

No reaction was seen between these lectins and chondrocytes of the growth plate. This suggests they do not express N or O-glycans with fucosyl termini or that these are not accessible. The latter is the case, because, after β-elimination, a strong reaction was seen between these lectins and chondrocytes of the normal growth plate. Pre-treatment with hyaluronidase disclosed lectin-binding glycans in proliferative chondrocytes, suggesting different positions for glycans fucosyl termini on the cells. A positive result was found by Farnum (1985) with UEA-I without any pre-treatment, which showed the presence of this carbohydrate residue. It is not clear why it was negative in the present study before pre-treatment; perhaps it was caused by the use of different methods or different concentrations of reagents used in this study.

Group 3 (CTA, ECA and PNA)

Chondrocytes of both the normal and abnormal growth plates showed reactions with ECA. These results show that N or O-glycans with β-galactosyl termini are produced in both and are probably not critical to the normal endochondral ossification process. ECA bound strongly to most of the pericellular area, suggesting that it is a strong marker for pericellular β-galactosyl residues. Only a few normal chondrocytes showed reactions with CTA, perhaps indicating that a subset of chondrocytes express the less highly branched oligomers on N-acetyllactosamine. PNA showed reactions with proliferative and hypertrophic chondrocytes, but not the resting cells, showing a different pattern of glycan production by these cells. Miosge et al (1998) found similar results with chondrocytes of normal fetuses. Takagi et al (1988) reported a reaction between PNA and all the chondrocytes of the growth plate. Velasco et al (1988) found binding between other lectins of this group and the chondrocytes cytoplasm of the growth plate. They suggested that this is due to the presence of galactosyltransferase.

Some of the binding sites for ECA were lost after pre-treatment with hyaluronidase, which may be due to a loss of some glycans because of an 'altered relationship' between PGs and plasma membrane, as Poole et al (1982) suggested hyaluronidase can induce. In addition, the ECA binding site was probably localised on alkali-stable N-glycans near to the hyaluronan. These were probably on link protein, which is made soluble by the

breakdown of hyaluronan by hyaluronidase and it is not released from its interaction with hyaluronan by β-elimination.

Group 4 (DBA, VVA, HPA and WFA)

Only WFA gave moderate to strong reactions with chondrocytes of both normal and abnormal growth plates. The pericellular area of positive cells was also stained with WFA. This showed that only restricted types of N-acetylgalactosamine residues were present in chondrocytes of the growth plate, and that these glycans did not affect the function of the growth plate. The negative results from the other lectins do not necessarily imply absence of their ligands (Farnum 1985) as they could have been caused by specific steric hindrance (Zimmermann and Thies 1984), or more probably, they were not accessible because of the density of the local matrix. Another possibility is that these lectins, such as DBA (140 kD), showed no reaction because they were too big to penetrate the matrix. Stronger staining was seen between WFA and chondrocytes of the normal growth plate after β-elimination, while it was lost after pre-treatment with hyaluronidase. This suggests that the binding sites for WFA are on glycans near to hyaluronan, probably on link protein, which were not affected by β-elimination, but were released with the link protein after pre-treatment with hyaluronidase.

Group 5 (DSA)

In the present study, the chondrocytes of the normal growth plate showed strong reactions with DSA. This shows that they are producing glycans containing oligomers of N-acetylglucosamine or N-acetyllactosamine, as Velasco et al (1988) also found. Chondrocytes of the abnormal growth plate showed no reaction with DSA, which suggests they are not capable of producing these carbohydrate residues, as their matrices were also negative.

Summary

The chondrocytes of the normal growth plate were functionally very active, but different zones showed various activities. In addition there was functional heterogeneity among the chondrocytes of both proliferative and hypertrophic zones (Simon and Cooke 1988). A difference was found in the carbohydrates of the resting chondrocytes from those found on and in the proliferative and hypertrophic chondrocytes (Farnum and Wilsman 1989, Abad et al 2002). Comparison of the normal and abnormal chondrocytes suggested that some of their products were not affecting the endochondral ossification processes. In addition, the metabolic activity of the chondrocytes of the abnormal growth plate was different from that found with normal chondrocytes, in respect of glycan synthesis.

5. Conclusions

Cartilage is an essential component of the skeleton, acting in skeletal development, stabilising the body and aiding free movement. Sternoclavicular joint (SCJ) and costochondral junction cartilages were analysed in the present study to determine their microanatomical features and molecular structures by using histological, immunohistochemical and lectin histochemical techniques.

