

**AN INVESTIGATION OF THE SUITABILITY OF THE PERI-
ARTICULAR OSTEOPHYTE AS AUTOGENOUS GRAFTS
FOR THE REPAIR OF ARTICULAR SURFACE DEFECTS.**

By

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A Thesis submitted for the degree of

Doctor of Medicine

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Statement of Originality

The work on which this thesis is based is my own independent work except where acknowledged.

Temitope Oluwagbenga Alonge
June 2000

Ethical Consideration.

Ethical committee approval was sought and obtained before osteophytes were retrieved from the patients undergoing total knee replacement for osteoarthritis.

Temitope Oluwagbenga Alonge

June 2000

Dedication

All honour, glory and majesty be unto Jesus Christ my LORD.

This work is dedicated to my lovely wife, Temitope Oluwatoyin Adunni Alonge and my wonderful children, Oreoluwa, Iyanuoluwa and Iyinoluwa Alonge. I am grateful to God for giving me such a wonderful family - I am blessed.

Temitope Oluwagbenga Alonge

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ABSTRACT : MD THESIS

AN INVESTIGATION OF THE SUITABILITY OF THE PERI-ARTICULAR OSTEOPHYTE AS AUTOGENOUS GRAFTS FOR THE REPAIR OF ARTICULAR SURFACE DEFECTS.

Temitope O Alonge

Osteophytes are intra-articular osteochondral outgrowths commonly found at the margins of synovial joints in response to a more central full thickness articular cartilage defects (FTCD). These defects are a significant cause of morbidity and it is probable that if left untreated they may lead to degenerative arthropathy. The aim of this study was to evaluate the suitability of osteophytes as repair tissues for FTCD. To effect a satisfactory repair, osteophytes should exhibit features that are similar to normal articular cartilage. Morphological, histological, immuno-histochemical, immuno-cytological, biochemical and mechanical studies were carried out to identify these similarities.

Compressive and shear stiffness values of 'white' osteophytes compared favourably with that of normal articular cartilage; the cartilage mantle of osteophyte has identical types and disposition of native collagen as are seen in normal articular cartilage; histological stains reveals hyaline like cartilage in the cartilage mantle of osteophytes; the chondrocytes in the cartilage mantle of osteophytes can be grown in monolayer culture and three-dimensional culture systems and in these culture systems, these cells behave like normal articular cartilage chondrocytes and the western blot analysis points to a cartilaginous ancestry for peri-articular osteophytes.

The localised expression of alkaline phosphatase, osteocalcin and c-fos in the chondrocytes in the deep layer of osteophyte cartilage mantle suggests that there are two types of cartilage in this cartilage mantle: resorbable and permanent. C-myc expression in the entire cartilage mantle of the peri-articular osteophyte is suggestive of an actively proliferating and hence probably reparative tissue.

The conclusions drawn from the experiments in this study strongly suggests that osteophytes may be suitable materials for grafting FTCD either as an osteochondral or cellular graft.

Temitope Oluwagbenga Alonge

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PUBLICATIONS ARISING FROM THIS RESEARCH.

1. **Alonge TO, Oni OOA, Morrison CJ.** A comparison of the compressive and shear stiffness of periarticular osteophytes, hypertrophic femoral intercondylar notch and normal articular cartilage. **J Musculoskeletal Res.** 1998; 2 (3) : 247-255.
2. **Alonge T O, Rooney P, Oni OOA.** The architecture of collagens in osteophytes. A study by immunohistochemistry. **J Musculoskeletal Res.** 1999; 3 (3) : 175-181.
3. **Alonge TO, Oni OOA.** An investigation of the frequency of co-existence of osteophytes and circumscribed full thickness articular cartilage defects. **In Press, African Journal of Medicine and Medical Sciences.**

Published abstracts.

1. **Alonge TO, OOA Oni, Fenwick S.** Collagen expression in osteophytes. Examination of decalcified material. **J Bone Joint Surg. [Supp II] 1998; 80-B : 180.**
2. **Alonge TO, I Boyd, OOA Oni.** The biology of peri-articular osteophytes; potential grafts for articular surface defects. **J Bone Joint Surg. [Supp II] 1998; 80-B : 180.**
3. **Alonge TO, Rooney P, Oni OOA.** A preliminary investigation of the behaviour of cells derived from osteophytes in monolayer culture. **J Bone Joint Surg. [Supp III] 1999; 81-B : 299.**

- 4. Alonge TO, Oni OOA.** Expression of c-myc, c-jun and c-fos oncogenes in peri-articular osteophytes. **J Bone Joint Surg. [Supp III] 1999; 81-B : 337.**
- 5. Alonge TO, Oni OOA.** A preliminary study of the fibrillary collagens of the peri-articular osteophytes using polarising light. **J Bone Joint Surg. [Supp III] 1999; 81-B : 337-338.**

PRESENTATIONS.

TO Alonge and OOA Oni.

Collagen expression in osteophytes. Examination of undecalcified material.

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TO Alonge, P Rooney and OOA Oni.

An investigation of the morphology of peri-articular osteophyte using the technique of cryofracturing and scanning electron microscopy.

2nd International Symposium on Cartilage repair - October 1997; Fribourg, Switzerland.

TO Alonge, P Rooney and OOA Oni.

The effect of collagen on cells derived from osteophytes.

The Nottingham Knee Symposium - October 1998; Nottingham, England.

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The effect of collagen on the cultural behaviour of cells derived from osteophytes.

British Orthopaedic Research Society / Section of Bioengineering, Royal Academy of Medicine in Ireland - October 1998; Dublin, Ireland.

TO Alonge, P Rooney and OOA Oni.

A preliminary investigation of the behaviour of cells derived from osteophytes in monolayer culture.

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A western blot analysis of the media in which cell derived from osteophytes have been previously grown.

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Osteophytes. An alternative source of chondrocytes for transplantation?.

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*The frequency of occurrence of osteophytes and circumscribed full thickness
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Boston, Massachusetts. United States of America.**

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*On the suitability of the peri-articular osteophytes as source of chondrocytes for
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Track 2 = Passage 3(2) cells propagated on collagen gel (precipitate);
Track 3 = Passage 3(3) cells propagated in collagen gel (precipitate);
Track 4 = Passage 3(3) cells propagated on collagen gel (precipitate);
Track 5 = Passage 3(3) cells propagated in collagen gel (supernatant);
Track 6 = Passage 3(3) cells propagated on collagen gel (supernatant);
Track 7 = Passage 3(2) cells propagated in collagen gel (precipitate);
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*An investigation of the frequency of co-existence of
osteophytes and circumscribed full thickness articular
surface defects in the knee joint.*

Introduction.

1.1 Articular cartilage

The articular cartilage is a unique tissue that covers the articulating ends of the bones of the synovial joints. It is a smooth, uniform, flexible elastic crust that prevents mutual abrasion between bone ends (in diarthroidal joints) thereby rendering motion safe and free (Hunter 1743). It is also referred to as hyaline articular cartilage denoting the pearly appearance and the structural organization that is peculiar only to it [Cruess 1982]. It is at most only a few millimeters thick but, surprisingly, it is very resilient with a high compressive stiffness which confers on the synovial joints the ability to transmit high load with low contact stresses and little frictional resistance [Buckwalter and Mankin 1997]. In so doing, the articular cartilage minimizes the peak stresses on the subchondral bone and little wonder Walt Whitman [1892] on the strength of the uniqueness and complexity of the diarthroidal joints claims that “the narrowest hinge in my hand puts to scorn all machinery”.

1.1.1 Embryology

Mckibbin and Holdsworth (1967) have shown that the articular cartilage is a unique tissue different from the rest of the epiphyses and not capable of participating in ossification. From the results of their experiment, they concluded that although the epiphyseal cartilage embryologically appears to be homogenous, in actual fact it is composed of two distinct moieties. The superficial layer is destined to become articular

cartilage and hence not ossifiable whilst the deeper layer can produce new cartilage and be converted to bone. Developmentally, the limb bud mesenchyme within the core of the ectodermal outgrowth initially appears as a homogenous population. This mesenchymal condensation marks the position of the future skeletal elements and this occurs prior to overt chondrogenesis (Archer *et al* 1982). With time, presumptive cartilage cells condense within this mesenchyme to form the pre-chondrogenic condensation (PCC) or anlage. Rooney (1984) has demonstrated that, at the time the cartilage anlage or PCC is well demarcated in the chick embryo, no evidence of joint formation was noticed. However, the PCC is not a pre-requisite for chondrogenic differentiation but it helps facilitate the process by which the rounded morphology of the cells are maintained and it is this morphology that favours chondrogenesis (Archer *et al* 1982). Barry (ICRS Boston 1998) has also demonstrated positive staining for fibromodulin in chondrocytes destined to become articular cartilage in the cartilage anlage of mouse embryo whilst the other epiphyseal chondrocytes had negative staining. The mechanism by which a subpopulation of chondrocytes within the homogenous mesenchymal embryonic rudiment evolves as articular cartilage is unknown (Archer 1994). However, the specificity of this type of chondrocyte and the articular cartilage that subsequently arises from it, and the features that distinguish it from epiphyseal growth cartilage could be due to the position or location of the predestined mesenchymal cells in the embryo or as a result of mechanical loading. The location of the chondrocytes within the PCC will no doubt have a bearing on their shape and matrix organization and this constitutes the 'intrinsic' factors while the epigenetic influences such as the mechanical loading constitutes the 'extrinsic' factors (Archer 1994). Haines (1947) has therefore proposed

that whereas the main mass of the epiphyses results from the chondrification of the blastema, which is the precursor of bone, the superficial contribution to this chondrification by the tissues of the inter-zone ultimately becomes the articular cartilage. Clearly this is contrary to the concept of cartilage resorption in the epiphyseal end and appositional growth of the articular cartilage from the synovial end.

1.1.2 Composition

Articular cartilage is an avascular, aneural and alymphatic tissue. Water is the largest constituent of the articular cartilage accounting for 70% to 80% of its total weight (Sun *et al* 1995). In response to stress or loading, the water in the articular cartilage shifts in and out of the tissue thereby allowing for deformation and subsequent recoil after the load has been removed (Miller 1996). Articular cartilage as a tissue, is made up of:

- a) cells (chondrocytes) and,
- b) extracellular matrix, which comprises of
 - (i) matrix fibers or collagens and
 - (ii) ground substance or 'filler'.

The chondrocytes are relatively few and they constitute 1-10% of cartilage tissue volume (Wirth & Rudert 1996) but they synthesize and regulate the composition of the extracellular matrix fibers and ground substance. The extracellular matrix fibers or collagens account for 10-20% of the wet weight of the articular cartilage. The predominant collagen type is type II and overall, the collagen fibers are responsible for the tensile strength of the articular cartilage. The ground substance is made up of protein polysaccharides or proteoglycans (PG) and these are responsible for the compressive

strength of the articular cartilage. The PG are composed of subunits known as glycosaminoglycans (GAGs) and these GAGs include chondroitin-4-sulfate, chondroitin-6-sulfate and keratin sulfate. The extracellular matrix (fibers and ground substance) synthesized by the chondrocytes in return maintains the homeostasis of the chondrocyte environment. In adult articular cartilage, the chondrocytes constitute almost 10,000 cells per cubic millimeters of tissue. The matrix domain on the other hand is about one chondrocyte per 120,000 cubic millimeters of matrix around it (Hunziker 1998- ICRS Boston)

1.1.3 Architecture

The chondrocytes in foetal life are numerous and are haphazardly arranged. This type of arrangement constitutes an isotropic organization. In adult life, the chondrocyte organization is more defined and is referred to as anisotropic organization. These two distinct patterns of organization accounts for a varied response to mechanical loading. The articular cartilage is not homogenous throughout its depth in terms of its chemical composition, chondrocyte organization and chondrocyte morphology and as such, distinct zones can be described (Jeffrey 1994). The superficial zone is made up of chondrocytes, which are morphologically flattened and somewhat similar to synovial lining cells. These superficial cells proliferate less rapidly and they also exhibit poor matrix synthesis. However, these superficial cells synthesize a peculiar protein that is similar to megakaryocyte stimulating factor (MSF) (Kuettner 1998-ICRS-Boston). The middle or intermediate zone is made of rounded, haphazardly disposed typical chondrocytes. In the deeper zones, the chondrocytes are arranged in a columnar fashion.

The extracellular matrix fibers are made up of collagens, which vary in diameter from 30-80nm and accounts for over 50% of the dry weight of the cartilage (Freeman 1973). The collagens are rod-shaped molecules which aggregate in a staggered array forming cross-linked fibers which provides strength and rigidity to this connective tissue. The ground substance on the other hand is constituted by protein-polysaccharide complex (proteoglycan [PG]), non-collagenous protein and lipid (Cruess 1982). The proteoglycans form aggregates (proteoglycan aggrecans) which when linked with hyaluronic acid resist compressive loading of the cartilage (Wirth & Rudert). The collagen matrix fiber network provides cartilage with its tensile strength and shape and counteracts the swelling pressure of the hydrophilic PG (Weckmann and Cabral 1996, Maroudas 1976, Jeffery 1994). There is a complex network of interaction between the matrix collagen fibers and the proteoglycan producing a woven network of fibers and ground substance. This interaction slows down the movement of water through the cartilage and thereby confers on the articular cartilage its viscoelastic properties (Cruess 1982, Mankin 1974). Therefore, the physiological and mechanical properties of articular cartilage seem to depend on the extracellular matrix fibers and the ground substances whose production and regulation in turn depends on the activity of the chondrocytes.

1.1.4 Articular cartilage turnover

The articular cartilage is probably designed to last the life span of the individual (Mankin 1982) and it appears to be well constituted to withstand the functional demands imposed upon it with a minimum of wear (Mankin 1963). The thickness of the articular cartilage in a particular part of the joint is also constant throughout life provided the articular

surface remains intact locally (Freeman 1973). The functions and features of the superficial layer of normal articular cartilage are similar to that of the skin and functionally it is therefore similar to the basement membrane. Nevertheless, constant use of the synovial joints must lead to some attrition of the articular cartilage and ultimately chondrocyte breakdown from the superficial layer (Mankin 1963). A steady state exists in normal articular cartilage where the rate of synthesis of matrix components is equal to the rate of breakdown.

Basically, two types of extracellular matrices are synthesised by the chondrocytes depending on their proximity to the chondrocytes. The matrix in the immediate surrounding of the cell is known as cell associated matrix. The matrices that are further away from the chondrocytes are known as the further removed matrix (territorial and extra-territorial matrices). The **cell-associated matrix** has a rapid matrix turnover of 15 days whilst the **further removed matrix** has a slower turnover of about 100 days. (Kuettner 1998 ICRS-Boston). Under normal circumstances, there is limited turnover of extracellular matrix (ECM) products in articular cartilage and this is possibly under the control of proteinases, for example metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Figure 1). In pathological conditions such as osteoarthritis (OA), this delicate balance breaks down and MMPs become more abundant and active, leading to breakdown of ECM and loss from the tissue. Aspartate and cysteine proteinases act within the cells (intracellular) whilst metalloproteinases (MMPs) and serine proteinases act outside the cells (extracellular). Various types of these enzymes have been isolated in chondrocytes in various diseases of the cartilage.

On the other hand, a variety of growth factors and cytokines present in the joints are able

to promote matrix synthesis and may help with repairing damaged cartilage tissue. These growth factors and cytokines act via receptors on the surfaces of the chondrocytes. The superficial layer in articular cartilage is rich in BMP-7 or osteogenic protein-7 (OP-1). OP-1 is a member of the TGF- β superfamily and has been shown to increase collagen and proteoglycan synthesis and therefore plays a role in cartilage repair (Grgic *et al* 1997). Following cartilage injury, there is an increase in proteoglycans (PG) synthesizes, but the loss of collagen from cartilage is irreversible and therefore the attempted repair does not lead to the restoration of normal articular cartilage. The fact that there is a lot of PG synthesized but limited repair is because the PG escapes from the cartilage since the collagen fibers that binds the PG are depleted. Normal articular cartilage injury can lead to cartilage breakdown and of all the factors so incriminated, only trauma has been conclusively shown to cause direct damage to the articular cartilage and this damage may then lead to osteoarthritis (Repo and Finlay 1977, Donohue *et al* 1983).

The hallmark of articular cartilage degradation is manifested in changes in the cells ('chondrone' formation), the cartilage (fibrillation/fissuring) and the subchondral bone (sclerosis). Injury to the chondrocytes leads to increased susceptibility to apoptosis, impairs cell viability and increases the loss of cell-associated matrix. The final common pathway for any insult to the articular cartilage appears to be an increase in interleukin-1 (IL-1) production. IL-1 acting on the chondrocytes reduces the ability of the cells to synthesis extracellular matrix and it also increases the release of proteinases. The aftermath of these two events is a decrease in the ability of the tissue to mount a repair process and instead increased breakdown of the articular cartilage.

1.1.4.1 Cellular response

Long before gross morphological changes are seen in damaged articular cartilage, the chondrocytes adjoining to the site of cartilage injury mount a reparative response evidenced by cellular proliferation. The relatively poor metabolic activity of chondrocytes limits the extent of this reparative process and the proliferating cells form cell clusters known as 'chondrones' (Archer 1994). The formation of 'chondrones' could also be due to physical lack of space and inability of the cells to move apart. This form of repair is ineffective and it tends to abort with time. The 'chondrone' formation has been described as a re-initiation of mitosis

1.1.4.2 Cartilage fibrillation

PG and the PG-collagen network are responsible for the viscoelastic properties of the articular cartilage and therefore any alteration in this network leads to a decrease in the ability of the articular cartilage to bear load. In these situations, normal cyclical physiological load may then lead to structural damage to the extracellular matrix proteins of the articular cartilage and ultimately the ground substance. The normal response of the articular cartilage to such insult is to increase the synthesis of PG and collagen to replace those that were lost (Kuettner ICRS Boston 1998). Unfortunately, the articular cartilage cannot restore the lost collagen fibres to the original state they were before damage and this makes the repair tissue inefficient. Repeated physiological load therefore leads to a breakdown of the extracellular matrix and this manifests initially as fissures in the substance of the articular cartilage and these changes are first apparent in the superficial

layer. With repeated load, these fissures propagate deep into the cartilage and multiple fissures leads to the formation of fibrillation. At this stage, the cartilage damage is irreparable by itself and this leads to osteoarthritis.

1.1.4.3 Subchondral bone changes

The viscoelastic property of the normal articular cartilage confers on it the ability to absorb physiological load and yet retain full recovery when the load is removed. This viscoelastic property therefore shields the subchondral bone from the effect of the loading of the articular cartilage. When the articular cartilage is damaged, this protective effect is lost and therefore, normal physiologic load is transmitted almost directly to the subchondral bone. The subchondral bone in return undergoes reactive hyperplasia and subsequent sclerosis. This sclerotic change is thought to further impair the nutrition of the articular cartilage leading to more damage to the articular cartilage. This vicious circle is probably one of the factors responsible for the progression of osteoarthritis.

1.1.5 Repair

Reparative response in a tissue is a consideration of the ability of the tissue to increase its rate of DNA and protein synthesis. Although chondrocytes are capable of active synthesis of DNA and extracellular matrix, this task is formidable for the small population of cells within the articular cartilage. These cells also have limited potential of metabolic activity [Mankin 1982]. The repair of articular cartilage depends on the depth of the lesion i.e. superficial (partial thickness cartilage defect) or deep (full thickness cartilage defect) lesion.

Superficial or partial thickness cartilage defect (PTCD).

In broad terms, superficial or partial thickness defects of the articular cartilage initiates an intrinsic response whether in the immature (young) or mature (adult) skeleton. This response is characterized by brief metabolic and enzymatic activities mediated by the original or resident chondrocytes (Mankin 1982, Wirth and Rudert 1996, Mankin 1963). Unfortunately, these responses are not enough to generate an adequate number of new chondrocytes or cartilaginous matrix for the full repair of even minimal lesion. This inadequacy is presumably more pronounced in skeletally mature animals (Mankin 1982) although Bennett and Bauer (1935) and Ghadially *et al* (1971) have shown that the age of the animal did not affect the healing of such lesions. Ultimately, this transient intrinsic metabolic and enzymatic response leads to the formation of a thin layer of fibrocartilage or fibrous tissue. This rather flimsy repair tissue covers the defect (Cheung *et al* 1978). Bennett and Bauer (1935) have also shown in their studies that the repair of superficial defects by proliferation of native chondrocytes is in part dependent upon the depth of the lesion; the deeper the defect, the more likely it is for this type of repair to take place. These deeper lesions which do not breach the subchondral bone plate, as described by Cheung *et al* (1978), are accompanied by the formation of a thin fibrous layer bridging the defect with formation of clones and cartilaginous matrix production by adjoining chondrocytes. This mechanism of cartilage regeneration by itself constitutes the intrinsic repair. Superficial or partial thickness defects in the articular cartilage of foetal lamb has been shown to heal completely in twenty-eight days with full restoration of its cellularity, extracellular matrix and architecture (Namba *et al* 1998). Similarly Calandruccio and

Gilmer have demonstrated complete healing of this lesion in the femoral condyles in six to ten weeks old mongrel puppies (Calandruccio and Gilmer 1962). Fisher (1939) has demonstrated rapid and complete healing of superficial laceration of the lateral region or area of the articular cartilage by hyaline-like cartilage in matured rabbits whereas when a similar lesion occurred in the central region or area, it was repaired by flimsy connective tissue. He was able to demonstrate a thin layer of synovium covering the lateral regions of the normal articular cartilage and therefore attributed the healing in this region to the presence of this vascular tissue. In all, studies in which partial or superficial thickness lesions were created such that the tidemark was not breached did not yield satisfactory repair (Calandruccio and Gilmer 1962).