The SCJ is superficial, easily accessed and, therefore, a valuable object for study. It is the only connection between the upper extremity and the trunk. Every movement of the arm results in the transmission of forces across the SCJ. Osteoarthritis is a common disorder in the SCJ, even though it is a non-weight-bearing joint, with hyaline articular cartilage and fibrocartilage both being affected, although the fibrocartilage is considered to protect the hyaline articular cartilage. OA changes not only the overall structure of cartilage, but also its molecular composition. Histological staining demonstrated and lectin histochemistry specified these changes.

This study provided evidence that amyloid depositions are common in different parts of the SCJ, such as articular cartilage, fibrocartilage, synovium and joint capsule. These amyloids, comprising β2-microglobulin, kappa and lambda light chains and P-component, were deposited without any history of haemodialysis or renal disease. Macrophage accumulations were found near to β2-microglobulin deposits in synovium and capsule, but not in cartilage.

Comparison of the growth plates in the normal costochondral junctions with those of cases of certain abnormalities showed morphological changes in some cases, but only molecular changes in others.

Through the use of the lectin histochemical technique, the present study demonstrated the existence of lectin-binding sites in the cells and matrices of the normal and diseased cartilages (articular cartilage, fibrocartilage and growth plate), indicative of the presence of certain carbohydrate residues in them. This study also provided evidence that there is some similarity between glycan expression in osteoarthritic cartilage and the growth plate. Altered glycosylation in the course of fetal skeletal disease was established in the present study by noting the changes in the lectin-binding patterns. These results demonstrated that during the expression of abnormalities, the affected tissues exhibited different patterns of glycans from those of normally developed specimens. In addition, some growth plates, known to be sites of secondary malformation accompanying the primary defect, although displaying a histologically normal appearance, also showed an

altered glycosylation pattern. This might indicate a possible general alteration in carbohydrate metabolism in the course of the development of several malformations.

The matrix of the mild to moderately osteoarthritic cartilage showed stronger reactions with most of the lectins than did other cartilages. Either there was exposure of lectin-binding sites by the disease mechanisms, or there was enhanced production of matrix molecules as part of an attempted repair process. Chondrocytes of the articular cartilage and growth plate showed nearly the same level of reaction with lectins, suggesting that chondrocytes of both cartilages have similar glycan expression. However, their matrices did not show the same reactions with specific lectins. This could be due to changes in the matrix as part of the developmental process in which the lectin-binding sites are "switched off". Positive results in the present study were interpreted to represent the existence of accessible lectin-binding ligands under the conditions of these experiments, but negative results do not necessarily imply the absence of lectin-binding sites, which may be due to an occupation of the ligands by endogenous lectins or other binding molecules, or be related to general steric hindrance in the matrix. In addition, studies of glycans in cartilage should be extended by the use of pre-treatment with enzymes, because very complex macromolecules in the matrix may not be easily accessible by lectins.

Of the lectin specificity groups used in this study, the group 5 lectins that bind to α-2-deoxy,2-acetamidogalactose were not particularly informative, with little or no reaction being give in either normal or abnormal cartilage. Of the other lectin groups, group 1 lectins with specificity for N-glycans, were interesting as there were clear differences in the staining of the matrices between normal and osteoarthritic cartilage, and LCA only showed differences between normal and abnormal growth plate cartilage. In the growth plate overall, the chondrocytes were stained by a larger number of lectins than the matrix. The lectins DSA and PNA intensely stained the normal growth plate, but did not stain the abnormal growth plate. This could be due to failure by the abnormal cartilage to produce Galβ1,3GalNAcα1 and 4GlcNAcβ1; this could be a significant reason for the malformation of the growth plate. After the pre-treatments, cartilages could be stained by a few lectins that had not done so before and vice versa, and many of the lectins stained the cartilage of the growth plate more intensely, which suggests that pre-treatments must be used in combination with lectin histochemistry in cartilage.

Overall, in terms of the hypothesis stated in the Aims and Objectives of this thesis, it is clear that there are changes in glycan expression in the articular cartilage of the sternoclavicular joint which allow a distinction to be made between mild/moderate

osteoarthritis and normal cartilage. The superficial location and accessibility of this joint would make it a possible cartilage biopsy site. The usefulness of this would depend on the relationship between osteoarthritis of this and other joints and on other joints showing identical changes in glycan expression.

Glycoprofiling also shows similarities and differences between articular healthy and diseased cartilage and normal and abnormal growth plate cartilage. In general the chondrocytes of the growth plate are more metabolically active in elaborating glycans and this might, in part, be an age-related phenomenon. It is not, therefore, possible from these studies to categorically assert that the osteoarthritic changes are a recapitulation of an endochondral ossification. In terms of glycan expression, this is probably too simple a concept since variation in expression is probably specific for location, function and disease.

Suggestions for Future Work:

1-Sternoclavicular joints could be examined in people who have had clinical problems which might affect their sternoclavicular joints (SCJs); examples would be fractured clavicle, malformed clavicle or malformed sternum i.e. pectus carinatum (pigeon chest) and pectus excavatum (funnel chest).

2-SCJs could be examined in quadruped animals, because in the human they are non-weight bearing and in these animals they are weight bearing. Changes e.g. amyloid deposits or OA in their SCJs could be sought possibly leading to suggestions for the relationship between weight bearing and these features.

3-SCJs could be examined in people who have used their hands extensively or have hung by their hands, such as swimmers or gymnasts, and compared with those with average use of the SCJ.

4-The composition of amyloid in the SCJ could be further explored by transmission electron microscopy of antibody labelled samples.

5-The temporomandibular joint (TMJ) could be examined and compared to the SCJ, using histochemistry and immunohistochemistry, as they have similar developments and structures, but one is non-weight bearing and the other is weight bearing. Amyloid deposits could be investigated in the TMJ in making this comparison.

6-A different set of lectins could be used in cartilage to complement the lectins already used and complete the investigation of the fine structure of glycosylation in articular cartilage and the growth plate. The use of a panel of lectins with overlapping ligands, particularly with a set of exoglycosidases, provides a means of analysis of glycans, which are larger than the sequences complementary to single binding sites. The set could contain GNA, NPA, ConA, SNA, LFA, CTA, RCA_{120}, STA, LEA, WGA, $BSA\text{-}IB_4$ and BSA-II.

7-In the longer term, more cases of abnormal growth plates could be acquired and studied.

8-Animal models of rare genetic abnormalities, such as genetic disorders of the skeleton in the mouse, could be studied by lectin histochemistry.

9-Pre-treatment with different enzymes e.g. chondroitinase ABC, combined with lectin histochemistry could be used to provide extra information on the structure of the glycans and their interactions in cartilage.

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Appendices

Appendix A

Sources of chemicals and materials

Reagents		Source
Acetone		BDH, Merck Ltd., Lutterworth, Leics
Aminopropyltriethoxysilane (APES)		Sigma Chemical Co. Ltd, Poole, Dorset
Anti CD68		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Avidin-peroxidase conjugate		Sigma Chemical Co. Ltd, Poole, Dorset
Biotinylated Lectins	HHA, MAA	Vector Laboratories, Peterborough, UK
	AAA	EY Laboratories, Inc., San Mateo, CA, USA
	PSA, LCA, E-PHA, LPHA, UEA-I, ECA, CTA, PNA, DBA, VVA, MPA, SBA, HPA, WFA, DSA	Sigma Chemical Co. Ltd, Poole, Dorset
Bovine serum albumin		Sigma Chemical Co. Ltd, Poole, Dorset
Calcium chloride		BDH, Merck Ltd., Lutterworth, Leics
Celestine blue		BDH, Merck Ltd., Lutterworth, Leics
Congo red		BDH, Merck Ltd., Lutterworth, Leics
Crude trypsin, type II-S		Sigma Chemical Co. Ltd, Poole, Dorset
DePeX		R.A.Lamb, London
DAB for lectin histochemistry		Aldrich Chemical Co. LTD., Poole, Dorset
DAB for immunohistochemistry		Sigma Chemical Co. Ltd, Poole, Dorset
Dimethylsulphoxide (DMSO)		Sigma Chemical Co. Ltd, Poole, Dorset
EDTA		Sigma Chemical Co. Ltd, Poole, Dorset
Eosin		Sigma Chemical Co. Ltd, Poole, Dorset
Goat anti-mouse		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Haematoxylin		BDH, Merck Ltd., Lutterworth, Leics
Hydrogen peroxide		BDH, Merck Ltd., Lutterworth, Leics
Hyaluronidase		Sigma Chemical Co. Ltd, Poole, Dorset
Human β2 microglobulin antigen		Sigma-Aldrich Company Ltd.Dorset, UK
Industrial methylated spirit (IMS)		Genta Medical, York
Methanol		Sigma Chemical Co. Ltd, Poole, Dorset
Methyl green		Sigma Chemical Co. Ltd, Poole, Dorset
Mouse anti human β2 microglobulin		Serotec Ltd, Oxford, UK
Mouse anti human AA amyloid		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Mouse anti human κ light chain		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Mouse anti humanλlight chain		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Normal swine serum		TCS Biosciences Ltd., Botolph Clayton, Buckingham
Normal goat serum		TCS Biosciences Ltd., Botolph Clayton, Buckingham
Picric acid		BDH, Merck Ltd., Lutterworth, Leics
Rabbit anti human P component		Novocastra Laboratories Ltd, Newcastle, Uk
Sirius red		R A Lamb, London
Sodium chloride		BDH, Merck Ltd., Lutterworth, Leics
Sodium hydroxide		BDH, Merck Ltd., Lutterworth, Leics
Sodium azide		Sigma Chemical Co. Ltd, Poole, Dorset
Stretavidin peroxidase		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Swine anti-rabbit		Dako Ltd., Angel Drove, Ely, Cambridgeshire
Toluidine blue		Sigma Chemical Co. Ltd, Poole, Dorset
Tris buffer saline (TBS)		Boehringer Mannheim, Ltd. Lewes, East Sussex
Xam		Sigma Chemical Co. Ltd, Poole, Dorset