Deep or full thickness cartilage defect (FTCD)

On the other hand, the repair of full thickness cartilage defects is characterized by the migration of cells that are foreign to the site of injury. Hunziker and Rosenberg (1996) have demonstrated migration of mesenchymal cells from the synovial membrane and subsynovial space, across the surface of the normal articular cartilage, into a cartilage defect pre-treated with chondroitinase ABC and transforming growth factor- β 1 in mature rabbits. These imported cells resulted in almost complete coverage of the lesions. Repair of full thickness cartilage defect extending into the subchondral bone and beyond is characterized by initial filling with fibrin clot and subsequent differentiation of the bone marrow cells to produce repair tissue which can vary from hyaline-like cartilage to fibrous tissue (Key 1931). Metsaranta *et al* (1996) and Nakajima *et al* (1998) have shown that the repair material following FTCD consisted of two types of tissues. At the

bottom of the defect were bone-derived cells which expressed high levels of type I collagen and osteonectin mRNA activity and they produced new osteoid. The superficial layer of the repair tissue showed a slow transition from a fibrin clot into undifferentiated mesenchyme with cells containing type III collagen mRNA. They also observed over time, that the repair tissue became fibrocartilaginous with small groups of cells turning on the transcription of type II collagen gene and acquiring a phenotype typical for hyaline cartilage. These two studies concluded that small clusters of cells in the repair tissue of FTCD are capable of turning on an apparently correct chondrocytic phenotype and that this lineage can be enhanced by regulatory factors. These types of repairs mediated by cells other than the existing resident chondrocytes is referred to as **extrinsic repair**. At the early stages of this repair process, the repair tissue exhibits features similar to hyaline-like cartilage (Bennett and Bauer 1935, Hunziker and Rosenberg 1996, Altman *et al* 1992) but with time this tissue undergoes degeneration (Shapiro *et al* 1993, Mankin 1982). This repair tissue therefore seems to be a poor substitute compared to the original normal articular cartilage, more so as it lacks the characteristic articular cartilage cell zoning, and this tissue also has markedly rough surfaces with sizeable areas of matrix devoid of cells (Ghadially *et al* 1971). It has been shown that this repair tissue, whether fibrocartilage or hyaline-like cartilage in composition is mechanically inferior to normal cartilage and is unable to withstand wear and mechanical loading (Mankin 1974, Chang *et al* 1996).

Full thickness or deep articular cartilage defect therefore constitutes a practical problem for the orthopaedic surgeon especially when this lesion occurs in younger patients. They have been known to cause joint dysfunction and pain (Resnick *et al* 1991). They may also

progress to osteoarthritis (Key 1931) and when this is severe, the usual treatment of choice is replacement of the arthritic surface with artificial prosthesis. Arthroplasties are commonly performed for patients over 60 years of age, but in the younger patients (less than 50 years) arthroplasty is not recommended most especially because of the limited life span of the prosthesis.

1.2 Articular cartilage defects

The problems of articular cartilage healing remain relevant to joint biology as well as to our understanding of the basic process of cartilage metabolism (Mankin 1982). Controversy abounds regarding the ability and or inability of articular cartilage to heal lacerative injuries or wounds involving the cartilage mantle. Hunter's observation in 1743 that "ulcerated cartilage is a troublesome thing and that, once destroyed, is not repaired" is still largely true to this day. It would appear that many factors may affect the repair of wounds in articular cartilage, but none seems to be more important than the depth (Ghadially *et al* 1971).

1.2.1 Pathology

Articular cartilage injuries are broadly classified as (a) superficial or partial thickness cartilage defect (PTCD) or (b) deep or full thickness cartilage defect (FTCD).

Partial thickness cartilage defect (Figure 2).

There is no general consensus regarding the exact definition of a PTCD. This type of defect has been variously defined as that: (a) which involves 100 μ m to 3mm of the cartilage mantle depth (Wirth and Rudert 1996, Namba *et al* 1998), (b) confined to the

cartilage mantle alone (Mankin 1962, Calandruccio and Gilmer 1962 and Shapiro et al 1993) and (c) extends to but not beyond the calcified cartilage layer (Mankin 1974, Cheung *et al* 1978) and therefore are analogous to clefts and fissures seen in osteoarthritis (Hunziker and Rosenberg 1996). While some authors are of the opinion that PTCs are relatively benign, static and with no evidence of progression to osteoarthritis (Mankin 1982, Bennette and Bauer 1935), others strongly believe that they tend to grow larger and deeper with time (Meachim 1963, Hunziker and Rosenberg 1996) and may ultimately contribute to the formation of osteoarthritis.

Full thickness cartilage defect (Figure 3).

On the other hand, a deep or full thickness lesion of the articular cartilage is almost universally accepted as that which breaches the subchondral bone plate and penetrates into the marrow (Mankin 1962, Shapiro *et al* 1993, Mankin 1982, Mankin 1974, Hunziker and Rosenberg 1996, Wirth and Rudert 1996 and Ghadially et al 1971) although Cheung *et al* (1978) chose to disagree with this view by defining it as a lesion which extends beyond the tidemark, through the calcified zone but not breaching the subchondral bone plate. The repair following full thickness cartilage defect is mediated by the vasculature of the subchondral bone plate. This response that is reminiscent of the response in other vascular tissue (Mankin 1962, Mankin 1974, Bennett and Bauer 1935) is characterized by a phase of necrosis, followed by inflammation and finally the phase of proliferation and repair (Mankin 1962, Mankin 1974).

It is apparent that the interpretation of the various experimental and clinical outcomes of PTC and FTC clearly depends on the definition given by the author(s) in question.

1.2.2 Pathogenesis

Articular cartilage responds to different forms of insults in diverse ways and some of the controversies in the literatures about these responses result from the confusion in terminologies which at times makes understanding of the process difficult (Mankin 1982). Superficial and deep articular cartilage lesions can be caused by various insults (trauma, inflammation and agents toxic to articular chondrocyte or to the cartilage mantle) and of course ageing.

1.2.2.1 Ageing

Age related changes in the tissues of the body starting as they often do at different periods of life, are of great anatomical, physiological and pathological interest. Fisher in 1939 carried out systematic post-mortem examination of sections of articular cartilage at different ages and found that degenerative changes began in the central or weight bearing area of the articular cartilage at a much earlier age. He also found that over 90% of persons between 60 and 70 years had visible fibrillation of the articular cartilage in this area. Bennett et al (1932) have shown, using the light microscopy that degeneration in the central or weight-bearing area of the articular cartilage actually begins in the third decade of life in large joints. These age related changes are probably related to the attrition of the superficial cartilage layer with recurrent joint motion. Smale *et al* (1995) examined paraffin-embedded whole knee joint sections of Wistar and Fischer 344 rat strains aged 6 (young adult) and 12 months (aged). The sections were stained with hematoxylin and eosin and Toluidine blue. The alterations observed in the articular cartilage mantle in these joints were graded as minimal, mild, moderate and severe depending on the extent

of the loss of Toluidine blue staining. They observed that young Wistar rats (6 months) were almost free of pathological changes whilst the older animals (12 months or older) had loss of PG staining accompanied by loss of chondrocytes, fraying and fibrillation of the cartilage surface. The age related changes began as PTCD and with time progressed to FTCD.

1.2.2.2 Inflammation

Inflammation of joints from whatever cause is accompanied by a large influx of neutrophils and cytokines into the joint. The neutrophils store large quantities of serine proteinases and metalloproteinases, neutrophil collagenase and gelatinase B. These enzymes can degrade cartilage if released close to the articular cartilage (Anderson 1985, Cawston and Rowan 1998). In most synovial fluids, TIMPs are in excess of MMPs. However, in septic arthritis, TIMP's activity is exceeded, polymorphs produce proteolytic enzymes and the bacterial activators produce excess plasmin. These enzymes are complimented by synovial inflammatory cytokines and ultimately lead to rapid cartilage destruction. The damaged cartilage is characterized by chondrocyte death and subsequently cartilage erosion. The depth of the cartilage erosion often depends on the virulence of the offending organism and the length of contact between the organism and the articular cartilage (Anderson 1985, Cawston and Rowan 1998).

1.2.2.3 Chemical injury

The viability and metabolic activity of the articular cartilage chondrocytes may be materially affected by systemic or locally administered chemical agents. Mankin and

Conger (1966) showed that administration of cortisol in either form and at varying doses into the rabbit knee joint produced a depression in the synthetic capabilities of the chondrocytes by up to 50% as evidenced by a decrease in the rates of incorporation of radiolabelled sulphate and glycine. They concluded from their study that this depression may lead to focal chondromalacia or incipient osteoarthritis. Mankin *et al* (1972) also demonstrated that administration of systemic corticosteroids in rabbits at dosages analogous to immunosuppression in humans, resulted in a decrease in the synthetic activity of articular cartilage chondrocytes as well as depletion of matrix proteoglycans. This may also predispose the articular cartilage to disintegration.

1.2.2.4 Agents toxic to chondrocytes

Some metabolic or systemic disorders such as acromegaly, alkaptonuric onchronosis, and gout amongst others may produce discrete and identifiable lesions of the articular cartilage (Anderson 1985). These lesions are almost always due to the toxic action of a metabolite present in excess quantity within the joint as a result of the metabolic disorder. Intra-synovial haemorrhage as seen in bleeding diathesis also predisposes the articular cartilage to direct toxic injury from the breakdown products of the blood. The chondrocytes have been shown to phagocytose the iron pigments and subsequently release lysosomal enzymes which then leads to matrix degradation. Repeated haemorrhage ultimately leads to superficial and later full thickness articular cartilage defect.

1.2.2.5 Trauma

Trauma is the one insult on the articular cartilage that is commonly reproduced in the laboratory as a cause of both superficial and deep cartilage defects (Repo and Finlay 1977). With a single traumatic episode above the physiological load on the cartilage or with repeated trauma to the cartilage at below physiological load, various cytokines are released by the chondrocytes into the synovial fluid, for example interleukin-1. This cytokine (IL-1) induces the release of proteinases which leads to cartilage degradation (Dingle 1991) and at the same time reduces matrix synthesis thereby impairing any attempt at repair (Kuettner ICRS Boston 1998).

1.2.2.6 Genetic predisposition

It has been the belief for over half a century that there is a strong genetic component to certain degenerative diseases of the articular cartilage leading to the development of osteoarthritis. This influence has been shown to increase to 65% in a twin study (Cicuttini and Spector 1996). The nature of the genetic influence may involve either a structural defect like the extracellular matrix (ECM) collagen or alteration in cartilage metabolism as a whole. At the carboxy or C-terminal of the individual strands of the triple helix of type II collagens the strands converge prior to forming the helix. Therefore any mutation that affects this process of convergence will affect the folding of the strands and this leads to the depletion of type II collagen. Since type II is the main ECM collagen in articular cartilage, the articular cartilage formed is then prone to injury or rather functions abnormally. If this mutation affects the N-terminal, on the other hand, the result will be the formation of abnormal collagen fibres (Olsen ICRS 1998). The findings outlined above can also affect the other collagen types, which cross-link with type II collagen, for

example type XI collagen.

1.2.3 Methods of treatment of full thickness articular cartilage defects (FTCD).

The consequences of full thickness articular cartilage defects has been recognized since the famous statement by William Hunter in 1743 that “from Hippocrates down to the present age, we shall find that an ulcerated cartilage is universally allowed to be a very troublesome disease; that it admits of a cure with more difficulty than a carious bone; and that, when destroyed, it is never recovered”. The sequelae of these lesions have also been demonstrated by Key (1931) using experimental models. The general consensus is that such lesions if left untreated will ultimately lead to the development of osteoarthritis (OA). In the older patient, arthroplasty is commonly offered when the OA is severe. However, in the younger patients where arthroplasty is not appropriate, the solution to this problem constitutes a nightmare to the orthopaedic surgeon.

There has been a long search for suitable materials that can be used as substitutes in the treatment of these debilitating lesions. The search has been frantic in the past 50 years and during this time frame, numerous graft materials have been suggested and tried with varying degrees of success (Minns *et al* 1995). The ultimate long-term aim is to achieve regeneration of a normal articular cartilage (i.e. the exact tissue) but the reality is that, to date, only attempts at repair has been achieved (i.e. a substitute material). A variety of approaches have been adopted to promote cartilage repair in focal full thickness cartilage defects. Successful stimulation of some sort of repair has been achieved in animal models and although some have been adapted for clinical practice, a good number have profound limitations. The restoration of FTCD can be achieved by one of three methods:

- (1) Osteochondral grafting
- (2) Stimulation of repair
- (3) Cartilage tissue engineering.

1.2.3.1 Osteochondral grafting.

This method of treatment involves the removal of articular cartilage with its underlying subchondral bone and then grafting this composite tissue into an already prepared recipient site i.e. the site of full thickness cartilage defect. The graft can either be an autograft when it is taken from one part of the joint and transplanted into another site in the same patient; or an allograft when the graft is taken from one individual and it is transplanted into a recipient site in another individual. If the transfer of a tissue is to a site where its function would be the same as its site of origin, the recipient site must provide optimum conditions for the survival and the physiological function of the transplant (Pap and Krompecher 1961). Some authors favour the use of autogenous osteochondral (OC) grafts (Pap and Krompecher 1961, Campbell *et al* 1963, Matsusue *et al* 1993, Roffman 1995, Outerbridge *et al* 1995, Bobic 1996, Hangody *et al* 1997, Hangody *et al* 1998) and all have reported excellent results.

Summary.

Advantages of autogenous osteochondral grafting:

- reliability of bony union.
- high survival rate of the grafted hyaline articular cartilage.
- no threat of rejection or disease transmission. (Matsusue *et al* 1993).

Disadvantage of autogenous osteochondral grafting:

- donor site morbidity.

- limitation of graft size.

The use of allograft osteochondral (OC) grafts is highly favoured by yet another group of surgeons (Wagner 1972, Meyers *et al* 1989, Convery *et al* 1996) who have equally reported excellent results.

However, to date, there is no evidence for successful, unlimited ingrowth and survival of allogeneic cartilage transplant (Wagner 1972) and moreover this method of treatment is not without apparent complications.

Summary:

Advantages:

- provision of large or abundant graft when required.

- no donor site morbidity.

Disadvantage:

- rejection of graft (Pap and Krompecher 1961, DePalma *et al* 1962,

Stevenson 1987, Stevenson *et al* 1989)

- storage and preservation of grafts increases its chances of rejection

(Stevenson *et al* 1989)

- disease transmission (Guhl *et al* 1991)

- degenerative changes in the cartilage mantle of the allograft (DePalma *et al* 1962)

1.2.3.1.1 Osteochondral shell grafting.

The use of osteochondral grafts with at least 3mm to 8mm of the subchondral bone is the

most popular choice but there are also advocates for the use of osteochondral shell allografts (Pap and Krompecher 1961, Wagner 1972, Chu ICRS Boston 1998). The shell consist mainly of articular cartilage with a very thin sliver of underlying subchondral bone and in some instances the amount of bone grafted is so small that it would appear to be an intact cartilage transplantation. Osteochondral shell may be an autograft or an allograft but the results obtained are not substantially better compared with the use of a graft with a larger subchondral bone. The complications and the precautions required are just as rife as for osteochondral grafting.

1.2.3.2 Stimulation of repair.

Cartilage or Chondrocyte transplatation.

It would appear very logical to replace articular cartilage defects with identical material. Afterall like begats like. This has formed the basis for the use of cartilage and cells derived from cartilage in the repair of full thickness cartilage defects (FTCD).

1.2.3.2.1 Intact articular cartilage:

Hjertquist and Lemperg (1972) grafted FTCD in the femoral heads of adult rabbits with intact costal cartilage from the 6th rib. The costal cartilage has been shown to be a suitable graft material because it is pre-stressed and the matrix is in a state of varying tension (Gibson 1965). However in all the cases reported by Hjertquist and Lemperg (1972), degeneration of the articular surface of the graft was also observed. This complication has been previously reported by Gibson (1965). Unfortunately, the degeneration is not confined to the graft or transplant tissue alone but it also affects the

neighbouring well-restored articular cartilage. The poor result obtained with the transplantation of intact cartilage has made this procedure unpopular. However, the use of foetal chondral allograft has been shown to produce satisfactory results in experimental models (Specchia *et al* 1996) but this procedure as a whole still has limited use in clinical practice.

1.2.3.2.2 Epiphyseal-derived chondrocytes transplantation;

Bently and Greer (1971) obtained isolated chondrocyte homografts following enzymatic dissociation of the femoral condylar articular and the metatarsal epiphyseal cartilage of young New Zealand White (NZW) rabbits. They then grafted FTCD in the lateral tibial articular surfaces of another set of NZW rabbits with the isolated chondrocytes. Eight weeks following grafting, they observed that the defects treated with epiphyseal-plate derived chondrocytes were completely filled with cartilaginous tissue which grossly resembled normal articular cartilage. On the other hand, defects treated with articular-cartilage derived chondrocytes healed by fibrocartilage with signs of graft rejection as well. They concluded that epiphyseal-plate derived chondrocytes were preferred as graft materials. This conclusion is probably not appropriate because homografts were used and the results obtained with the articular cartilage-derived chondrocytes may be due to the rejection.

Itay *et al* (1987) have also used homologous epiphyseal-derived chondrocytes in biological resorbable immobilisation vehicle (BRIV) to treat FTCD in Leghorn roosters with remarkable success. Their rationale for the use of embryonal epiphyseal

chondrocytes with a higher mitotic rate and embedding these cells in a relatively immunogenically tolerant extracellular matrix was that this composite tissue and cells would provide an adequate cellular mass to repair the defect. The concept of using epiphyseal-derived chondrocytes as outlined above (Bentley and Greer 1971 and Itay *et al* 1987) are plausible but the ethical problems that surrounds this method will be enormous apart from the risks of rejection.

1.2.3.2.3 Normal articular cartilage-derived chondrocyte transplantation;

Chesterman and Smith (1968) treated FTCD in the humeral heads of Dutch rabbits with chondrocytes obtained by enzymatic dissociation of slivers of normal cartilage or slivers of normal cartilage pre-treated with papain. They observed that the defects treated with isolated chondrocytes alone were partly filled by synovial membrane and fibrocartilage. The floor of the defects treated by papain-softened cartilage glistened in places as though new cartilage had been formed and histology of the repair tissue confirmed hyaline like cartilage and fibrocartilage formation. On the basis of their findings, they proposed the possibilities of therapeutic chondrocyte transplantation and cartilage banking as a back-up.

The poor result obtained by Chesterman and Smith following isolated chondrocyte transplantation could be due to: (a) senescence of the few surviving transplanted chondrocytes in the recipient sites (since the chondrocytes were not expanded in culture); (b) inability of the surviving chondrocytes to mount enough metabolic response to effect cartilage matrix production and propagation of effective repair and (c) loss of chondrocytes into the joint cavity following transplantation as they did not provide any

restriction to the movement of the chondrocytes.

Peterson *et al* (1984) addressed the above issues with their pioneering experiment on rabbits. First they expanded the enzymatically dissociated chondrocytes by propagating them in monolayer culture, covered the created FTCD with a membrane or tissue (synovium, periosteum, fascia, blood clot or tendon) before injecting the expanded chondrocytes under the membrane or pouch. The successful outcome of this research has been the catalyst of more animal experiments and ultimately clinical application in humans.

Grande *et al* (1989) made significant modification on the technique of Peterson *et al* (1984). Firstly they transplanted relatively large number of chondrocytes (expanded), the average being 1.04×10^6 . This was to ensure that even if some chondrocytes died, there would still be enough cells left to effect repair. Secondly they used autologous periosteum as the covering pouch to contain the implanted cells at the transplanted sites. Although only 25% of the repair tissue following this expanded autologous chondrocyte implantation had excellent results as evidenced by complete healing of the defect, their small sample size makes it difficult to interpret this results. On the whole, the outcome of this study has been encouraging and has formed the basis for its clinical application.

The source of the chondrocytes in the experimental works of Peterson *et al* and Grande *et al* has been from samples of articular cartilage obtained from the osteochondral grafts created. This will be inapplicable in clinical practice since the basis for the research is to find ways of treating such defects. Other sources of chondrocytes have been sought over

the years e.g. bone marrow derived mesenchymal chondrocytes (Butnariu-Ephrat *et al* 1996). Although the authors reported low yield of chondrocyte enriched bone marrow derived cells in the goat study, they reported a 100% yield with human bone marrow.

Brittberg *et al* (1994) reported on the outcome of autologous chondrocyte implantation in the treatment of FTCD in human knees. They relayed the procedure of Grande *et al* but obtained cartilage slices from the upper medial femoral condyles arthroscopically at the first surgery. In clinical practice the autologous periosteal graft was harvested from the upper medial tibia and more recently from the lower femur. Again in clinical practice, the edges of the periosteal pouch are sealed with fibrin glue to prevent leakage of the implanted cells. The biopsies of the repair tissue in about 70% of the patients treated by Brittberg group had intact articular surfaces and a hyaline-like appearance, whereas in about 25% fibrocartilage was the main repair tissue. This laudable treatment modality is expensive, technically demanding and it is not readily available to most patients. The ultimate fate of the defect created during the harvesting of the cartilage slices remains unknown although it is believed that this iatrogenic injury will not lead to any long-term morbidity. In the wake of this reality, research is still continuing to find alternative sources of chondrocytes in clinical practice for use in autologous chondrocyte implantation.