Appendix B

Data sheets for immunohistochemistry reagents

Monoclonal Mouse Anti-Human Amyloid A

Clone mc1 **Code No. M 0759 Lot 029.** Edition 24.04.01

Intended use For in vitro diagnostic use.

DAKO Monoclonal Mouse Anti-Human Amyloid A, Clone mc1, for use in immunocytochemistry. The antibody labels amyloid A (AA) in tissues, aids in the identification and classification of AA-amyloidosis.

Immunocytochemical staining using DAKO Monoclonal Mouse Anti-Human Amyloid A in combination with Congo Red staining (1), or particularly Congo Red fluorescence, (2) is much more sensitive than Congo Red staining alone. Interpretation must be made within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Introduction Amyloidosis is a group of diseases that have in common the extracellular deposition of fibrillar proteins with a specific biochemical conformation known as ß-pleated sheets. Approximately 20 different precursor proteins that may be deposited as amyloid fibrils have been identified (3). Amyloid proteins deposited in the tissues can be identified by Congo Red staining, and chemically classified via amino acid sequence studies, by immunochemistry, or via immunocytochemistry (1). Amyloid A (AA) is an extracellular deposited insoluble fibrillar protein, highly resistant to proteolytic degradation, and produced from the precursor protein serum amyloid A (SAA) (3). Before the therapy of a suspected amyloid disease can be planned, both the presence of amyloid and its chemical origin must be known (2).

Reagent provided Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 0.05 mol/L Tris/HCL, pH 7.2, and containing 15 mmol/L NaN3. Clone: mc1 (4, 5). Isotype: IgG2a, kappa. Mouse IgG concentration: 355 mg/L. Total protein concentration: 14.3 g/L.

Immunogen An equal mixture of human amyloid A coupled to horseradish peroxidase and human amyloid A coupled to high molecular weight kininogen (5, 10).

Specificity In micro-ELISA the antibody reacts with amyloid AA and the serum precursor of amyloid A indicating crossreactivity among amyloid-A proteins and the precursor serum amyloid protein A. In contrast, no reactivity to other non-AA amyloid fibril proteins or human serum proteins, such as albumin, transferring, and IgG is seen (4, 5). In immunocytochemistry, the antibody labels tissues from AA patients, but shows no reactivity with a host of unknown antigens in tissue sections of various organs (5), nor with amyloid types A□A□and amyloid fibril proteins in familial amyloid polyneuropathy and senile cardiovascular amyloidosis, respectively. Neither are Alzheimer's amyloid plagues, amyloid in microangiopathy, lichen amyloidosis, and senile islets of Langerhans labelled by the antibody (5-7).

Precautions 1. For in vitro diagnostic use. 2. The NaN3 used as a preservative is toxic if ingested. NaN3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.

Storage Store at 2-8 □C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the user must verify the conditions. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact DAKO Technical Services.

Specimen preparation Paraffin sections: The antibody can be used on formalin-fixed, paraffin-embedded tissue sections. Heat-induced epitope retrieval in 10 mmol/L citrate buffer, pH 6.0, or in DAKO Target Retrieval Solution (code No.S 1700) is recommended. The tissue sections should not dry out during the treatment or during the following immunocytochemical staining procedure.

Frozen sections and cell smears: The antibody can be used on frozen sections (5).(2)

Staining procedure Dilution: DAKO Monoclonal Mouse Anti-Human Amyloid A Component, code No. M 0759, may be used at a dilution range of 1:50-1:100 when applied on formalin-fixed, paraffin-embedded sections of human kidney from a patient with amyloidosis A and using secondary amyloides and using 15 minutes heat-induced epitope retrieval in 10 mmol/L citrate buffer pH 6.0, and 30 minutes incubation at room temperture with the primary antibody. Optimal conditions may vary depending on specimen and preparation method, and should be determined by each individual laboratory. As a negative control, DAKO Mouse IgG2a, code No. X 0943, diluted to the same mouse IgG concentration as the primary antibody is recommended. Visualization: Sensitive staining techniques, such as DAKO LSAB+/HRP kits and DAKO EnVisionTM+/HRP kits are recommended. Follow the procedure enclosed with the selected visualization kit. Automation: Not tested. **Product-specific limitations** Cross-reactivity with the serum precursor of amyloid A has been observed (5, 6).

Performance characteristics Amyloid A labelled by the antibody has an extracellular localization in the majority of cases. Normal tissues: The antibody does not label normal tissue (5). Abnormal tissues: The antibody labelled amyloid A in renal and rectal biopsies of patients with inflammatory pediatric disease (1). Tissues from patients with a clinical diagnosis of rheumatoid arthritis, sporadic Muckle-Wells syndrome,

idiopathic polyneuritis, idiopathic amyloidosis, familial Mediterranian fever, and Still's syndrome were also labelled (5).

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Monoclonal Mouse Anti-Human Lambda Light Chains

Clone N10/2 **Code No. M 0614 Lot 108.** Edition 09.11.00

Presentation Monoclonal mouse antibody supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing fetal calf serum) dialysed against 0.05 mol/LTris/HCl, pH 7.2 containing 15 mmol/L NaN_3. Mouse Ig concentration: 200 mg/L. Isotype: IgG1, kappa. Total protein concentration: 7.0 g/L.

Storage 2 - 8 □C.

Immunogen IgG purified from normal human serum.

Specificity/reactivity The DAKO antibody has been tested by means of immunoblotting after agarose gel electrophoresis against a panel of myeloma proteins, and reacted with those containing lambda light chains irrespective of Ig isotype. No reaction was obtained with kappa
chains. In cryostat sections, the antibody labels B cell follicles in human lymphoid tissue. The mantle zones show a mosaic pattern of labelling, representing B cells carrying lambda immunoglobulin on their surface, whilst in the germinal centres immune complexes bound to follicular dendritic cells give a coarse meshwork pattern of staining. Plasma cells are not sharply stained in cryostat sections since immunoglobulin tends to diffuse from the cytoplasm of these cells. Extracellular immunoglobulin (e.g. within vessels, or bound to connective tissue) will also be stained in cryostat sections. In routinely fixed, paraffin-embedded tissue the antibody gives strong labelling of lambdapositive plasma cells, and cells which have absorbed exogenous immunoglobulin (e.g. Reed-Sternberg cells). The antibody also reacts with surface immunoglobulin on normal and neoplastic B cells in peripheral blood. This is of great value in demonstrating the monoclonal nature (light chain restriction) of lymphoid neoplasms.

Staining procedures Formalin-fixed and paraffin-embedded sections. Can be used on formalin-fixed, paraffin-embedded tissue sections. Antigen retrieval, such as by heating in DAKO Target Retrieval Solution, code No. S 1700, is mandatory. The slides should not be allowed to dry out during this treatment or during the following immunohistochemical staining procedure. For tissue sections sensitive staining techniques are recommended, such as the LSAB or the EnVision™system. The antibody may be used at a dilution of 1:75 - 1:100 with the LSABsystem when tested on formalin-fixed, paraffin-embedded sections of human tonsil. Enzymatic digestion with proteolytic enzymes such as trypsin, pronase and protease K can be used as an alternative to the above antigen retrieval.(2)
Frozen sections and cell smears. Can be used for labelling acetone-fixed cryostat sections or for fixed cell smears. For staining cell smears, the APAAP method is recommended. The antibody may be used at a dilution at 1:75 - 1:100 in the APAAP technique, when tested on acetone-fixed cryostat sections of human tonsil. These are guidelines only; optimal dilutions should be determined by the individual laboratory. Methodology: The antibody can be used manually as well as on automated immunostaining instruments.