1.2.3.2.4 Three-dimensional culture technique.

Efforts to expand the treatment options for full thickness articular cartilage defect have increasingly focused on the implantation of cell-polymer constructs which are also known as three-dimensional culture system. In clinical practice, a 3-dimensional culture

technique or system for the regeneration of cartilage implants depends on the following:

- (1) cell density
- (2) scaffold porosity
- (3) scaffold degradation rate
- (4) scaffold thickness (Freed *et al* 1993)

High cell density is required for regeneration of compact functional cartilage tissue (Bruckner *et al* 1989). The high chondrocyte density is accompanied by a high glycosaminoglycan (GAG) production. The clinical relevance of this is that the higher the GAG content of the 3-dimensional graft, the less the deformation that occurs when it is subjected to compressive stress (Freed *et al* 1993). Scaffolds with high porosity enhances rapid proliferation of chondrocytes and in addition promotes rapid production of GAG. They provide minimal diffusional constraints during chondrocyte growth. Scaffolds with a high degradation rate enhances chondrocyte proliferation and subsequently promote cartilage regeneration in the 3-dimensional cultures. The main function of the scaffold is to give mechanical integrity in the first 2 to 3 weeks of culture during which time, the proliferating cells elaborate enough extracellular matrix. Subsequently the scaffolds should biodegrade sequentially to permit the regeneration of compact tissue and minimise host inflammatory response following transplantation.

Thin scaffolds are readily resorbable and allow for uniform distribution of chondrocytes and their surrounding extracellular matrix. In addition, chondrocytes and GAG densities have been found to be significantly higher for thin than for thick scaffolds (Freed *et al* 1993).

Freed *et al* (1993) have also suggested that an ideal 3-dimensional culture system should

be based on a small quantity of donor cartilage thereby limiting the damage to the donor site. It is very important to use 3-dimensional scaffolds with clinically relevant dimensions and structures and in practice, these types are more reproducibly formed using synthetic scaffolds. The scaffolds that have been used for 3-dimensional cultures with satisfactory results include:

(a) *Collagen gel* (Yasui *et al* 1982, Kimura *et al* 1983, Wakitani *et al* 1989

Kawamura *et al* 1998).

(b) *Agar gel*. (Bujia *et al* 1993)

(c) *Polyglycolic acid*. (Freed *et al* 1993, Bujia 1995)

(d) *Carbon fibres*. (Brittberg *et al* 1996)

1.2.3.2.5 Scaffolds:

Muckle and Minns (1989) have reported the use of carbon fibre pads to repair full thickness articular cartilage defects in 47 patients having demonstrated successful outcome with animal experiments. They observed that the repair tissue formed following this procedure had smooth conforming surfaces continuous with the adjacent articular cartilage. The frictional resistance of the repair tissue was noticed to be lower than that of fibrillated or degenerate cartilage. No foreign-body reaction was found with the carbon fibre implantation and this raises the possibility that this technique may find a place in the management of full thickness articular cartilage.

Brittberg *et al* (1994) also used carbon fibre pads and rods as implants for the treatment of far more extensive full thickness articular cartilage defects in 37 patients. The mean implanted area was 5.5cm² and they obtained over 80% good or excellent results

according to the clinical evaluation modalities used. The histology of the repair tissue revealed dense carbon fibre-anchored fibrocartilage and again this fibrocartilage was found to exhibit lower frictional resistance than that of degenerate cartilage. The outcome of this study suggests that FTCD in young patients that is considered too large for OC or autologous chondrocyte implantation can be treated with carbon fibre pads or rods.

1.2.3.2.6 Multiple drilling through the subchondral bone.

Multiple drilling through the subchondral bone plate is one of the commonest operative procedures performed world-wide for the treatment of full thickness articular cartilage defects (FTCD). This procedure which was popularised by Pridie (1959) and later by Insall (1967, 1974) relies on the ability of the pluripotent stem cells of the bone marrow differentiating into cartilaginous tissue. Pridie and Insall reported clinical cases in which the resultant repair cartilage were presumed to be hyaline-like, but there were no histological proofs. Mitchell and Shepard (1976) have shown that the early repair tissue following this procedure is indeed hyaline-like but with time (over a period of 12 months) the repair cartilage degenerate. The changes in the repair cartilage were the same whether the lesion was in a weight bearing or non-weight bearing surface. Although Salter et al (1980) attempted to improve the quality of the repair cartilage with the use of continuous passive motion, the findings reported above question the rationale for the continual use of this procedure.

1.2.3.2.7 Subchondral abrasion.

Subchondral abrasion involves the use of a motorized burr to abrade the entire cartilage

mantle through the bone plate down to the subchondral bone where vascularity is just observed. This procedure, which was popularized by Johnson (1986), is also one of the most commonly used methods in the treatment of FTCD. The nature of the repair tissue formed after subchondral abrasion still remains controversial. Kim *et al* (1991) have shown that the repair cartilage following subchondral abrasion is also hyaline-like in the early phase of repair and this is more pronounced with continuous passive motion (CPM) augmentation. Unfortunately, this study period (12 weeks) is too short to make any conclusions regarding the usefulness of subchondral abrasion in the management of FTCD as the behaviour of the repair cartilage can not be validated over time.

1.2.3.2.8 Perichondral grafting.

The ability of the perichondrium to transform into a cartilaginous tissue when transplanted to cover FTCD has long been recognised (Skoog *et al* 1972, Engkvist and Ohlsen 1978, Amiel *et al* 1985). The cartilaginous potential of the rib perichondrium has also been demonstrated to be superior to that of the ear perichondrium. Despite these, the ability of the repair cartilage generated following perichondrial grafting to perform the functions of the articular cartilage that it replaces remains in doubt. Engkvist (1979) evaluated the repair cartilage following free perichondral grafting in two groups of rabbits. In the early group, the repair cartilage was harvested and evaluated between 2 and 8 months following the procedure. In this group, he observed that the repair cartilage was evenly smooth and histologically, the cellular arrangement was found to be haphazard although the cells stained intensely with safranin O. In the late group, the repair cartilage was evaluated between 12 months and 17 months. In this group, he

observed that the repair cartilage was pale, grey, non-glistening and ulceration was noticed in the centre of some of the specimens. Histologically, although the cartilage mantle was still thick and with prominent tide mark, there were hallmarks of moderate to advanced degenerative changes. Bouwmester *et al* (1997) treated FTCD in 88 patients with free costal autologous perichondral graft. They obtained good results in 91% of carefully selected cases with isolated focal defects. In an attempt to improve the durability of perichondrium-derived cartilage, Chu *et al* (1997) obtained perichondrocytes by enzymatic dissociation of perichondrial tissue. The cells were then propagated in a polylactic acid 3-dimensional culture system before transplanting into FTCD in rabbits. The repair cartilage that resulted from this procedure was found to be similar to normal articular cartilage morphologically, histologically, and biochemically.

1.2.3.2.9 Periosteal grafting.

The periosteum is a unique tissue in that it has the potential to exhibit osteogenic and chondrogenic capabilities (Ritsila *et al* 1973). The chondrogenic potential of the periosteum was validated by Rubak (1982). He treated FTCD created in the femoral grooves of 6-month-old rabbits with free autologous periosteal grafts with the cambium layer facing the defects. He was able to demonstrate that the repair tissue showed evidence of cellular proliferation in the cambium layer as early as 4-7 days and that the central portion of the repair cartilage that bore more load resembled hyaline-like cartilage than the other parts of the repair tissue. The morphology of the repair cartilage was maintained for over 12 months with no evidence of degeneration. O'Driscoll *et al* (1988) have even demonstrated that this good result can be improved if CPM was instituted in

the immediate post-operative period. They also observed complete obliteration of the gap between the repair cartilage and the adjoining normal articular cartilage following repair of FTCD. Rubak *et al* (1982) have gone a step further to confirm, using tissue isolation technique, that the cartilaginous repair tissue formed following free periosteal transplantation indeed originated from the periosteum. The chondrogenic potential of the periosteum was utilised clinically by Niedermann *et al* (1985) in the treatment of patients with osteochondritis dissecans (OCD) and osteonecrosis.

1.2.3.2.10 Chondral shaving.

Chondral shaving involves the excision or debridement of the damaged area of articular cartilage leaving as smooth a surface as possible. This procedure is carried out using a powered or motorised shaver and again it is one of the popular methods in the treatment of FTCD (Ewing 1990). The nature of the repair tissue formed after chondral shavings also remains controversial. Kim *et al* (1991) observed that following this procedure, the bed or the remaining cartilage showed evidence of progressive cartilage necrosis characterised by superficial fragmentation and fibrillation. Cloning of the chondrocytes in the remaining cartilage was also noticed and this apparently represents a failed attempt of tissue repair. Electron microscopy also revealed degeneration of the bed of the remaining cartilage with remarkable incongruity of the presumed smooth surface. The lack of repair tissue following chondral shaving may be due to the trauma inflicted on the adjoining chondrocytes by the instrument during the procedure. There does not seem to be any advantage, therefore in using this procedure in clinical practice.

1.2.3.2.11 Microfracture

This technique pioneered by Steadman (1992) to treat FTCD involves breaching of the subchondral bone plate with a sharp pointed awl in place of the high-speed drill. This procedure is embarked upon after thorough debridement of the damaged cartilage down to the bone plate as any remnant unhealthy cartilaginous tissue impairs the formation of a durable repair cartilage. The aim is to allow for the egress of marrow cells to migrate into the defects and the seemingly organised blood clot in and around the lesion paves the way for chondrogenic and osteogenic repair of the lesions. However, he found that the combination of continuous passive motion and microfracture gave a satisfactory outcome after three years even in high level athletes. Although this technique appears to be a refinement of subchondral drilling in that it minimises the damage that can be done with the later method, the repair tissue is still fibrocartilage. Coletti *et al* (1972) have shown that this fibrocartilage is mechanically inferior to that of normal articular cartilage, yet the functional results reported by Blevin *et al* (1998) in high-level athletes in the United States of America following this procedure is very promising.

1.2.3.2.12 Synovial transplantation.

Rothwell (1990) treated FTCD with a composite tissue consisting of synovium, sub-synovial fat and the periosteum. The grafts were anchored to the bed of the defects with sutures and the animals were sacrificed at various intervals up till 2 years. The grafted defects were examined grossly and histologically. At three months, the deeper layer of the repair tissue had undergone metaplasia into variably differentiated fibrocartilage. At one year, the entire repair tissue was mainly fibrocartilage and at two years, the repair

cartilage had deteriorated with areas of degeneration of the fibrocartilage and dedifferentiation into a more fibrous tissue. The short life span and subsequent metaplasia of the initial cartilaginous repair tissue needs to be improved upon for this technique to merit any clinical application.

1.2.3.2.13 Meniscal transplantation.

Shagaldi *et al* (1991) treated FTCD with glutaraldehyde-fixed bovine meniscal xenograft, glutaraldehyde-fixed bovine costal cartilage and viable media stored osteochondral allografts. The repair tissues were assessed grossly, histologically and mechanically at 6 and 12 months. They observed a cartilaginous repair tissue with satisfactory congruency with the adjacent normal articular cartilage at 6 months in lesions that had meniscal xenograft. Costal cartilage xenografts also produced excellent repair tissues at 6 months, but degenerative changes were seen in these lesions at 12 months. Successful allografts were found to be congruent at 6 months with no evidence of degeneration of the repair cartilage. Meniscal xenografts were also observed to be mechanically durable and the repair cartilage that it produces bonded firmly with the host. Sumen *et al* (1995) have reported good results following the treatment of FTCD with autologous meniscus in an animal model. They have suggested the use of frozen allograft menisci in the treatment of these lesions. The follow-up period for the assessment of these results were rather short and the logistics of obtaining and storing harvested human menisci is by no means easy. Therefore the use of this repair tissue in clinical practice, although feasible, will need a lot more research.

1.2.3.2.14 Polyurethane (Polyester) and Polytetrafluoroethylene grafts.

Messner and Gillquist (1993) treated FTCD in the femoral condyles of mature rabbits with either polyester (Dacron) or polytetrafluoroethylene (Teflon) felts. At three months the surfaces of the repair tissues evaluated were irregular. Flakes of these synthetic materials were found in the joints and these resulted in synovitis. Histology of the repair tissue revealed ingrowth of fibrous tissue and bony trabeculae from the underlying bone marrow into the base of the implant and the periphery of the implants. The repair tissue did not integrate with the adjacent cartilage and no hyaline-like cartilage was observed in any of the repair tissues. They concluded that these synthetic materials were not satisfactory substitutes for normal articular cartilage.

1.2.3.2.15 Autologous osteoperiosteal graft.

The use of this composite graft for the repair of FTCD in rabbits as reported by O'Driscoll and Slater (1986) relied on the chondrogenic potential of the periosteum and the mechanical strength of bone. The response was enhanced by the use of continuous passive motion. The neocartilage so generated from the periosteum in turn was endowed with a subchondral bony core, which provided support for the neocartilage. The surface integrity of the repair cartilage was smooth and intact and the neocartilage formed was also observed to be firmly bonded to the adjacent articular cartilage at all interfaces. The authors advocated the use of this "biological resurfacing" technique in clinical practice based on this experimental findings. Although, the basis of their composite graft is very logical, the follow-up period is so short that the long-term survival of the neocartilage particularly to mechanical load remains uncertain.

1.2.3.2.16 Spongialization.

This procedure entails en bloc resection of the diseased articular cartilage with its corresponding subchondral bone leaving the ‘spongiosa’ of the cancellous bone exposed (Ficat *et al* 1979). The rationale behind this procedure, which is an advancement on the Pridie’s operation, was based on the fact that the subchondral bone in longstanding FTCD is dense, ischaemic and of poor quality. The depression created in the articulating surface of the joint following this procedure also serve to protect the repair cartilage formed from excessive pressure arising from the opposing cartilage. This procedure also decompresses the subchondral bone, which is usually very sensitive to pressure transmitted by the softened or degenerate cartilage. In general, the repair tissue following this procedure is poorly organised highly cellular and less fibrous compared to normal articular cartilage. Electron microscopy of the repair tissue confirms that it is mainly fibrocartilaginous. The long-term benefit of this method of treatment remains uncertain.

1.2.3.2.17 Callo-osseous graft.

The concepts of using this technique was based on the hypothesis that medullary callus generates hyaline cartilage prior to being eroded by vessels. This graft is also presumed to have a matrix which provides a sound scaffold for cell migration, proliferation and differentiation and it (matrix) is also rich in natural collagen, growth factors and a secured sound base of cancellous bone. Takahashi *et al* (1995) treated FTCD in the patella groove of the femora of mature Japanese strain rabbits with this technique. The retrieved femora were evaluated histologically, biochemically and by

immunohistochemistry. Histology of the repair cartilage at two weeks revealed the appearance of chondrocytes in the callus part of the graft and in-between the trabeculae. With time, the proliferating chondrocytes had formed a hyaline-like cartilage repair tissue with a tide-mark noted in some sections. The thickness of the repair cartilage tended to diminish with time but there were no episodes of bony substitution of the superficial cartilage layer. Immunohistochemistry of the repair cartilage showed predominantly type II collagen from 8 weeks with some residual staining for types I and III collagens. They concluded that callo-osseous graft appeared to have the potential to repair osteochondral or FTCD and that medullary callus may form articular cartilage rather than bone if placed in an appropriate environment. In clinical practice, the application of this type of technique will be greatly restricted because of the inability to procure callus when needed.

1.2.3.2.18 Osteotomy.

The articular cartilage is resistant to impactive loading and it also shields the subchondral bone from excessive loading. With a degenerate or damaged articular cartilage as occurs in FTCD, this protective effect is lost. This then leads to subchondral sclerosis with concomitant reduction in articular cartilage perfusion and a vicious circle is set in motion. Reduction of subchondral bone sclerosis after high tibial osteotomy has been reported (Maquet 1985). Odenbridge *et al* (1992) carried out high tibial osteotomy for patients with FTCD mainly in the medial compartment of the knees secondary to genu varum deformity. Two years following the osteotomy they observed an improvement in the joint cartilage status in these patients particularly those patients that had over-correction of

their varus deformities. This improvement was evidenced by an increase in the reparative activity of the chondrocytes. Fibrocartilage was the main repair tissue even when there was eburnation prior to the osteotomy. In addition, there was an increase in the neocartilage thickness. The adjoining articular cartilage also had increased cellularity. These findings clearly show that cartilage regeneration occurs following high tibial osteotomy and moreover, this regeneration is correlated with the degree of knee re-alignment after the surgery.

1.2.3.2.19 Drugs.

Joint pain is one of the commonest presenting symptoms in patients with FTCD. Most often, these patients are prescribed one of the many non-steroidal anti-inflammatory drugs (NSAIDs). The choice of which NSAID to use depends on many variables not the least the prescribers preference which is often not based on any scientific facts. There are three types of NSAID depending on their ability to aid cartilage regeneration. There are those which are: (a) capable of stimulating cartilage matrix synthesis for example aceclofenac and tenidap (b) unable to effect any major influence on matrix synthesis for example piroxicam and nabumetone and (c) inhibitors of cartilage matrix synthesis for example indomethacin, ibuprofen and naproxen. Gonzalez *et al* (1994) and Dingle and Parker (1997) have demonstrated that aceclofenac stimulates the synthesis of glycosaminoglycans (GAG) in degenerate articular cartilage. This stimulatory effect is ascribed to the inhibition of the activity of interleukin-1 (IL-1) thus allowing the expression of indigenous growth factor activity. Thus aceclofenac may promote articular cartilage repair in patients with FTCD and this may prove to be a temporary measure at

least prior to the use of one of the more invasive techniques described earlier. The intriguing part in the outcome following the use of aceclofenac will be to ascertain the quality of the 'repair' cartilage by way of biopsy. The use of this NSAID may be preferred to the others if the joint pain is attributed to cartilage defect.

1.2.3.2.20 Gene therapy.

Genetic predisposition has been known to account for some form of abnormality in the extracellular matrix of the articular cartilage (Cicuttini and Spector 1996). This can ultimately lead to FTCD which in turn could progress to osteoarthritis. The advent of genetic engineering now enables the synthesis of therapeutic gene products which can act within or outside the cells to turn on the production of the deficient constituent of the articular cartilage (Evans ICRS 1998). Gene transfer to chondrocytes followed by intra-articular transplantation may allow for functional modulations of chondrocyte biology and enhance repair of damaged articular cartilage. Using this modality of treatment, a gene delivery system such as the viruses can be used to initiate and sustain the release of the needed constituents of the articular cartilage (Baragi *et al* 1997). This method of treatment is still in the experimental stages and it is hoped that a suitable and controlled system that could deliver a sustained release of necessary genetic information will soon be found.

1.2.3.2.21. Chondroproliferative agents.

There has been a lot of suggestion for the use of growth factors to increase chondrocyte proliferation at the site of FTCD. Hepatocyte growth factor (HGF), a multifunctional

factor which promotes proliferation and morphogenesis in epithelial cells, has been shown to play an important role in cartilage metabolism. Wakitani *et al* (1997) created FTCD in rabbit femoral condyles and injected the joints with HGF (saline was injected into the control joints). They evaluated the healing of the defects grossly and histologically. They observed excellent healing in the defects treated with HGF with hyaline-like cartilage compared with the control. They concluded that growth factors may play a complementary role (if not singly) in the repair methods already highlighted.

1.2.3.3 Cartilage tissue engineering.

This system utilises a bio-reactor. A bio-reactor is a system in which isolated expanded chondrocytes can proliferate and elaborate cartilage matrix *in vitro*. The use of a bioreactor (magnetically stirred flasks, air-lift reactors or rotating microgravity bioreactors) enables adequate control and manipulation of the culture system. This system has been used to augment a polyglycolic acid 3-dimensional culture systems to aid the production of bio-artificial cartilage (Ratcliffe ICRS 1998). He was able to demonstrate that the bio-artificial cartilage elaborated cartilage-specific collagen types and the histology of this cartilage was identical to that of articular cartilage. This system is still experimental and expensive but appears very promising for the future.

The fact that there are so many methods proposed for the treatment of FTCD is a reflection that no one treatment method is optimal and moreover no one method is universally accepted. There is no doubt that whilst excellent outcomes will be reported with the use of some methods, a combination of methods may provide the best outcome

for this difficult but real problem.

1.3 Osteophytes

The word ‘osteo’ denotes bone and ‘phyte’ (‘phyton’) in Greek means “to come into being” (The concise Oxford dictionary 1982). This clearly indicates that osteophytes are not a normal integral part at the sites where they are found and that they are not present at birth. Churchills Illustrated Medical dictionary (1989) defines an osteophyte as a bony outgrowth seen most often in osteoarthritic joints and located adjacent to the eroded articular cartilage. It also claims that osteophytes can arise either by endochondral or intramembranous ossification (of a presumably cartilaginous precursor). Aigner *et al* 1995, in a bid to highlight its basic composition defines an osteophyte as a neoplastic cartilaginous and osseous protrusion growing at the margins of osteoarthritic joints. Osteophytes have been called chondro-osteophytes or osteochondrocytes (Fisher 1922, Matyas *et al* 1997) and these synonyms are a reflection of the heterogenous nature of this tissue. In total contrast to the above definition, Harrison *et al* 1953, have described osteophytes as any new bone and marrow formed within a degenerate articular cartilage but this definition does not appear to be popular.