Monoclonal Mouse Anti-Human Kappa Light Chains

Clone A8B5 **Code No. M 0730 Lot 077.** Edition 06.03.01

Presentation DAKO-Kappa, A8B5 is a mouse monoclonal antibody supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing fetal calf serum) dialysed against 0.05 mol/L Tris/HCl, 15 mmol/L NaN3, pH7.2. Mouse Ig concentration: 110 mg/L. Isotype: IgG1, kappa. Total protein concentration: 14.3 g/L.

Storage 2-8 □C.

Immunogen IgG purified from normal human serum.

Specificity/reactivity The DAKO antibody has been tested by means of immunoblotting, after agarose gel electrophoresis, against a panel of myeloma proteins and reacts with those containing kappa light chain irrespective of the Ig isotype. No reaction is obtained with lambda chains. In cryostat sections, the antibody labels B cell follicles in human lymphoid tissue. The mantle zones show a mosaic pattern of labelling, representing B cells carrying kappa immunoglobulin on their surface, whilst in the germinal centres immunocomplexes bound to follicular dendritic cells give a coarse meshwork pattern of staining. Plasma cells are not sharply stained in cryostat sections since immunoglobulin tends to diffuse from the cytoplasm of these cells. Extracellular immunoglobulin (e.g. within vessels, or bound to connective tissue) will also be stained in cryostat sections. In routinely fixed, paraffin-embedded tissue this antibody gives strong labelling of kappa-positive plasma cells, and cells which have absorbed exogenous immunoglobulin (e.g. Reed-Sternberg cells). The antibody also reacts with surface immunoglobulin on normal and neoplastic B cells in peripheral blood. This may be of value in demonstrating the monoclonal nature (light chain restriction) of lymphoid neoplasms.
Staining procedures Formalin-fixed and paraffin-embedded sections: can be used on formalin-fixed, paraffin-embedded tissue sections. Enzymatic digestion with proteolytic enzymes such as pronase should be performed before staining. The slides should not be allowed to dry out during this treatment or during the following immunohistochemical staining procedure. For tissue sections, a variety of sensitive staining techniques is suitable, including im-munoperoxidase procedures, the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique and avidin-biotin methods such as LSAB methods. Following **enzymatic digestion** the antibody may be used at a dilution of 1:50 - 1:100 with the LSAB methods when tested on formalin-fixed paraffin-embedded sections of human tonsil. Frozen sections and cell smear: can be used for labelling acetone-fixed cryostat sections or for fixed cell smears. For staining cell smears, the APAAP method is recommended. The antibody may be used at a dilution at 1:50 - 1:100 in the APAAP technique and avidin-biotin methods such as the LSAB methods, when tested on acetone-fixed cryostat sections of human tonsil. These are guidelines only; optimal dilutions should be determined by the individual laboratory. Flow cytometry: the antibody is well-suited for flow cytometry (indirect technique) using DAKO Rabbit Anti-Mouse Immunoglobulins/FITC, code No. F 0313. Automation: the antibody can be used on automated immunostaining systems.

Amyloid P Protein - rabbit polyclonal antibody NCL-PCOMp

Specificity Human amyloid P protein also known as P component. Traces of contaminating antibodies have been removed by liquid-phase absorption with human plasma proteins. Specificity has been ascertained by crossed-immunoelectrophoresis.
Antigen used Purified P component from human plasma. **for immunisations**
Preparation Lyophilised Ig fraction purified from rabbit serum diluted in PBS with 1% BSA containing 15mM sodium azide. Reconstitute with 1ml of sterile distilled water as indicated on vial label.
Effective on frozen tissue Not evaluated.
Effective on paraffin wax Yes **embedded tissue**
Recommendations on use Immunohistochemistry: Typical working dilution 1:200 - 1:300. Trypsin digestion of paraffin sections is recommended. 60 minutes primary antibody incubation at 25oC. Standard ABC Technique. Also effective in immunodiffusion techniques.
Positive controls Human kidney with amyloidosis.
Staining pattern Amyloid deposits in the glomeruli.
Storage and stability Store unopened lyophilised antibody at 4oC. Under these conditions, there is no significant loss in product performance up to the expiry date indicated on the vial label. The reconstituted antibody is stable for at least two months when stored at 4oC. For long term storage, it is recommended that aliquots of the antibody are frozen at -20oC (frost-free freezers are **not** recommended). Repeated freezing and thawing must be avoided. Prepare working dilutions on the day of use.
Legal consideration NCL-PCOMp is recommended **FOR RESEARCH USE ONLY.**
Application Amyloid consists mainly of rigid, non-branching protein fibrils, together with rod-like aggregates of a pentagonal-shaped glycoprotein called amyloid P protein. Amyloid P protein, also known as P component, comprises 10 per cent of amyloid tissue and is present in all but the central nervous system forms of amyloid. Amyloid P protein is a constituent of normal basement membranes and the microfibrillary elastic fibre network. NCL-PCOMp is effective on formalin-fixed, paraffin-embedded material and provides an alternative choice to NCL-AMP (suitable only for tissue fixed in 70 per cent ethanol).
References
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Breathnach S M, Bhogal B, Dyck R F, et al.. Immunohistochemical demonstration of amyloid P component in skin of normal subjects and patients with cutaneous amyloidosis. British Journal of Dermatology. **105**: 115-1174 (1981).
Dyck R F, Lockwood C M, Kershaw M, et al.. Amyloid p-component is a constituent of normal human glomerular basement membrane. J.Exp. Med. **152**: 1162-1174 (1980).