1.3.1 What are osteophytes?

Osteophytes are extremely variable and irregular in shape (Aigner *et al* 1995) and they tend to grow in any area of low joint stress (Harrison *et al* 1953). The presence of marginal or central osteophyte is the earliest sign and an essential radiological feature in

considering the presence or absence of osteoarthritis in a diarthroidal joint (Pottenger et al 1990). Osteophyte formation is presumed to be a response to the subtle changes in the biochemical composition and stiffness characteristics of the articular cartilage (Buckland-Wright *et al* 1991). The presence, number and size of marginal osteophytes have been found to increase as part of an active disease process i.e. along with the progression of the arthritic changes (Buckland-Wright *et al* 1991). These along with other changes in the joints such as subchondral sclerosis, subchondral cyst, altered articulating ends of bone and the presence of periarticular ossicles formed the basis for grading osteoarthritis as none (Grade 0) to severe (Grade 4) (Kellgren and Lawrence 1957). Moreover, the presence of osteophytes in a joint more than any other pathological feature distinguishes osteoarthritis from other arthritides (Matyas *et al* 1997, Jeffrey 1975). Osteophytes appear to be an intrinsic feature of osteoarthritis (OA) and this may suggest that osteophyte formation may be caused by the same factors leading to OA, yet osteophytes are not consistently more common or more pronounced in severe OA (Resnick *et al* 1991).

1.3.2 Composition of osteophytes.

Morphogenic heterogeneity is a hallmark of mature osteophytes, but basically an osteophyte is thought to be composed of:

- a) mesenchymal fibrous connective tissue
- b) fibrocartilage,
- c) hyaline cartilage,
- d) hypertrophic cartilage and

e) bone (Colin 1949, Aigner *et al* 1995, Jeffery 1975, Matyas *et al* 1997,).

The mesenchymal connective tissue occupies the superficial layer beneath which are located rounded fibrocartilaginous cells. Deep to this is the hyaline cartilage zone with characteristic round Saffranin O and Toluidine blue strongly positive cells. Deeper still is a zone of rounded hypertrophic chondrocytes some of which show degenerative changes with signs of cartilage calcification and vascularisation in its innermost layer. The hypertrophic cartilage layer lies on top of trabecular bone, which is continuous with the juxtar-articular bone of the adjoining articular cartilage. (Aigner *et al* 1995, Jeffery 1975, Harrison *et al* 1953). Fibrocartilage is thought to account for most of the cartilage component of osteophytes and it is this zone that is continuous with the adjacent articular cartilage (Aigner *et al* 1995). The cells of the fibrocartilaginous zone are morphologically transitory between the fibrous mesenchymal connective tissue and hyaline cartilage cells (Aigner *et al* 1995).

1.3.3 Sites of predilection.

Joints vary in their susceptibility to the formation of osteophytes. The hip joint for instance is more prone to osteophytes formation than the other large joints of the body (Jeffrey 1975, Resnick *et al* 1991). Neuropathic joints on the other hand are notorious for forming very large osteophytes in the early stages of the degenerative disease (Figure 4) which is usually rapidly progressive (Anderson 1985). Presumably, the lack of pain sensation leads to unrestricted and unguarded loading of these joints with subsequent acceleration of the degeneration and therefore osteophyte formation (Knaggs 1932). Osteophytes have been found to develop in some joints following massive autogenous

osteocondral graft. This has been attributable to minor degenerative changes which accompany these types of grafts. Osteophyte formation has been shown to be more pronounced with allografts, especially frozen allografts (Stevenson *et al* 1989).

1.3.4 Types of osteophytes.

In clinical practice, two types of osteophytes are recognisable: marginal and central (Pottenger *et al* 1990). They are both commonly associated with degenerative joint diseases and their presence in and around the joints gave rise to the older terminology for osteoarthritis : ‘hypertrophic arthritis’.

1.3.4.1 (a) Marginal osteophyte (MO) (Figure 5).

This type of osteophyte, also known as border osteophyte (Key 1931) is formed in the margins of the joints at the point where the outer edge of the articular cartilage is continuous with the synovium, periosteum or perichondrium. Thus, it arises from the non-load-bearing or low contact stress region of the joint (Resnick *et al* 1991). In osteoarthritic joints, marginal osteophytes often merge with or overgrow the original articular cartilage (Fisher 1922, Jeffrey 1975, Aigner *et al* 1995). Characteristically, marginal osteophytes tend to grow into the “free” part of the articular or joint space thereby following a path of least resistance (Jeffrey 1975, Harrison *et al* 1953). The prominence and sizes of marginal osteophytes, along with other radiological parameters have been used in the classification of the severity of osteoarthritis in some joints of the body as earlier stated (Kellgren and Lawrence 1957).

1.3.4.2 (b) Central osteophyte (CO) (Figure 6).

This type of osteophyte which is thought to be uncommon, is also erroneously known as “flat” osteophytes. Unlike marginal osteophytes, central osteophytes are predominantly articular. They usually present as slightly elevated ridges within degenerating articular cartilage where reparative tissue is seen overlying pre-existing necrotic cartilage. On plain radiographs, central osteophytes are button-shaped excrescences with a sharp margin and they are continuous with the underlying bone. The origin and growth of this osteophyte has been shown to depend on the proliferation of blood vessels into the degenerate articular cartilage from the subchondral bone marrow (Harrison *et al* 1953). The necrotic cartilage tissue acts as a nidus for calcification giving rise to central osteophytes (Bullough 1997). Therefore, central osteophytes are thought to arise from endochondral ossification in the remnant of hyaline cartilage in the degenerating joint but their pathogenesis has been considered to be similar to that of marginal osteophyte (Ahlback 1968). Central osteophytes are often encountered in the intercondylar region of anterior cruciate deficient knees where they presumably arise because of the instability of the joint and subsequently repeated impact between the intercondylar region and the tibial spine. Abraham-Zadeh *et al* (1994), reported an incidence of 9.8% for central osteophytes on plain radiographs and 14.3% on MRI. They found that central osteophytes (CO) were associated with marginal osteophytes (MO) in 95% of cases, and with a predilection for the medial femoral condyles in the knee. In over 70% of cases, they were able to demonstrate overt damage to the articular cartilage underlying the central osteophytes. They also found that the average height of the COs was 1.5mm, and also that larger COs are associated with larger MOs (Figure 7).

1.3.4.3 (c) Capsular osteophytes.

This type of osteophyte is commonly found in atrophic or neuropathic joints such as Charcot's joints. In these joints, extensive destruction of the joint which arises from the withdrawal of atrophic function, leads to greater destruction of the articular cartilage and bone (Knaggs 1932). This causes extensive flaking of the articular cartilage into the synovial fluid and these flakes form cartilaginous nodules which subsequently undergo calcification. Lloyd-Roberts (1953) referred to this type of osteophyte as chondromata as he strongly believed that osteophytes arose from intra-articular cartilage debris. Capsular osteophytes as the name implies, are commonly attached to the capsule, but sometimes they may break off and become free in the joint giving rise to loose bodies (Knaggs 1932)

1.3.4.4 (d) Epiarticular osteophytes or Buttreassing (Figure 8).

This type of osteophyte is common in the medial and the posterio-medial surfaces of the head of the femur. They are thought to arise as subperiosteal bone caused by the elevation of the periosteum away from the neck of the femur by a traction force transmitted through the posterior-inferior capsular reflection in the neck of the femur (Lloyd-Roberts 1953). Jeffrey (1975) described epi-articular osteophytes as the growth of a marginal osteophyte to involve the surface of the femoral head. This growth can either be cranial when it arises from the peripheral margin of the femoral head or caudal when it arises from the margin of the fovea capitis. In either instance, the original cartilage in the femoral head becomes buried underneath the osteophyte and is partly eroded by bone

(Jeffery 1975).

1.3.4.5 (e) False or pseudo-osteophytes.

Not all bony outgrowths or protrusions at the margins of joints are osteophytes.

Enthesophytes are protrusions arising from the bony attachments of joint capsules (Figure 9) and articular ligaments (Resnick *et al* 1991). They arise from tractional forces e.g. the 'seagull' sign in the interphalangeal joint of the fingers and the bony spike found at the tibial attachment of the anterior cruciate ligament (Resnick *et al* 1991).

Syndesmophytes are bony protrusions found at the sites of attachment of ligaments but they do not have cartilage caps nor do they have any bearing with the joint margins. They are strictly extra-articular (Resnick *et al* 1991).

1.3.5 Potential causes of osteophytes.

A variety of factors have been implicated in the genesis of osteophytes, but as it stands, no single pathway appears to be universally accepted. It is quite possible that a combination of these possible causes comes into play in the formation of this unique tissue.

1.3.5.1. Mechanical stresses.

Mechanical stresses when applied to connective tissue cells stimulates intracellular metabolism. The type of response depends on the type of stress applied. Shear stress for instance has been shown to stimulate mesenchymal cells to produce fibrous tissue; dilatation stress apparently promotes the formation and maintenance of cartilage while

compression/tensional stresses stimulate new bone formation (Resnick et al 1991).

Mechanical stresses caused by joint instability, for example anterior cruciate ligament (ACL) disruption (Marshall 1969, Gilbertson 1975) may cause stretching of the synovial membrane and capsular insertion and/or shear stress between the articulating bones. This in turn may trigger fibrocartilaginous proliferative activities in the marginal zone. This marginal zone represents the area marked by the transition from articular cartilage to synovium and periosteum. Endochondral ossification of the ensuing fibrocartilaginous bud gives rise to the bony core of mature osteophyte. This theory is plausible because immobilisation of the joints of experimental animals after ACL transection prevented osteophyte formation (Resnick *et al* 1991).

1.3.5.2. Intra-articular debris.

Degenerate articular cartilage has a tenuous connection with the deeper layers and it is therefore prone to being dislodged into the joint (Jeffery 1975, Lloyd-Roberts 1953). The debris so formed can not be digested by the synoviocytes and they either lie on the surface or are buried within the synovial membrane. The presence of this debris results in synovial hyperplasia and consequently synovial and capsular fibrosis (Lloyd-Roberts). The progressive fibrosis exerts relentless traction on the synovial reflection at the marginal zone triggering fibrocartilaginous proliferation and osteophyte formation. There is therefore an overlap with the mechanism of osteophyte formation as a result of mechanical stress. Experimentally, intra-articular injection of cartilage homogenates has been found to lead to osteophyte formation (Resnick *et al* 1991). This possibility may be discounted however, because as shown by Marshal (1969) and Gilbertson (1975),

1.3.5.5. Compensatory hyperplasia.

The loss of articular cartilage (and chondrocytes) in the central area has been shown to induce compensatory chondrocyte proliferation and concomitant hyperplasia in the lateral or marginal area (Fisher 1922, Key 1931, Fisher 1939). This hyperplasia appears to pave the way for the formation of marginal osteophytes. This phenomenon is also thought to occur in the surviving chondrocytes adjoining the degenerate cartilage even in the central areas. The hyperplastic cartilage now covers the degenerate cartilage leading to the formation of central osteophytes (Resnick *et al* 1991).

1.3.6 Pathogenesis of marginal osteophytes.

Although the formation of osteophytes is nearly always accompanied by loss of hyaline articular cartilage, clinical observations and experimental studies of animal models have not yet demonstrated a direct relationship between the formation, number and/or size of osteophytes and the extent of articular cartilage damage (Marshall 1969, Gilbertson 1975). Osteophyte formation has been shown to correlate with aging and not necessarily as an early sign of OA (Moskowitz and Golberg 1987). Pathological occult articular cartilage damage has been shown to be evident histologically as early as the third decade of life in the absence of any joint dysfunction (Fisher 1939). This finding has been confirmed by the changes in the gross morphology and composition of apparently normal articular cartilage with age (Bullough 1997). The earliest detectable proliferative changes in the knee of man has been shown to occur in the marginal area long before articular cartilage erosion or subchondral bone sclerosis occurs (Marshall 1969). This finding which was confirmed in animal experiments (Marshall 1969, Gilbertson 1975) suggest

that there may be a population of pluripotential cells at the marginal zone which are responsive to mechanical, inflammatory, angiogenic and humoral stimuli. Numerous histopathologic evidence supports the fact that osteophyte formation is preceded by fibrocartilaginous proliferation in the marginal zone which is then followed by endochondral ossification at its base as a result of vascular invasion (Key 1931, Fisher 1922 and 1939, Marshal 1969, Gilbertson 1975, Jeffrey 1975, Lloyd-Roberts 1953, Aigner *et al* 1995, Matyas *et al* 1997) (Figure 10).

Key (1931) created full thickness articular cartilage defect (FTCD) in the central area of the femoral condyles of mature rabbits and observed cartilaginous nodules at the lateral region of the condyles. Subsequent bony infiltration of these cartilaginous nodules from their bases lead to the formation of what he then referred to as cartilaginous exostosis or border osteophytes. Bennett and Bauer (1932) created FTCD in the femoral condyles of adult dogs and observed that from 4 weeks following the creation of the defects, all the joints had measurable effusion, with associated synovial hypervascularity, hyperplasia and moderate lymphocytic and phagocytic infiltration. They found that osteophyte formation at the margins of the condyles were preceded by the formation of raised, scalloped nodular cartilaginous proliferations and that these elevations were due in part to the formation of new subchondral bone. They concluded that the formation of marginal osteophyte was accompanied by degeneration and atrophy of articular cartilage in the more central area of the articular cartilage. Fisher (1922, 1939) observed that the lateral region of articular cartilage was better nourished than the central area and that this region is covered by a thin layer of highly vascularised villous synovial tissue. He and other

investigators (Caladruccio and Gilmer 1962) noted that in response to FTCD in the central area of articular cartilage, the lateral region exhibited compensatory hyperplasia which ultimately lead to the formation of marginal osteophytes.

Occult articular cartilage damage without gross morphological changes may appear histologically as a change in the appearance and distribution of chondrocytes within the cartilage mantle (McDervitt *et al* 1977). The damaged chondrocytes are thought to release certain chemicals and hormones which then alter the pH of the tissue and the synovial fluid (Resnick *et al* 1991, Miller 1996) causing a disruption in the structure of the articular cartilage and ultimately trigger the formation of osteophytes. However, McDervitt *et al* (1977) believe strongly that osteophyte formation and biochemical changes in the articular cartilage are concurrent rather than sequential processes. Colombo *et al* (1983) and Pelletier *et al* (1995) have demonstrated that paramethasone acetate and triamcinolone both glucocorticoids markedly reduced the incidence and severity of osteophytes formation in the knee joints of experimental animals. Colombo *et al* (1983) have also observed that the administration of pirofen, a non-steroidal anti-inflammatory drug (NSAID) also led to a decrease in the size but not the incidence of osteophytes. Presumably, the anti-inflammatory properties of the glucocorticoids and the NSAID resulted in the lower incidence and number of osteophytes. This hypothesis is unlikely because tribenoside, a sclerosing agent and tamoxifen, an anti-oestrogen both cause a considerable and dramatic reduction in the number of osteophytes. Since these drugs have very few properties in common, Colombo *et al* (1983) concluded that it was impossible to assign a mechanism of action for these drugs and that these actions are

probably not related to the anti-inflammatory properties alone. On the other hand, Pelletier *et al* (1995) have suggested that the action of triamcinolone hexacetonide in reducing the formation of osteophytes may be due to a protective effect of this drug on the articular cartilage lesions.

Osteophyte formation has long been observed to occur frequently in patients suffering from gout. The involvement is not confined to the gouty joints alone and this phenomenon is more prevalent in obese gouty patients (Resnick *et al* 1991). Insulin is a potent growth factor for connective tissue. Insulin-resistant type-II diabetics have diminished availability of insulin and this has been shown to lead to a decrease in osteophyte formation. This reduction may be due to the attenuation of the chondrogenesis and osteogenesis required for osteophyte formation (Resnick *et al* 1991, Horn *et al* 1992, Schouten *et al* 1993). These findings add to the credibility of inflammatory and hormonal manipulations as essential factors in the pathogenesis of osteophytes.

Synovial hyperaemia and hyperplasia are common features of primary OA both in humans (Harrison *et al* 1953, Lloyd-Roberts 1953) and in experimental OA (Key 1931, Bennet and Bauer 1932, Fisher 1939). This vascular response is believed to occur in parallel rather than precede osteophyte formation (Moskowitz and Goldberg 1987). This, (vascular response) along with mechanical stresses, articular cartilage damage and the subsequent release of chemicals and humoral transducers may trigger off the formation of osteophytes in the marginal zones of the articular cartilage. The earliest histological appearance of marginal osteophyte following anterior cruciate ligament transection consisted of a focal collection of mainly spindle-shaped fibroblast-like cells in the

marginal area (McDervitt *et al* 1977). This is then followed by differentiation into a mixture of woven bone and chondrocytes as early as two weeks after experimentally induced OA (McDervitt *et al* 1977, Moskowitz and Goldberg 1987). These early osteophytic spurs are composed mainly of cartilaginous tissue with fibrocartilage in the upper zone and hyaline cartilage at the base. As the osteophyte matures, endochondral ossification of part of the basally located hyaline cartilage occurs and the resultant bony tissue has a trabecula pattern at this stage (McDervitt *et al* 1977, Moskowitz and Goldberg 1987). Key (1931) also observed that in some instances, ossification of the cartilaginous spur began in the center as opposed to the base.

Irrespective of the trigger factor, the final common pathway in the formation of osteophyte appears to be the proliferation of the fibrous mesenchymal or synovial tissue in the marginal zone, chondrogenic differentiation of the resultant hyperplastic mesenchymal tissue and ultimately the formation of fibrocartilage. This fibrocartilage then differentiates into hyaline cartilage, part of which further differentiates and hypertrophies to form hypertrophic chondrocytes (Figure 11) and some ossify to form the trabeculae bone of osteophytes (Figure 12) (Key 1931, Marshal 1969, Gilbertson 1975, Resnick *et al* 1991, Aigner *et al* 1995, Matyas *et al* 1997). This pathway constitutes a differentiation gradient with a fibrous mesenchymal superficial layer followed by a gradation of cartilage differentiation in the deep layer (Aigner *et al* 1995). The chondrocytes in the fibrocartilage zone (the uppermost part of the deep cartilage layer) has been shown to retain the ability to undergo cellular proliferation and differentiation even in mature osteophyte (Jeffrey 1975, Matyas *et al* 1997). In all, the various cells and

tissue types and their organisation in osteophytes appears to correlate with those seen in normal developing foetal epiphysis (Aigner *et al* 1995) and fracture callus (Matyas *et al* 1997).

Since osteophytes are homogenous tissues consisting of fibrous tissue, fibrocartilage, hyaline cartilage and bone (Key 1931, Fisher 1939, Marshal 1969, Gilbertson 1975, Aigner *et al* 1995, Resnick *et al* 1991), it is possible that mechanical forces may play a role in shaping the differentiation of the various regions of osteophyte (Figure 12). The interplay of the various causes of the mechanical stresses at various joints of the body and at different parts of the same joint is probably responsible for the wide variety of osteophytes encountered in clinical practice (Figure 13).

1.3.7 Clinical relevance of osteophytes.

Osteophytes are considered to be failed attempts by the body to repair or remodel degenerating articular cartilage (Fisher 1922, Harrison *et al* 1953, Jeffrey 1975, Aigner *et al* 1995). In general, osteophyte formation is limited to those areas within the joints that do not transmit weight. Moreover, osteophytes arising from the margins of diarthroidal joints often merge with or overgrow the original articular cartilage. Sometimes, the cartilaginous surface is similar to that of the normal articular cartilage and in these situations, the osteophytes may form part of the articulation of the joint (Fisher 1922, Aigner *et al* 1995). Pottenger *et al* (1990) demonstrated that marginal osteophytes are essential in the stabilization of severely arthritic knee joints by reducing varus-valgus instability, although this is more relevant with relatively large osteophytes. However, in

clinical practice, osteophytes are generally implicated as important causes of local joint pain, deformity, nerve compression and restriction of joint movements (Anderson 1985, Fisher 1922, Jeffery 1975, Moskowitz and Goldberg 1987) and its retention has been known to cause post-operative pain following total knee arthroplasty (Dennis and Channer 1992).

Aims of the study.

Osteophytes are osteochondral intra-articular outgrowths produced in response to a full thickness damage of the articular cartilage (Key 1931, Fisher 1939, Petterson *et al* 1984). Osteophytes have therefore been presumed to be repair tissues (Aigner *et al* 1995). To fulfill this role, osteophytes must have some similarities to the normal articular cartilage (cellular and morphological) and at the same time have proliferative capabilities.

The cartilage mantle of osteophytes comprises of two types of chondrocytes as determined by the isoforms of type II collagen expressed (Matyas *et al* 1997). Type IIA collagen positive cells, located in the superficial layer were considered to be embryonic-like whereas type IIB collagen positive cells located in the deeper layer of the osteophytic cartilage mantle were considered to be mature. This observation implies that a population of chondrocytes within an osteophyte may have the potential for rapid proliferation and/or differentiation. Since osteophytic chondrocytes have the potential to proliferate and differentiate, the possibility exists that these properties could be utilized for articular cartilage repair. This study was conducted to evaluate these possibilities.