Serotec Product Data Sheet

MOUSE ANTI HUMAN BETA 2 MICROGLOBULIN (1) MCA1115

Clone Number: B2

Volume/Quantity: 1mg

Product Form: Purified IgG - liquid

Preparation: Purified IgG prepared by affinity chromatography on Protein A from tissue culture supernatant

Buffer: Phosphate buffered saline

Preservatives Stabilisers: 0.09% Sodium Azide

Approx. Protein Concentrations: IgG concentration 1.0 mg/ml

Immunogen: Human beta lymphoblastoid cell line (FAU)

Isotype: IgG1 (Mouse)

Specificity: Specific for human beta-2 microglobulin. Useful for studies in myeloma and other conditions. Can be used in combination with MCA1116 for immunoassays since both antibodies recognise different epitopes on the beta-2M molecules.

Species Cross Reactivity: Not tested.

Applications:

	Yes	No	Not Tested
Flow Cytometry			.
Immunohistology - frozen			■
- paraffin			■
- resin			■
ELISA	■		
Immunoprecipitation			■
Western Blotting			.
Radioimmunoassay			■

Where this antibody has not been tested for use in a particular technique this does not necessarily exclude its use in such procedures. It is recommended that the user titrates the antibody for use in their own system using appropriate negative/positive controls.

References:

1. Liabeuf, A. *et al.* (1981). An antigenic determinant of human beta 2 microglobulin masked by the association with HLA heavy chains at the cell surface analysis using monoclonal antibodies. J. Immunol. 127: 1542-1548.

Storage Conditions: Store at +4°C or at -20°C if preferred. This product should be stored undiluted.Storage in frost-free freezers is not recommended.

Avoid repeated freezing and thawing as this may denature the antibody.

Should this product contain a precipitate we recommend microcentrifugation before use.

Shelf Life: 12 months from date of despatch.

Health and Safety Information: (A full Health and Safety assessment is available upon request)

This product contains sodium azide: a POISONOUS AND HAZARDOUS SUBSTANCE which should be handled by trained staff only.

FOR RESEARCH PURPOSES ONLY. NOT FOR THERAPEUTIC OR DIAGNOSTIC USE

Serotec Offices contact details: http://serotec.oxi.net/asp/offices.html
www.serotec.com Serotec Ltd, 22 Bankside, Station Approach, Kidlington, Oxford, OXON OX5 1JE, UK serotec@serotec.co.uk Tel: +44 (0)1865 852700

Appendix C

Table C1. Summary of HHA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	4	1-2	1-2	1	1	1	1	1	1	4	1	0	1	1	1
M OA	4	4	4	1	1	1	1	0	0	4	2	0	1	1	1
S OA	4	1	1	1	1	1	1	1	1	4	1	0	1	1	1

Table C2. Summary of PSA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	2-4	2-3	4	1-4	2-3	4	1	2-3	4	0-2	2	0	2-4	2-4	2-4
M OA	2-4	4	3-4	1-4	4	3-4	1-4	4	3-4	0-2	2	0	2-4	2-4	2-4
S OA	2-4	2-3	3-4	1-4	2-3	3-4	1-4	2-3	3-4	0-2	2	0	2-4	2-4	2-4

Table C3. Summary of LCA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	1	1	0	0	0	0	0	0	2	2	0	1	1	1
M OA	0	3-4	3-4	0	4	0	0	0	0	2	2	0	1	1	1
S OA	0	3-4	3-4	0	4	0	0	0	0	0	1	0	1	1	1

0 = no staining, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong, intense staining