The ultimate aim of this project is to determine whether osteophytes could be used either as an osteochondral graft or as a source of expanded culture chondrocytes in the repair of full thickness articular cartilage defects. To address the aim of this project therefore, four main aspects have been studied:

- 1) to ascertain the relationship between full thickness articular cartilage defects (FTCD) and the production of osteophytes by carrying out a prospective epidemiological evaluation;

- 2) to compare the mechanical and ultra-structural properties of osteophytes and normal articular cartilage and to determine whether intact osteophytes would be mechanically suitable as an osteochondral graft;
- 3) to determine the sites of “activation” within osteophytes by investigating the location of the native osteophytic collagen and oncogene product synthesis by immunocytochemical techniques;
- 4) to evaluate the cellular and biochemical properties of osteophytic chondrocytes in two- and three-dimensional tissue cultures with a view to utilizing cultured osteophytic cells as alternative source of chondrocytes in autologous chondrocyte implantation.

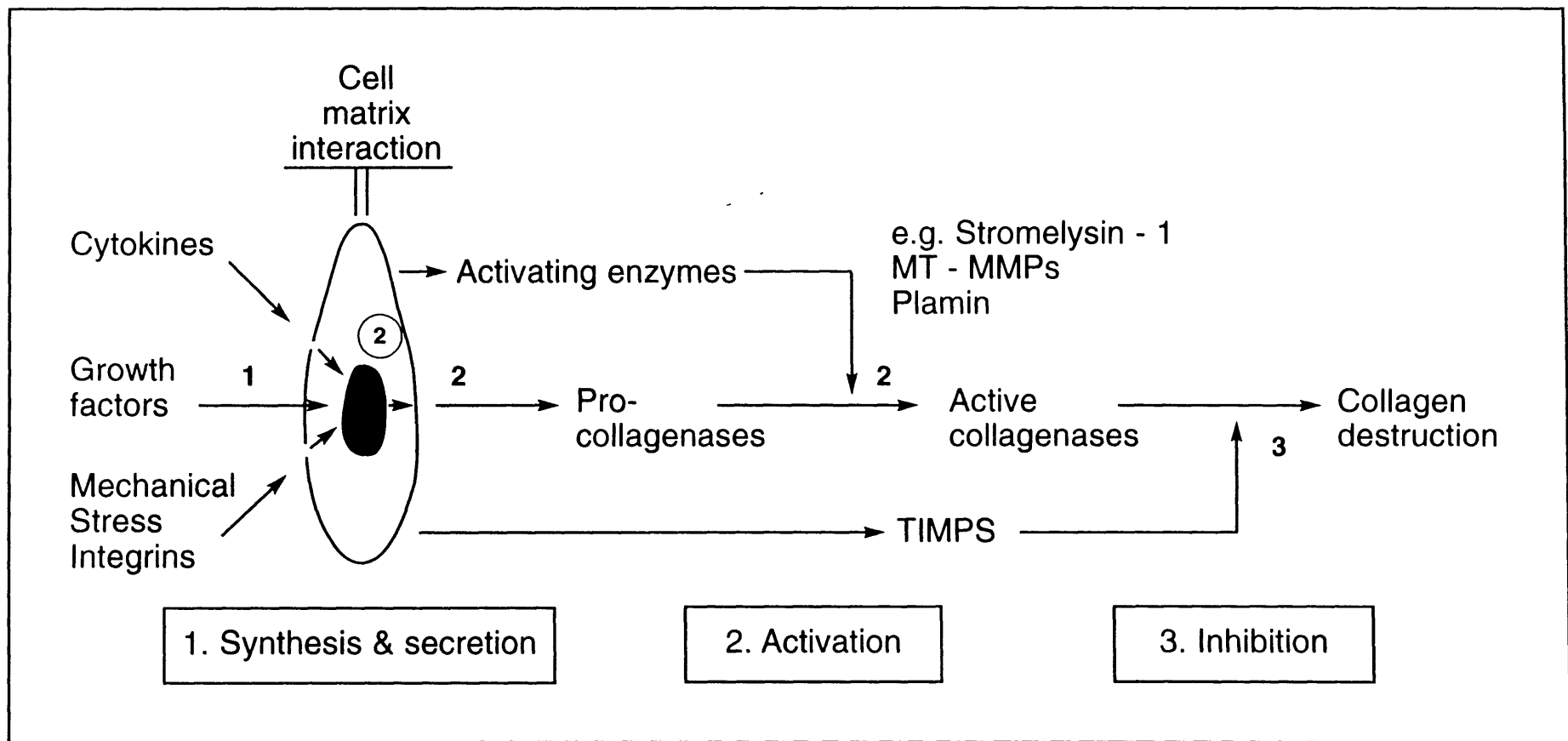


Figure - 1 Collagen breakdown in Normal Articular Cartilage

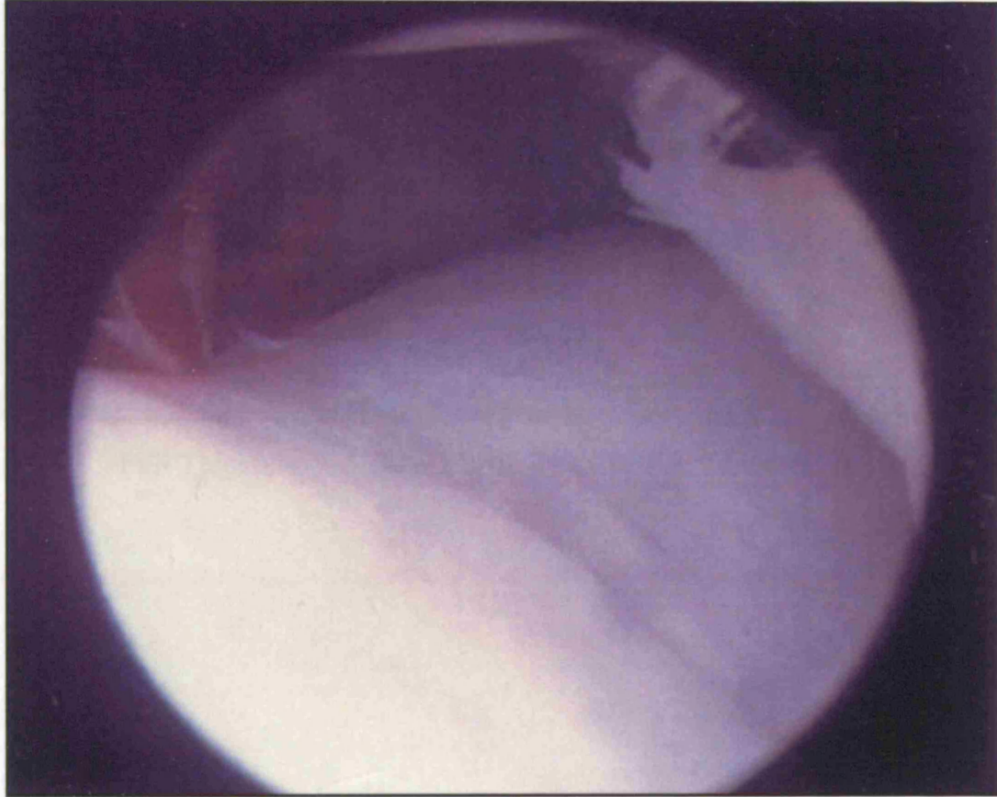


Figure 2 - Partial Thickness Articular cartilage Defect in the trochlear (arthroscopic picture)

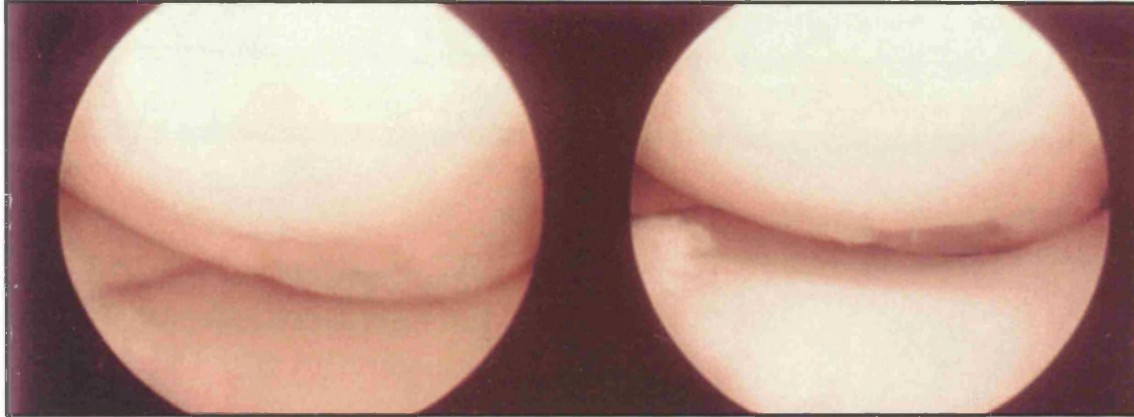


Figure - 3 Full Thickness Articular Cartilage Defect in the lateral femoral condyle

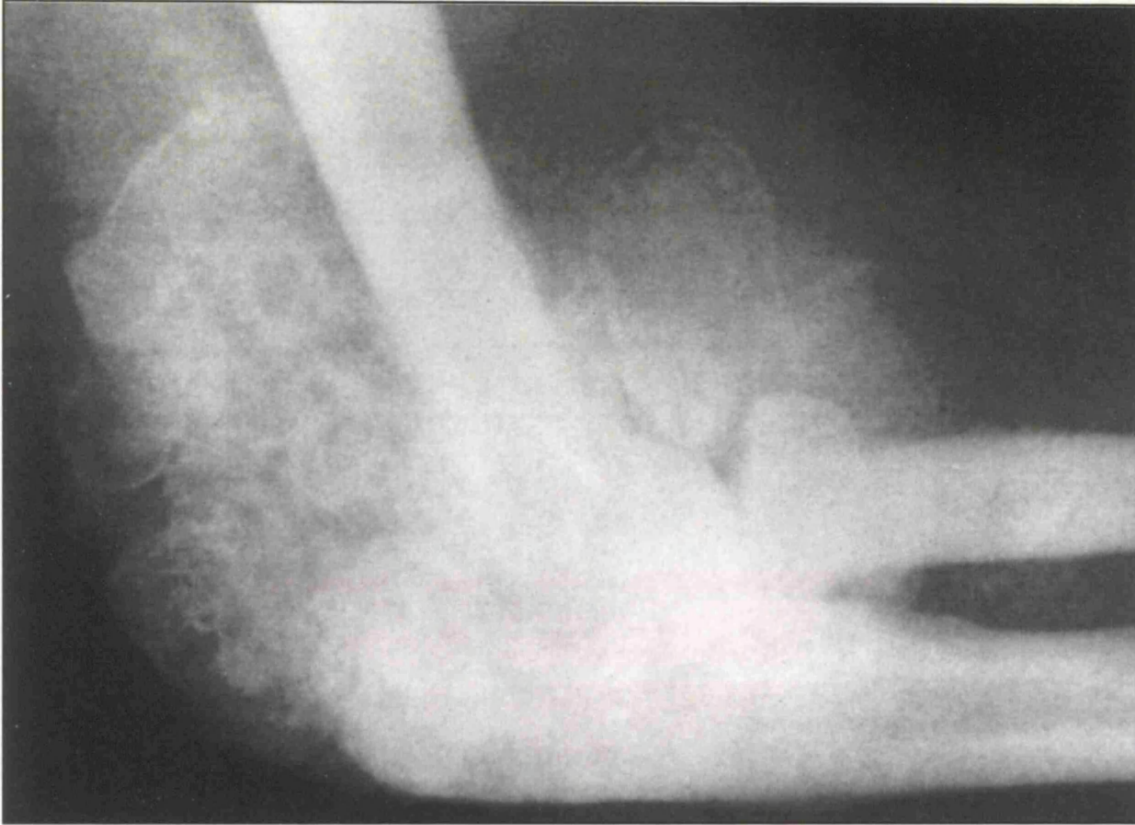


Figure - 4 Osteophyte formation in a neuropathic joint (elbow).
(*A Textbook of Radiology and Imaging, vol:1, Sutton. D., 1993*).

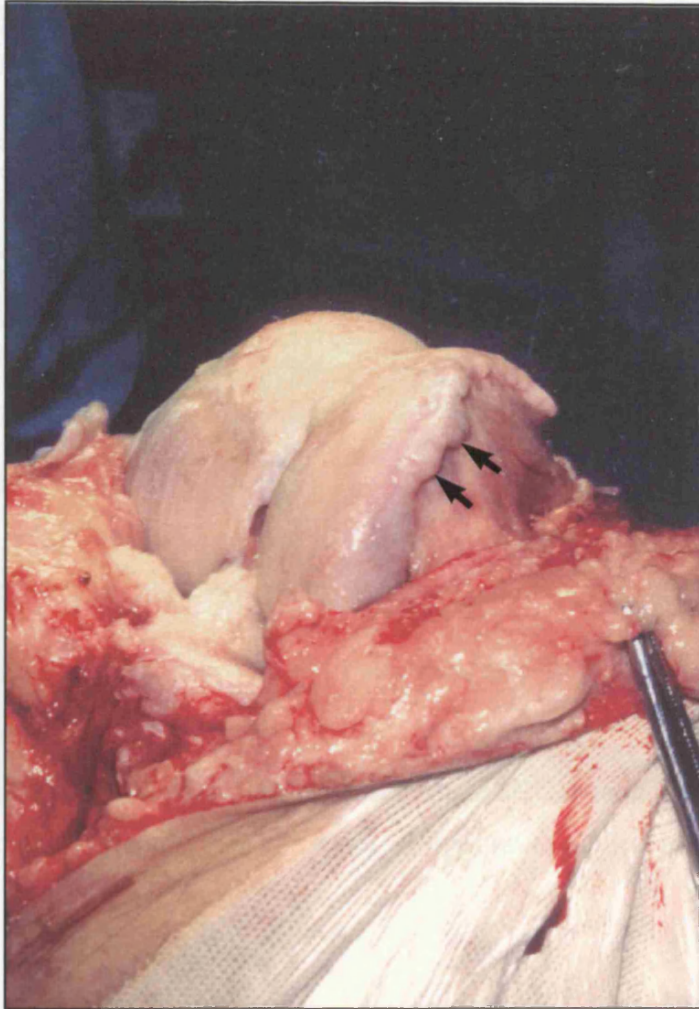


Figure - 5 Photograph of a Marginal Osteophyte.

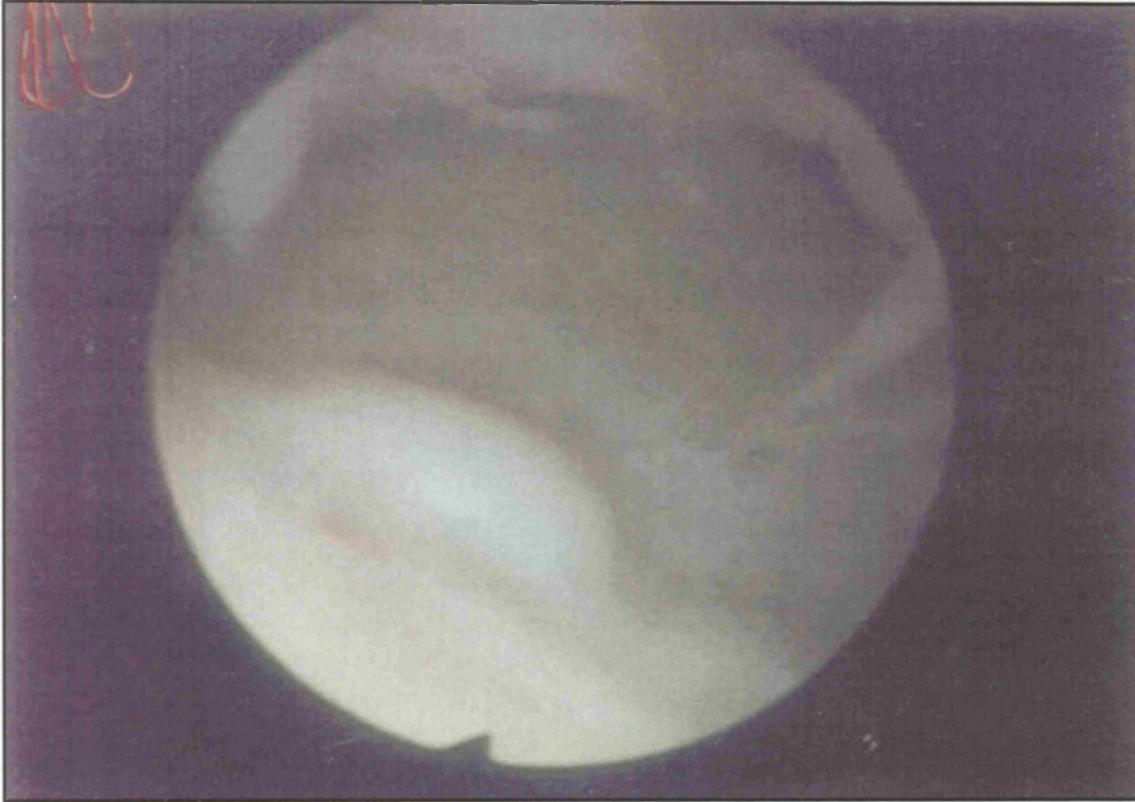


Figure 6 - Central Osteophyte in the medi al femoral condyle (arthroscopic picture)



Figure - 7 Central osteophyte medial femoral condyle (*Bone and Joint Imaging, Resnick. D., 1989*).



Figure - 8 Epiarticular osteophyte or buttressing around the femoral neck (arrows).
(*Textbook of Diagnostic Imaging. Putman C. E. and Ravin C. E., Ed), 1994).*

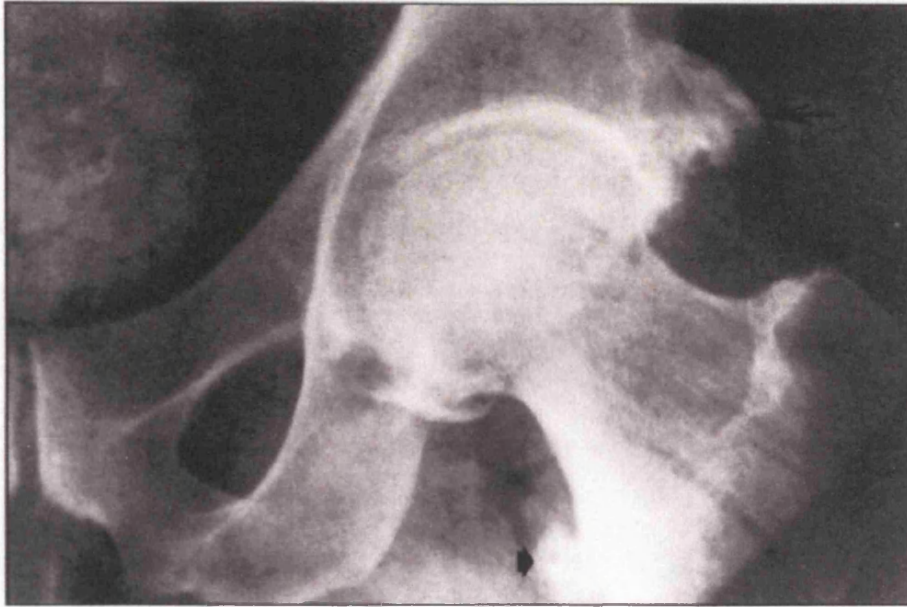


Figure -9 Enthesophytes around the hip joint (arrows). (Textbook of Diagnostic Imaging. Putman C.E. and Ravin C.E., (Ed), 1994).

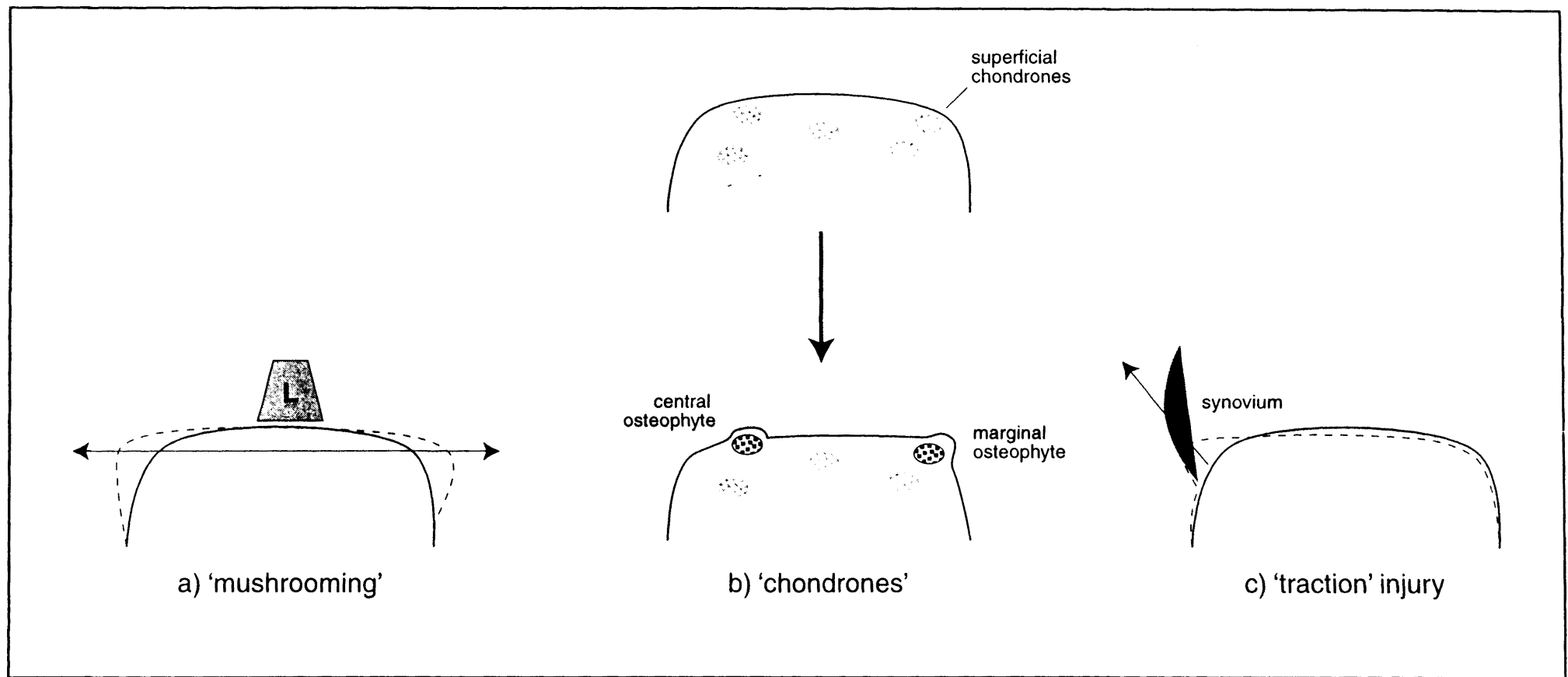


Figure - 10 Concepts of osteophyte formation

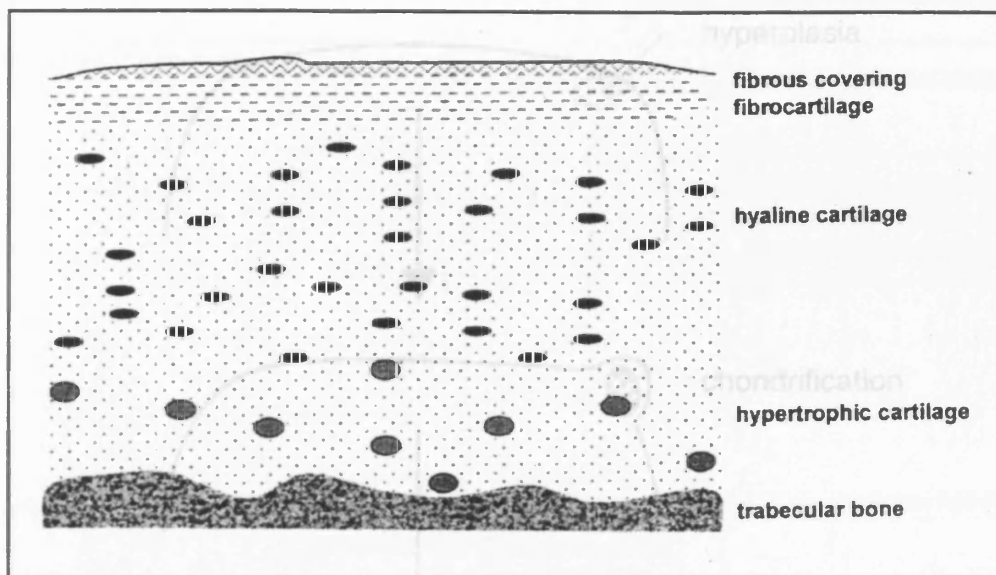


Figure - 11 The osteophyte cytoarchitecture



Figure - 12 Pathogenesis of osteophytes

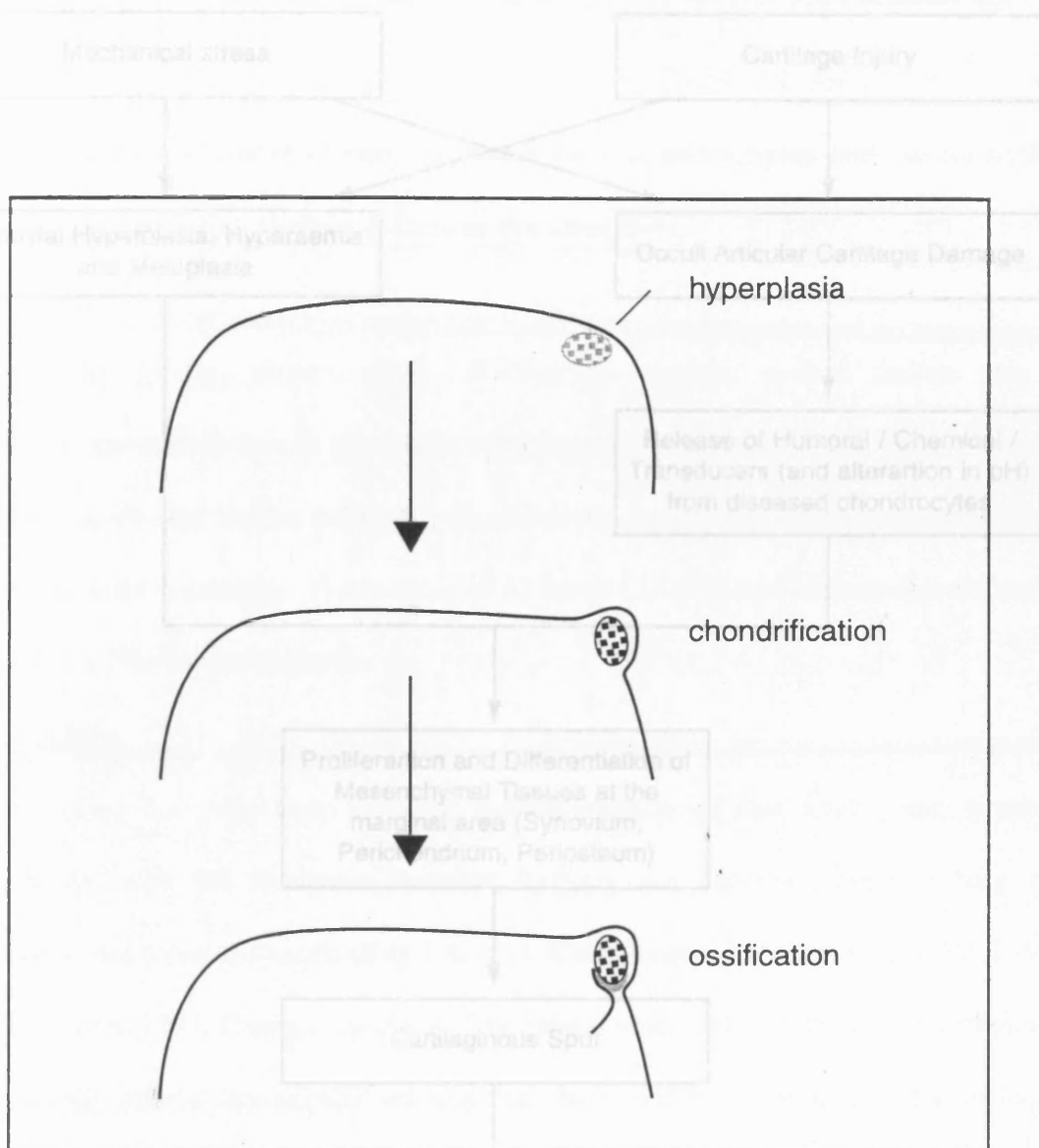
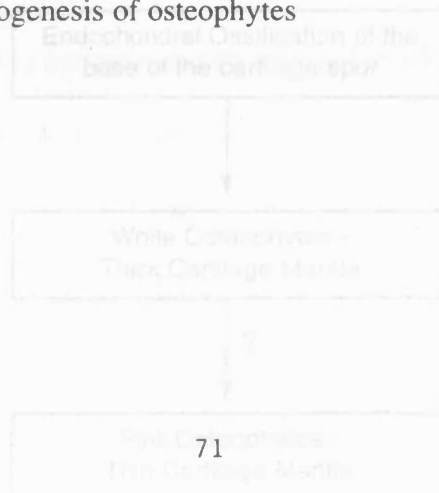


Figure - 12 Pathogenesis of osteophytes



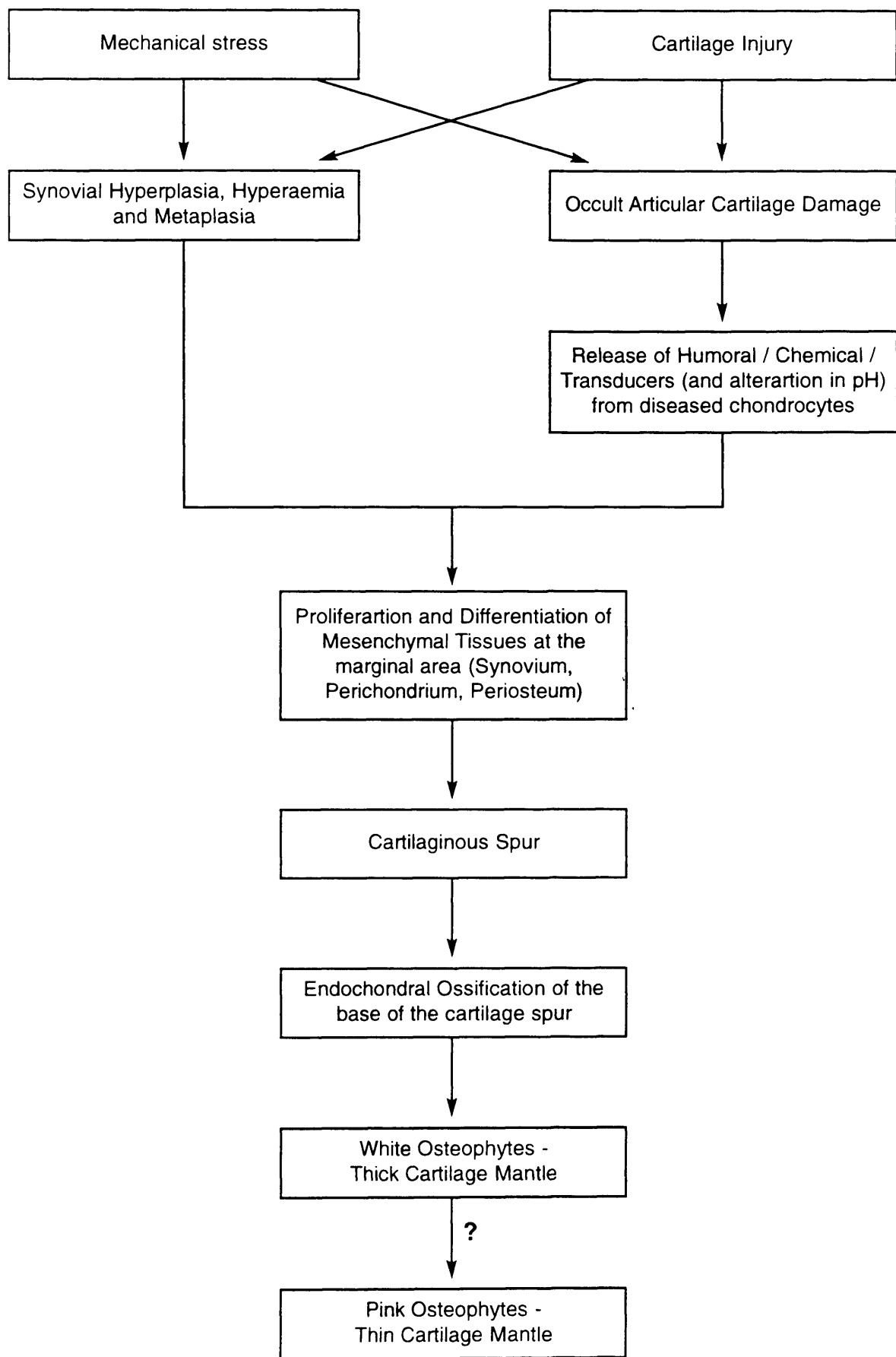


Figure 13 - Schema of Osteophytes Aetiopathogenesis

An investigation of the frequency of co-existence of osteophytes and circumscribed full thickness articular surface defects in the knee joint.

Summary.

Materials for grafting circumscribed full thickness articular surface defects may be obtained from osteophytes. In this study, the frequency of co-existence of these two intra-articular lesions was studied prospectively in patients undergoing therapeutic arthroscopy for painful knee conditions. Thirty-three of 88 knees (37.5%) had surface defects and 23 of these (69.7%) had osteophytes.

Introduction.

Circumscribed full thickness articular surface defects of the knee joint which is synonymous with full thickness chondral fracture, are important because they may eventually lead to osteoarthritis (Key J A 1931, Calandruccio R A, and Gilmer W S 1962, Shapiro F *et al* 1993, Chang *et al* 1996). The natural repair tissue in these circumstances is qualitatively inferior to normal articular cartilage and may be unable to withstand physiological loads. Consequently, some form of treatment is usually recommended especially in young people. The methods currently regarded as most likely to be successful are autologous chondrocyte transplantation (Brittberg *et al* 1994) and autogenous osteochondral grafting (Pap K and Krompecher S 1961, Hangody L and Karpati Z 1994, Hangody *et al* 1997). Materials for these procedures, are usually obtained from the normal areas of the affected joint and in so doing, new surface defects are created. These iatrogenic lesions may themselves become pathological in the future. Hence, osteophytes

are now being considered as potential source of graft materials (Matyas *et al* 1997, Oni O and Morrison C 1998) but they can be used as grafts only if they were present in the diseased joint. The purpose of this study was to find out how frequently osteophytes co-existed with full thickness articular surface defects.

Case series and methods.

Patients undergoing therapeutic arthroscopic surgery of the knee for a variety of painful conditions during a six-month period from 1 September 1997 were prospectively studied. At operation, the presence and location of full thickness articular surface defects were recorded together with the presence or absence of osteophytes. To be accepted into the study group, a surface defect had to be:

- a. circumscribed (i.e. with surrounding margins of normal cartilage, visually and by probing);
- b. more than 5mm in diameter for ease of visualisation and measurement using the tip of the arthroscope probes;
- c. eburnated (i.e. be completely devoid of cartilage covering, exposing the subchondral bone) or
- d. if visually intact, have a gritty bony sensation on probing at one or more location (s).

In order to eliminate potential, obvious or known causes of osteophytes and/or defects, knees with bilateral disease, demonstrable ligamentous laxity (Clancy *et al* 1982, Indelicato P and Bittar E 1985) or generalised articular cartilage disease (Harrison *et al* 1953) were excluded. Also excluded were patients in which arthroscopy was part of the

definitive procedure such as a tibia tubercle transfer.

Results.

A total of 88 knees fulfilled the criteria for the study of which 33 (37.5%) had 62 circumscribed full thickness surface defects. There were 22 males and 11 females aged between 18 and 60 years (mean = 36 years). As shown in Figure 14, there were 21 medial femoral condyle (33.9%) and 24 (38.7%) patello-femoral lesions. The lateral femoral condyle (LFC) and the medial (MTP) and lateral (LTP) tibia plateau were less frequently affected. Table 1 shows that in 23 of the affected knees (69.7%), osteophytes were observed at the joint margins. Figure 15 shows location of the osteophytes in the knee joints. In 5 knees (14.7%) osteophytes were located at the margins of the medial femoral condyle (MFC) alone, 3 joints (8.8%) had concomitant patella and medial trochlear marginal osteophytes and the same number had concomitant medial femoral condyle, patellar and medial trochlear marginal osteophytes. Almost 80% of the osteophytes were observed at the margins of the medial femoral condyle and the patello-femoral compartment.

Table 1.

Relationship between defects and osteophytes.

	No	%
With osteophyte	23	69.7
Without osteophyte	10	30.3

Discussion.

These results suggest that the medial femoral condyle (MFC) and the patello-femoral articulation (PFJ) are the areas of the knee most susceptible to articular cartilage defects. The reason(s) for this is not entirely clear. There are a number of possibilities that may be responsible for these findings. One, the excursion of the MFC is smaller than that of the LFC during flexion/extension (Oni O 1998), and therefore, there may be less lubrication medially. Two, there may be a relative diminution of extraosseous as well as intraosseous blood supply to the MFC compared to the LFC. Presumably this could make the MFC more vulnerable to vascular insults (Reddy A and Frederick R 1998). Three, the more rounded shape of the MFC (Muller W 1982) may result in a reduction in the contact area between it and the tibia plateau. As a consequence of this, there may be an increase in the contact stresses in this part of the knee compared to the lateral compartment. With regards to the patello-femoral joint, it is practically load bearing at all times regardless of whether a person was standing, sitting or lying.

The study also reveals a close association between osteophyte formation and surface defects. There are strong experimental corroborations for this finding. Key (1931) created full thickness defects measuring 3mm by 6mm in the femoral condyles of mature rabbits and between 8 days and 7 months observed progressive degenerative changes in the joints with marginal osteophyte formation (Key J 1931). Fisher (1939) created full thickness cartilage defect in the central area of the articular cartilage and observed the formation of

osteophytes in the lateral or marginal areas of the joint (Fisher A 1939). Peterson *et al.* (1984) created full thickness cartilage defects in the condyles of matured rabbits and observed osteophyte formation in 28.6% of defects that were not treated by autologous chondrocyte transplantation (Peterson *et al* 1984). Other workers, notably Grande *et al.* (1989) and Outerbridge *et al* (1995), have also observed a strong association between full thickness cartilage defect and marginal osteophyte formation (Grande *et al* 1989, Outerbridge *et al* 1995). Thus, in clinical practice, there is a potential source in affected knees from which materials may be obtained for either chondrocyte transplantation or for osteochondral grafting (Matyas *et al* 1997, Oni O and Morrison C 1998). It may not be necessary at all in most instances to obtain materials for grafting from normal areas of the knee.

The process by which surface defects stimulate osteophyte formation is not known with any certainty but the mechanisms may be speculated. The formation of a full thickness surface defect involves the necrosis of tissue and subsequent mechanical wear. It is known that the debris generated cause an inflammatory reaction. Synovitis frequently accompanies cartilage defects (Bennett *et al* 1932) and this is often in conjunction with synovial hyperplasia (Key J 1931, Fisher A 1939, Bennett *et al* 1932). The inflammatory process may cause the synthesis of leukotrienes and other growth factors (Kumar *et al* 1992) that may be mitogenic for cartilage and other mesenchymal cells. Inflammation may cause an irritative metaplasia in the marginal tissues and the altered tissues may subsequently proliferate and differentiate along chondrogenic and osteogenic pathways

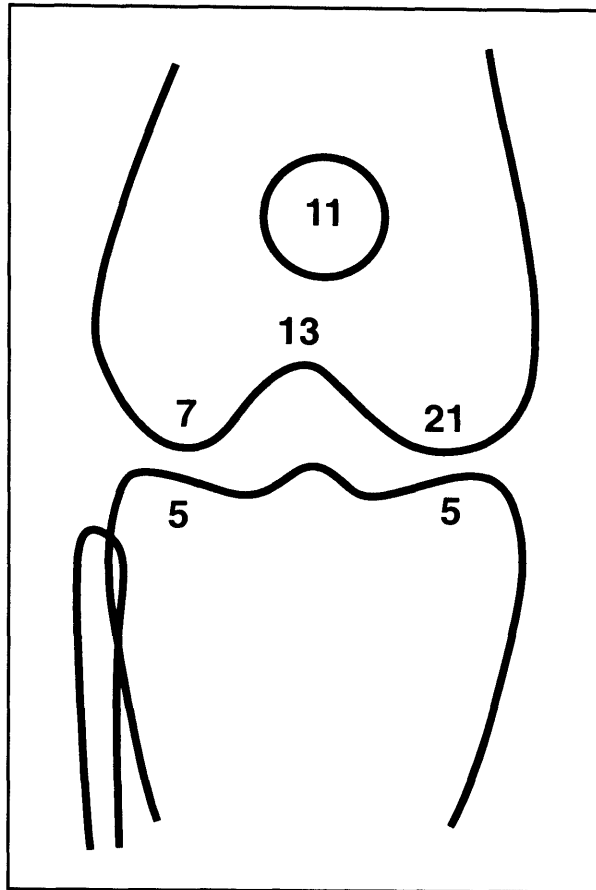


Figure 14 Location of Defects

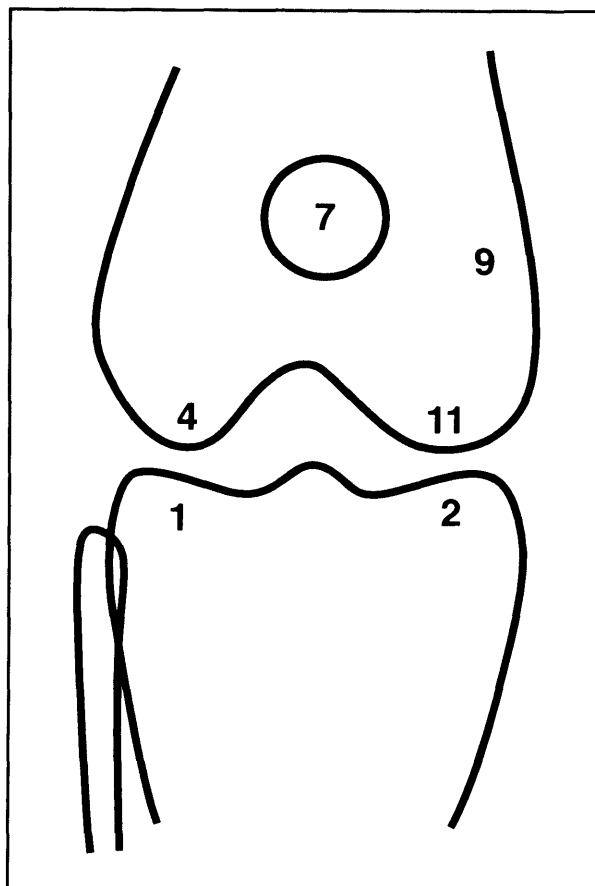


Figure 15 - Location of Osteophytes

PART 2 – OSTEOPHYTES AS OSTEOCHONDRAL GRAFTS?