N: normal, M OA: mild to moderate osteoarthritis, S OA: severe osteoarthritis

Cho: chondrocytes, Ost: osteocytes, Trab: bone trabeculae, TM: territorial matrix, ITM: interterritorial matrix

Table C4. Summary of E-PHA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	3-4	2-4	2-4	3-4	2-4	2-4	0	0	0	0-2	2	1	3-4	1	1
M OA	3-4	2-4	2-4	3-4	2-4	2-4	0	0	0	0-2	2	1	3-4	1	1
S OA	3-4	2-4	2-4	3-4	2-4	2-4	0	0	0	0-2	2	1	3-4	1	1

Table C5. Summary of L-PHA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	0	0	0	0	0	0	0	0	1	2	0	1	2	0
M OA	0	4	4	0	0	0	0	0	0	1	1	0	1	2	0
S OA	0	4	4	0	0	0	0	0	0	0	1	0	1	2	0

Table C6. Summary of UEA-I lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	1	1	0	1	1	0	1	1	0	2	0	1	2	2
M OA	0	4	4	0	1	1	0	1	1	0	2	0	1	2	2
S OA	0	1	1	0	1	1	0	1	1	0	1	0	1	2	2

0 = no staining, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong, intense staining

N: normal, M OA: mild to moderate osteoarthritis, S OA: severe osteoarthritis

Cho: chondrocytes, Ost: osteocytes, Trab: bone trabeculae, TM: territorial matrix, ITM: interterritorial matrix

Table C7. Summary of MAA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	2	2	2	0-1	1	1	0-1	1	1	1	1	4	1-2	2-4	2-4
M OA	1	3-4	3-4	2-4	3	3	3-4	3	3	2	2	4	1-2	2-4	2-4
S OA	1	3-4	3-4	2-4	3	3	3-4	3	3	2	2	4	1-2	2-4	2-4

Table C8. Summary of ECA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	2	1-2	2	2-4	0	0	0-4	0	0	1	1	0	1	2-4	2-4
M OA	2	1-2	1-2	2-4	4	4	0-4	4	4	2	1	0	1	2-4	2-4
S OA	0-4	1-2	1-2	0-4	1	1	0-4	1	1	0	1	0	1	2-4	2-4

Table C9. Summary of PNA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0
M OA	0	1	4	0	1	4	0	1	4	0	1	0	1	0	0
S OA	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0

0 = no staining, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong, intense staining

N: normal, M OA: mild to moderate osteoarthritis, S OA: severe osteoarthritis

Cho: chondrocytes, Ost: osteocytes, Trab: bone trabeculae, TM: territorial matrix, ITM: interterritorial matrix

Table C10. Summary of DBA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	0	2	0	0	2	0	0	2	0	0	0	1	0	0
M OA	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
S OA	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

Table C11. Summary of VVA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
M OA	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
S OA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Table C12. Summary of MPA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone								
	Cho	Matrix		Cho	Matrix		Cho	Matrix		Cho	Matrix	Ost	Trab		
		TM	ITM		TM	ITM		TM	ITM						
N	0	1	1	0	1	1	0	1	1	2	1	1	1	0	0
M OA	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
S OA	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0

0 = no staining, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong, intense staining

N: normal, M OA: mild to moderate osteoarthritis, S OA: severe osteoarthritis

Cho: chondrocytes, Ost: osteocytes, Trab: bone trabeculae, TM: territorial matrix, ITM: interterritorial matrix

Table C13. Summary of HPA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone			Cho	Matrix	Ost	Trab		
	Cho	Matrix		Cho	Matrix		Cho	Matrix							
		TM	ITM		TM	ITM		TM	ITM						
N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M OA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S OA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table C14. Summary of WFA lectin histochemistry in articular joint tissue

	Articular cartilage									Fibrocartilage		Subchondral bone		Syno-vium	Capsule
	Superficial zone			Middle zone			Deep zone			Cho	Matrix	Ost	Trab		
	Cho	Matrix		Cho	Matrix		Cho	Matrix							
		TM	ITM		TM	ITM		TM	ITM						
N	0	0	0	0	0	0	0	0	0	0	0	0	1-2	0	2
M OA	0	0	0	0	0	0	0	0	0	0	0	0	1-2	0	2
S OA	0	0	0	0	0	0	0	0	0	0	0	0	1-2	0	2

0 = no staining, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong, intense staining

N: normal, M OA: mild to moderate osteoarthritis, S OA: severe osteoarthritis

Cho: chondrocytes, Ost: osteocytes, Trab: bone trabeculae, TM: territorial matrix, ITM: interterritorial matrix