***Chapter 3* – A comparison of the compressive and shear stiffness of peri-articular osteophytes, hypertrophic femoral intercondylar notch and normal articular cartilage.**

***Chapter 4* – A comparative histology of the peri-articular osteophytes and normal articular cartilage.**

***Chapter 5* – The architecture of collagens in osteophytes. A study by immunohistochemistry.**

***Chapter 6* – A study of the fibrillary collagen of the cartilage mantle of osteophytes using polarising light.**

***Chapter 7* – The ultrastructure of the peri-articular osteophytes – an evaluation by scanning electron microscopy.**

A comparison of the compressive and shear stiffness of peri-articular osteophytes, hypertrophic femoral intercondylar notch, and normal articular cartilage.

Summary.

The compressive and the shear stiffness of peri-articular osteophytes, hypertrophic femoral intercondylar notch and normal articular cartilage were compared by obtaining force-indentation curves and by applying a shear force to the cartilage/bone interface. The primary stiffness (at 0-100N load) was similar for osteophytes (391N/mm), intercondylar notch (400N/mm) and normal articular cartilage (401N/mm). By contrast, the secondary stiffness (at 100-500N load) was much higher for the intercondylar notch (493N/mm) compared to the osteophytes (410N/mm). There were two varieties of osteophytes, 'pink' and 'white'. Compared with 'pink' osteophytes, 'white' osteophytes exhibited features similar to a progressively hardening material and would appear to be a more mechanically suitable material as an osteochondral graft for full thickness articular cartilage defect. The shear characteristics of all the samples were similar.

Introduction.

Full thickness articular cartilage defects are frequently encountered during arthroscopy for various knee pathologies and may be responsible for unexplained knee pain, effusion and limitation of joint movement. It is presumed that if these defects are left unattended, they will ultimately lead to osteoarthritis. The management of focal full thickness cartilage defect in the weight bearing surfaces in the joints of young patients still remains largely unresolved. There is, to date, no universally accepted operative procedure for this disabling problem when it is seen in young people. Drilling of the defects down to bleeding subchondral bone in the hope that fibrocartilage will cover the defect is commonly embarked upon. The mechanical properties of this repair tissue have been studied and found to be inadequate in weight bearing joints (Coletti *et al* 1972, Engkvist 1979). Full thickness articular cartilage defects may sometimes be found in conjunction with peri-articular osteophytes and in an anterior cruciate deficient knee, there is in addition femoral intercondylar notch hypertrophy (Dahlstedt *et al* 1990). Notchplasty is an essential step in the operative procedure carried out during intra-articular anterior cruciate ligament reconstruction. It would therefore seem reasonable that the

bone removed during notchplasty with its surface reminiscent of articular cartilage could be used as an osteochondral graft for repairing full thickness cartilage loss if encountered (Bobic 1996). In this study, the mechanical 'qualities' of the normal articular cartilage, intercondylar notch and the periarticular osteophytes have been investigated.

Materials and Method.

All the specimens were obtained from osteoarthritic (OA) knee joints in elderly patients undergoing total knee replacement. OA knees were chosen for the study in order to be able to obtain osteophytes of large enough size for meaningful testing. Osteophytes were obtained from the margins of ten femoral articular surfaces (trochlear) with an osteotome. The hypertrophic femoral intercondylar notch was similarly harvested with the knee at 90 degrees of flexion. Visually normal articular cartilage samples were taken from the least involved part in the compartment(s). Using an osteotome, the specimens were cut into 10mm square blocks with approximately 2mm of underlying subchondral bone. They were frozen in normal saline until study when they were thawed out in their closed plastic containers at room temperature.

1. Standardisation.

(a) To determine cartilage thickness, transverse sections of specimens were obtained. With the aid of a magnifying glass, the cartilage-bone junction was identified and using a sharp dissection, the cartilage mantle was removed and a vernier calliper was used to measure its thickness.

(b) To determine the ideal setting times and stiffness of the base material, epoxy resin was prepared and poured into plastic moulds and the resin blocks transferred unto a material testing machine and indentation testing carried out at intervals of 30 minutes, 60 minutes and 90 minutes and force-indentation curve was obtained. The stiffness values were obtained and then compared until a constant value was obtained.

2. Compressive stiffness.

For compressive stiffness, each specimen was embedded in epoxy resin in a plastic mould with the cartilage surface uppermost (Figure 16). While setting, the flat end of a metal spatula was placed on the surface of the specimens, flush with the top of the plastic mould to prevent the specimens from tilting and to keep them in place as centrally as possible until the resin is firm.

The embedded specimens were removed from the moulds and transferred to an electronic materials testing machine (Hounsfield, Redhill), with a 5mm diameter flat tip indenter and a computerised output. An increasing load (to a maximum of 500N) was applied over 10 seconds at a displacement rate of 50mm/minute and a force-indentation curve was automatically generated (Figure 17). The primary stiffness values were calculated for 0-100N of applied load and the secondary stiffness for 100-500N of applied load. Calculations were made from the gradient of the curve and also at various values of force matched with displacement. The results for each group of specimens were pooled and the means were statistically compared using student's t-test and chi-square methods.

In order to determine the intrinsic, size independent properties of the materials tested, stress-stiffness curves were obtained for each material. Stress was defined as;

$$\text{Stress } (S) = F/A.$$

where F = force (N) and A = area of application of force (m^2)

$$\text{Young's modulus } (E) = \text{Stress/Strain } (\text{N/m}^2)$$

where strain (e) = $\delta l/l_0$, δl = change in length and l_0 = original length.

Stiffness (K) = F/d (N/mm) where d = displacement in mm.

$$E = k.l_0/A,$$

Since E and l_0 are fixed values for each sample, and A is constant for all cases,

$$E \propto K.$$

Therefore, for an elastic material a stress-stiffness plot should generate a linear curve.

During handling and testing the specimens were kept moist with drops of water.

3. Shear.

For shear testing, (Figure 18) the plastic mould was filled half way with epoxy resin and allowed to settle for about 10 minutes. The specimen was then embedded in the centre with the cartilage surface uppermost and the cartilage/bone interface approximately level with the surface of the first layer of epoxy resin in the cups. Thereafter, the resin was allowed to set for a further 80 minutes. Next, a circular shaped polytetrafluoroethylene non-clinging film with a 1cm by 1cm square hole cut in the centre was placed over the specimen so that the cartilage mantle of the specimen protrudes through the film. Another mixture of epoxy resin was poured to fill the cup i.e. cover the protruding cartilage portion of the specimens and the cling film.

This was again allowed to set for 90 minutes. This experimental model ensures that the bony portion of the specimen is firmly embedded in one block of resin, while the cartilage mantle is firmly embedded in the other. The non-cling polytetrafluoroethylene film ensures very low friction between the two resin blocks and there is no direct contact between the two blocks.

The resin-specimen-resin composite was removed from the plastic cup and transferred into the shear apparatus attached to the Hounsfield material testing machine (Figure 19). A shear force was applied until failure and the output was automatically generated by computer (Figure 20).

Results.

Visually, two types of osteophytes were identified namely, 'pink' and 'white' (Figure 21a and 21b). The mean values of the thickness of the cartilage mantles were: 3mm for normal articular cartilage, 2mm for "white" osteophytes, 1mm for femoral intercondylar notch and less than 1mm for "pink" osteophytes.

With the application of compressive force, the cartilage of the "pink" osteophyte did not recover after indentation but that of the "white" osteophyte, intercondylar notch and normal

cartilage demonstrated some recovery. In all cases, the force-indentation curve revealed that deformation increased non-linearly from 0-100N of force applied but with increasing load, the subsequent increase in deformation was linear for the “white” osteophyte, intercondylar notch and normal articular cartilage specimens.

Following the application of a compressive force, two types of force-indentation curves were obtained from the osteophytes. One set of osteophytes (“pink” osteophytes) exhibited features of progressively softening material with increased loading. (Figure 22). A second set of osteophytes (“white” osteophyte) produced a distinct primary (0-100N) and secondary (100-500N) stiffness with features reminiscent of a progressively hardening material. (Figure 23).

The stress-stiffness curves (Figure 24) reveal that for normal articular cartilage and “white” osteophytes, stiffness increased linearly with increasing stress, but the gradient of the curve is steeper for the “white” osteophyte. By contrast, stiffness values were practically unchanged with increasing stress for the femoral intercondylar notch while for ‘pink’ osteophytes, stiffness values decreased with increasing stress.

All specimens failed at a shear force prior to 450N. Flakes of cartilage came off with the top resin block but the cartilage/bone interface was not disrupted in any test. The findings were similar in the osteophytes, the intercondylar notch and normal articular cartilage.

Discussion.

According to these results, two types of responses are generated when osteophytes are subjected to compressive loading. At 0-100N loads, an instantaneous indentation was produced in all the specimens and this is thought to be due to mass movement of water from the matrix and collagen (Kempson *et al* 1971). At 100-500N loads there was increasing deformation and this could be due to the inherent resilience of the material. The primary stiffness of both types of osteophytes, intercondylar notch and normal articular cartilage appear to be similar. The secondary stiffness at a force between 300N-500N is similar only for the 'white' osteophytes, intercondylar notch and normal cartilage. All these tissues appear to exhibit features of a progressive hardening material whereas the secondary stiffness of the 'pink' osteophytes differs in that this tissue exhibits progressive softening properties. The stress-stiffness curve (Figure 24) also reveals that the normal articular cartilage and the 'white' osteophyte exhibit a

linear/elastic property whilst the ‘pink’ osteophyte exhibits elastic/plastic characteristics. The intercondylar notch on the other hand, hardens initially but then yields subsequently.

These results raise the possibility that there are fundamentally two distinct and different types of osteophytes, namely; “pink”, which has a thin cartilage mantle and “white” which has a thicker cartilage mantle. The size of the cartilage mantle of the osteophytes and the differing intrinsic mechanical properties (components and structural architecture) may be responsible for the observed variation in stiffness. In this respect, it may be noted that numerous workers have demonstrated a relationship between the chemical composition (McCuthen 1962), thickness (Kempson *et al* 1971, Obeid *et al* 1994), and location (Kempson *et al* 1971) of cartilage and its mechanical properties.

The two types of osteophytes appear to reflect stages in the maturation of the osteophyte. Central osteophytes for example appear to form from a cartilage bud (Figure 25). This is then invaded by creeping endochondral ossification. Presumably in the early stages of this process of invasion, there is a larger cartilage mantle and the osteophyte will appear whitish in colour. By contrast, in the later stages, the cartilage mantle will be much smaller and the subchondral

bone will become visible through it thus giving the osteophyte a pink colour. There is no reason to suppose that peripheral osteophytes are not produced in the same manner.

None of the materials tested had the exact properties of normal articular cartilage. However, the “white” osteophyte appears to be mechanically similar to the normal articular cartilage compared with the “pink” osteophyte and therefore, the “white” osteophyte may be the only type of osteophyte useful for osteochondral grafting. Furthermore, since the “pink” osteophyte appears to progressively soften with increasing stress, it may not be suitable for grafting in load bearing areas. By contrast, in this study the intercondylar notch appears to harden with increasing stress up to a point and thereafter behaves like bare bone.

Following grafting, the subchondral bone of osteophytes ought to integrate readily into the cancellous bone at the graft site. There may be a potential for graft hypertrophy and remodelling in the “white” osteophytes because of the thick cartilage mantle, which theoretically contains more chondrocytes. In an articular environment, cyclical loading during joint motion may help restore the congruity of the joint by conforming the “white” osteophyte graft to the joint contour.

Numerous surgical solutions have been offered for this disabling problem including joint debridement, chondroplasty, microfracture, drilling, shaving, osteochondral grafting (Hangody *et al* 1997) and most recently autologous chondrocyte transplantation (Brittberg *et al* 1994) but no one method has been shown to be definitive. I therefore propose the use of osteophytes as a suitable material for osteochondral grafting of full thickness cartilage defect. Osteophytes are repair tissues, which are usually found in abundance in association with degenerate and degenerating joints. Since the mechanical properties of 'white' osteophytes compare favourably with that of a normal articular cartilage and since this tissue is readily available, its use as an autogeneous osteochondral graft will obviate the need for a staged surgery with its attendant cost and complications.



Figure 16 - Photograph showing a specimen embedded in resin.

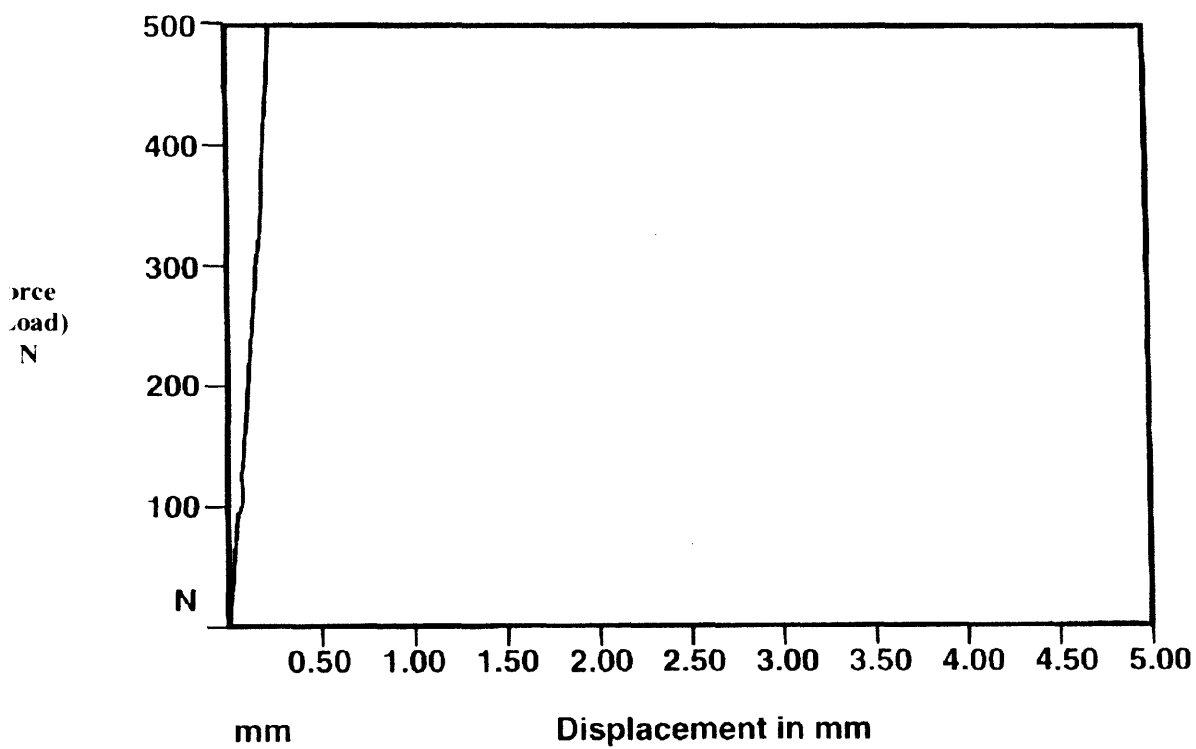


Figure 17 – A typical Force – indentation curve (Epoxy resin)

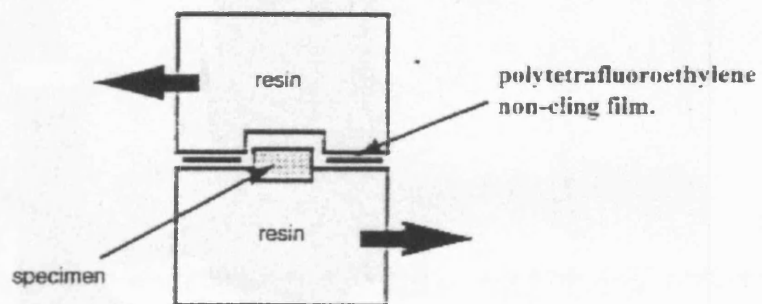


Figure 18 – Experimental set-up for measuring the shear modulus of cartilage



Figure 19 - Photograph of shear apparatus attached to material testing machine.

Shear Force-Displacement curve for normal articular cartilage, intercondylar notch and osteophyte

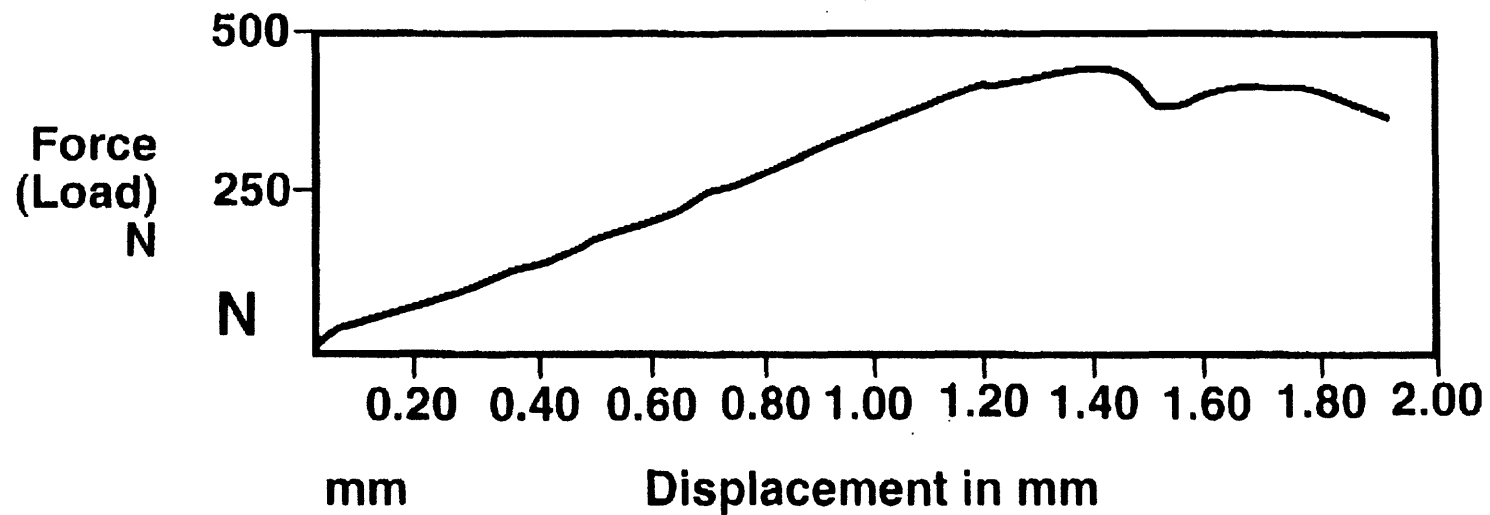


Figure 20

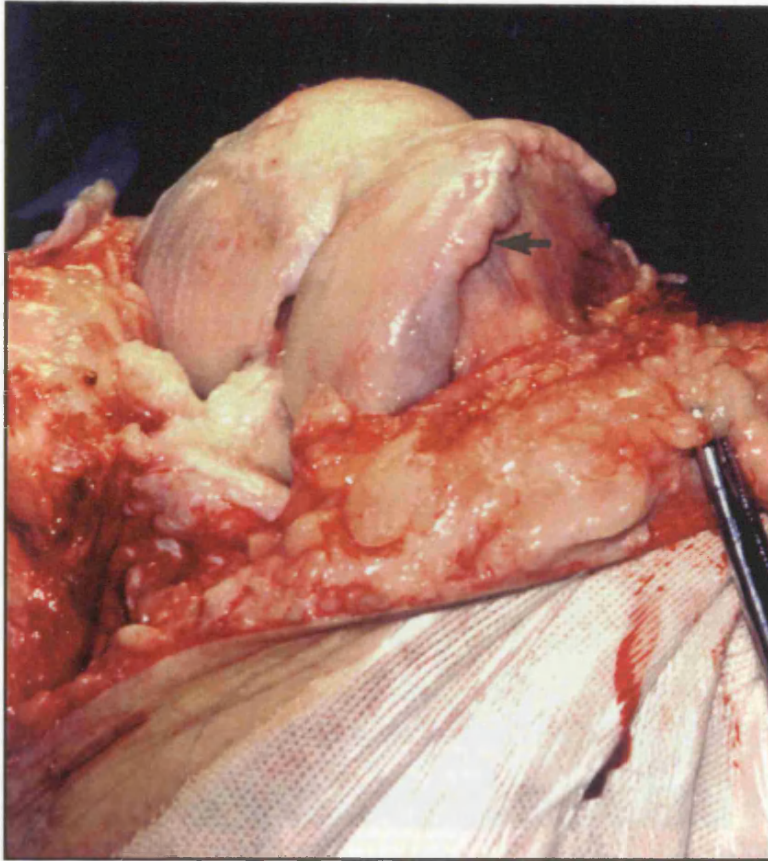


Figure 21a Photograph of a 'pink' osteophyte.

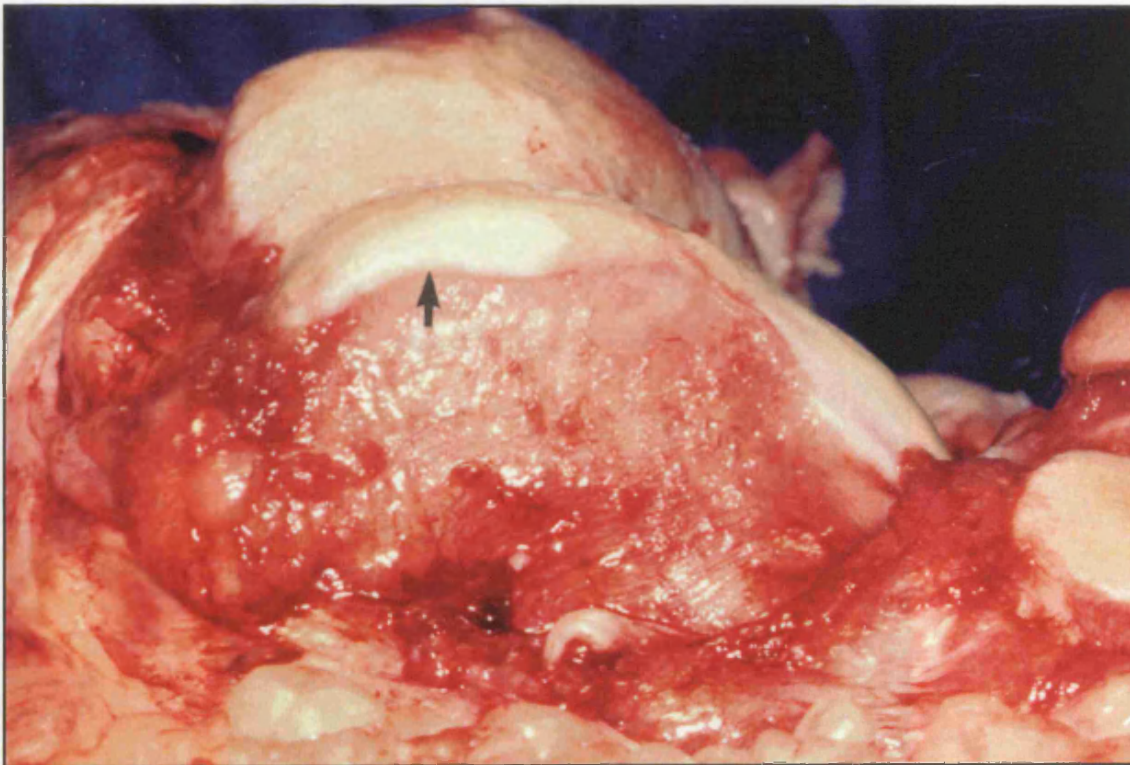


Figure 21b Photograph of a 'white' osteophyte.

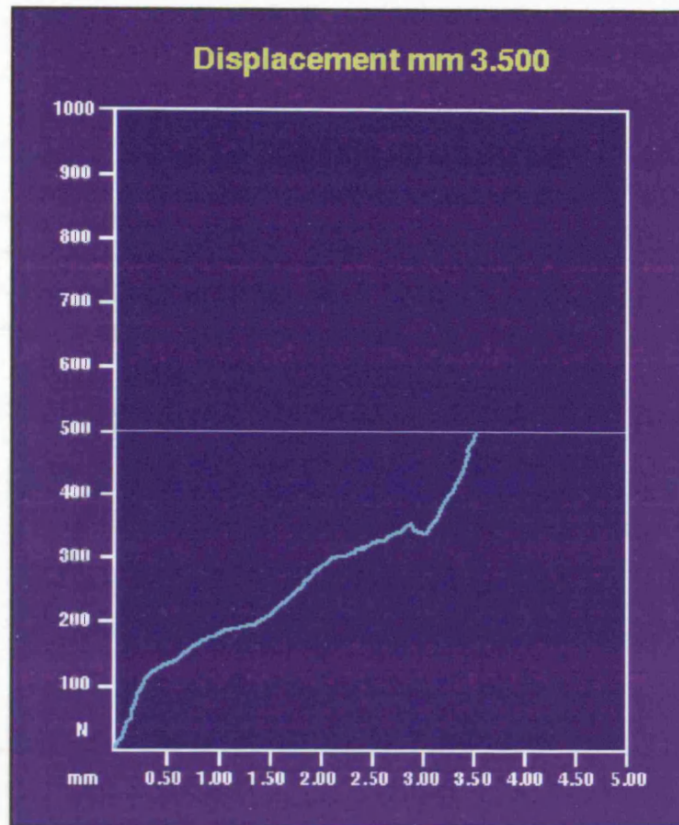


Figure 22 Force-Displacement curve for a typical 'pink' osteophyte.

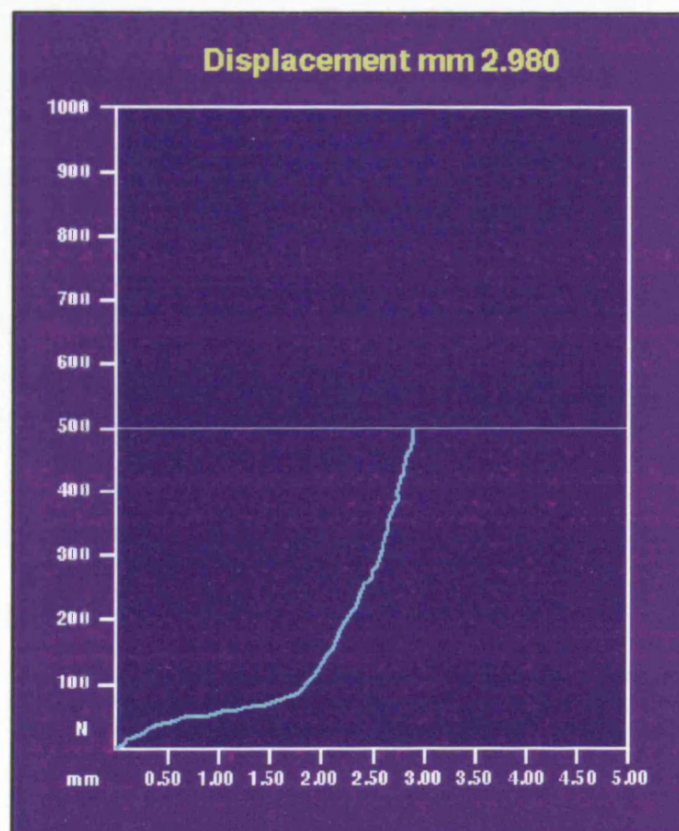


Figure 23 Force-Displacement curve for a typical 'white' osteophyte.

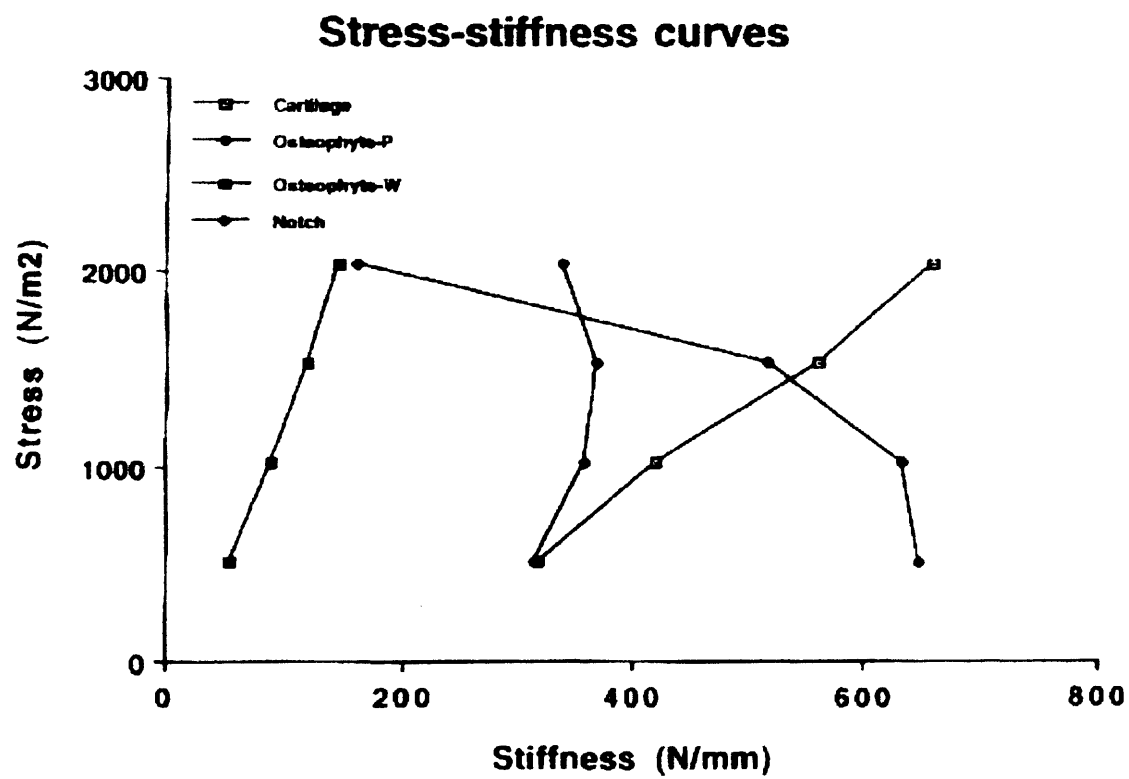


Figure - 24

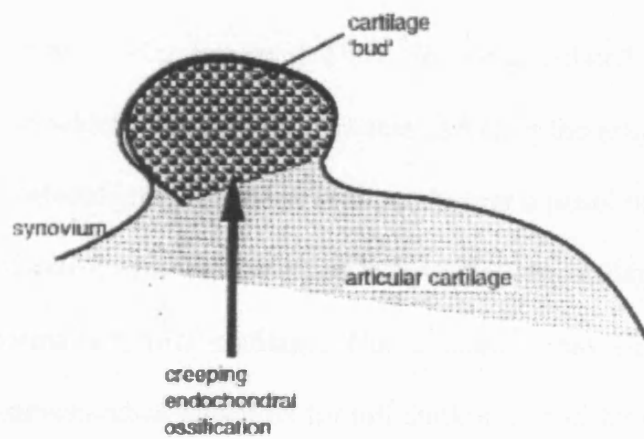


Figure 25 – Osteophyte formation by 'budding'

A comparative histology of the peri-articular osteophytes and normal articular cartilage.

Summary

Osteophytes are intra-articular tissues that are closely associated with normal articular cartilage in joints in which there is full thickness defect in the articular cartilage. In this study, sections of osteophytes have been evaluated using a panel of tinctorial stains. The cartilage mantle of osteophyte was observed to have similar cartilaginous matrix staining as was seen in normal articular cartilage. This similarity may suggest that osteophyte may be an ideal osteochondral substitute for full thickness articular cartilage defect.

Introduction

Osteophytes are intra-articular osteochondral outgrowths commonly found in association with degeneration of the articular cartilage in synovial joints and they are mostly found at the margins of the joints (Key 1931, Aigner *et al* 1995). An osteophyte is a heterogeneous tissue (Matyas *et al* 1997), extremely variable and irregular in shape (Aigner *et al* 1995) and they tend to grow into areas of low joint stress (Harrison *et al* 1953). They are formed in an area of the joint where a number of different types of tissues converge (articular cartilage, synovium and periosteum). The contribution of each of these tissues or cell types to the formation of osteophyte is not known with certainty. Contrasting theories exist regarding the formation of osteophyte. Bennett and Bauer (1937) believe that 'traction injury' at the marginal area is a pre-requisite for osteophyte formation. Moskowitz and Goldberg (1987) proposed the concept of synovial hyperaemia and hyperplasia of the marginal mesenchymal tissue (in response to a more central full thickness articular cartilage defect) to form a cartilaginous bud, which then

undergoes endochondral ossification. 'Mushrooming' of the marginal articular cartilage due to loading of a damaged central articular cartilage has also been proposed (Resnick *et al* 1991). More recently, 'chondrone' or chondrocyte cluster formation in the marginal area in response to a more central cartilage damage has also been suggested (Mitchell *et al* 1992).

Articular cartilage on the other hand is a smooth, uniform, flexible elastic crust (Hunter 1743) often referred to as hyaline cartilage denoting its pearly appearance (Cruess 1982). The articular cartilage is not homogeneous throughout its depth in terms of its chemical composition, chondrocyte organisation and chondrocyte morphology and as such, distinct zones can be described (Jeffrey 1994).

Osteophytes have been referred to as repair tissues in response to full thickness articular cartilage defects (Aigner *et al* 1995) although they are located in the non-weight bearing part of the articular cartilage. To carry out the task of repair, it is to be expected that osteophytes should bear some similarities to the tissue it is presumed to be repairing. Therefore, the aim of this study was to evaluate the similarities and dis-similarities between these two intra-articular osteochondral structures using a panel of stains.

Materials and methods

Osteophytes were obtained from the trochlear and femoral condyles of 10 patients aged between 66-88 years undergoing total knee replacement for osteoarthritis (OA).

Articular cartilage was also obtained from the visually normal compartment in 10 patients undergoing total knee replacement for osteoarthritis (OA) in which the degenerative change affected one compartment mainly.

Decalcified specimens

Specimens were fixed in formalin, decalcified, routinely processed and embedded in paraffin. Comparative 7µm sections were obtained and stained using: Haematoxylin and Eosin (H&E), Sirius red (SR), Toluidine blue (TB), Safranin O (SO), Alcian blue (AB), van Gieson (VG) and Ralis and Ralis universal tetrachrome stain. The choices of these stains were made to evaluate the cartilaginous components on osteophytes and normal articular cartilage using the normal cartilage as the reference model.

Undecalcified specimens

In a parallel study, specimens were also prepared and stained according to the Tripp and MacKay's technique (T&M) for identifying mineralisation, bone and osteoid.

Result.*Macroscopic examination*

On excision, osteophytes were observed to be cartilage capped bony outgrowths with abundant subchondral bone (Figure 26).

For the purpose of this study, the cartilage mantle of normal articular cartilage and osteophyte has been divided into three layers or zones: superficial, intermediate and deep layers. The superficial layer is that which is adjoining the joint cavity; the deep layer is that which abuts on the subchondral bone and the intermediate layer is between the superficial and the deep layers. This division into layers however, lacks distinct demarcation.

Haematoxylin and Eosin (H & E). (Figure 27a & 27b).*Normal articular cartilage.*

The chondrocytes in the superficial layer were flat and transversely oriented while the intermediate layer chondrocytes are haphazardly distributed. The chondrocytes in the deep layer are arranged in columns in the longitudinal direction and tidemark was seen in all the sections.

Osteophyte

Generally, the chondrocytes appear to be fewer but the cellular arrangement was similar to that of the normal articular cartilage. In the deep layer, some of the longitudinal cellular columns contain up to six cells. No tidemark was seen in any of the sections.

Tripp and Mackay. (Figure 28a & 28b).

Normal articular cartilage.

The superficial layer of the cartilage mantle had a lighter shade of red staining whilst the intermediate and deep layers had an orange staining. A thin layer of wavy black staining was observed demarcating the subchondral bone from the cartilage mantle. Patchy areas of black stains were also seen in the subchondral bone.

Osteophyte.

The entire cartilage mantle had a uniform orange staining. A much thicker and broader band of black staining was seen demarcating the cartilage mantle above from the subchondral bone. This suggests a much thicker band of calcifiable cartilage in osteophyte.

Ralis and Ralis. (Figure 29a & 29b).

Normal articular cartilage.

The superficial layer of the cartilage mantle had a bluish-pink staining background but the intermediate and deep cartilage layers were slightly deeper with the same pattern of staining. The subchondral bone matrix had a uniform brick red staining whilst the

osteoblasts had a deeper shade of red staining. The subchondral bone marrow stained yellow and no deep red staining was seen in the subchondral bone.

Osteophyte.

The superficial layer had a predominant pink staining with few specks of red staining but in general, the cartilage mantle was stained blue. The subchondral bone matrix also had a brick red staining which tended to vary in intensity within the same section.

van Gieson (Figure 30a & 30b).

Normal articular cartilage.

A uniform sheet of thick orange-red staining was observed in the entire cartilage mantle although more evident in the superficial layer. Dark staining of the chondrocytes nuclei were observed and the red blood cells and the bone marrow stained yellow. The subchondral bone trabeculae had a pale orange stain.

Osteophyte.

Bundles of orange-red staining were observed in the cartilage mantle and this had no uniformity and did not cover the entire surface area.

There was dark staining of the chondrocyte nuclei and the bone marrow and red blood cells also stained yellow. However, the subchondral bone had a brick red staining.

Sirius red. (Figure 31a & 31b).

Normal articular cartilage.

Intense red staining of the matrix of the entire cartilage mantle was observed. The calcified cartilage and the subchondral bone on the other hand had an orange staining.

Osteophyte.

Patchy or sparse red staining of the matrix of the cartilage mantle was observed and these were mainly around the chondrocytes. The deep layer of the cartilage mantle and the subchondral bone also has an orange staining.

Toluidine blue. (32a & 32b).*Normal articular cartilage.*

The entire cartilage matrix had an intense purple stain and the chondrocytes were distinctly buried in the matrix. There was a reduction in the intensity of the purple staining both above and below the tidemarks. There was a pale blueish staining of the subchondral bone.

Osteophyte.

The entire cartilage mantle also stained purple but the intensity of the stain was less than that observed in the normal articular cartilage. The matrix around the deep chondrocytes adjoining the subchondral bone also had an intense purple staining. The subchondral bone had a pale blue staining.

Alcian blue. (Figure 33a & 33b).*Normal articular cartilage.*

The cartilage matrix in the superficial layer had a faint bluish staining, but the staining was more intense in the matrix of the intermediate layer. In general, the blue pericellular extracellular matrix staining increased from the superficial layer to the deep layer.

Osteophyte.

In the superficial layer, the cartilage matrix also has a faint blue staining. There was also an increase in the intensity of the matrix staining in the intermediate layer and these had a band-like configuration around the chondrocytes. The blue pericellular extracellular matrix staining also increased from the superficial to the deep layer.

Safranin O. (Figure 34a & 34b).*Normal articular cartilage.*

The superficial layer had a faint orange staining of the matrix whereas the intermediate and deep layers of the cartilage mantle had an intense but uniform orange staining. The chondrocyte nuclei had dark staining. The subchondral bone had a faint orange staining.

Osteophyte.

The superficial layer also had a faint orange staining of the cartilage matrix, but in the intermediate and the deep layer, the cartilage matrix had a more intense orange staining. The chondrocyte nuclei also had dark staining and the subchondral bone also had a faint orange staining.

Discussion

From this study, haematoxylin and eosin stain will be referred to as the 'basic' histological or 'reference' stain and this (Proctor 1989) clearly shows that the cellular arrangement of the chondrocytes in the cartilage mantle of normal articular cartilage and peri-articular osteophytes are similar. The uniform demarcation of the cartilage mantle from the subchondral bone by a band of mineralised bone at the cartilage-subchondral bone interface as shown by the Tripp and Mackay technique (Tripp and Mackay 1972) are similar in both normal articular cartilage and osteophytes. This finding seems to support the hypothesis of Moskowitz and Goldberg (1987) of a cartilaginous front or bud (following mesenchymal tissue proliferation) invasion of this bud by blood vessels and subsequently regulated endochondral ossification in the pathogenesis of osteophyte.

It is generally agreed that osteophyte is a composite tissue comprising fibrous tissue, fibrocartilage, hyaline cartilage and bone (Key 1931, Aigner *et al* 1995, Resnick *et al* 1991, Fisher 1939, Marshall 1969, Gilbertson 1975). The fibrous and fibrocartilage components are located in the superficial layer, the hyaline cartilage segment is located deep to the fibrocartilage and this hyaline cartilage segment abuts on the subchondral

bone (Moskowitz and Goldberg 1987). However, this study has shown that the cartilage mantle of osteophyte stains positively for cartilage matrix as revealed by Alcian blue (Steedman 1950), Toluidine blue (Bancroft and Cook 1994), Safranin O (Lane *et al* 1997), Ralis and Ralis (Ralis and Ralis 1975) and Sirius red (Llewellyn 1970) staining. Although the intensity of the stains appears to be less in the osteophytic cartilage, it is possible that the predominant cartilage type in osteophyte is that which is found in articular or hyaline cartilage.

The collagen staining (van Gieson 1889) was similar in both normal articular cartilage and osteophyte although the collagen load or quantity appears to be larger in articular cartilage. This is apparent in that the orange staining of the collagen in articular cartilage was a thick uniform sheet of stain whilst in the osteophytes; the stain was in distinct bundles with uncovered areas in the cartilage mantle.

Although osteophytes are repair tissues, this study has shown that these osteochondral tissues are very similar to normal articular cartilage. Since the co-existence of osteophytes and full thickness cartilage defects has been well documented (Key 1931, Peterson *et al* 1984), the results of this study therefore raises the possibility that osteophytes can be used as substitutes (osteochondral graft) for damaged articular cartilage when these two are found in the same joint. Although the cartilage stains showed some similarities between osteophytes and normal articular cartilage, which is mainly hyaline cartilage, I believe therefore that osteophytic cartilage also has hyaline component. Although the cartilage stain intensities were more in the normal articular cartilage, yet osteophytes may still be better substitutes for damaged articular cartilage.

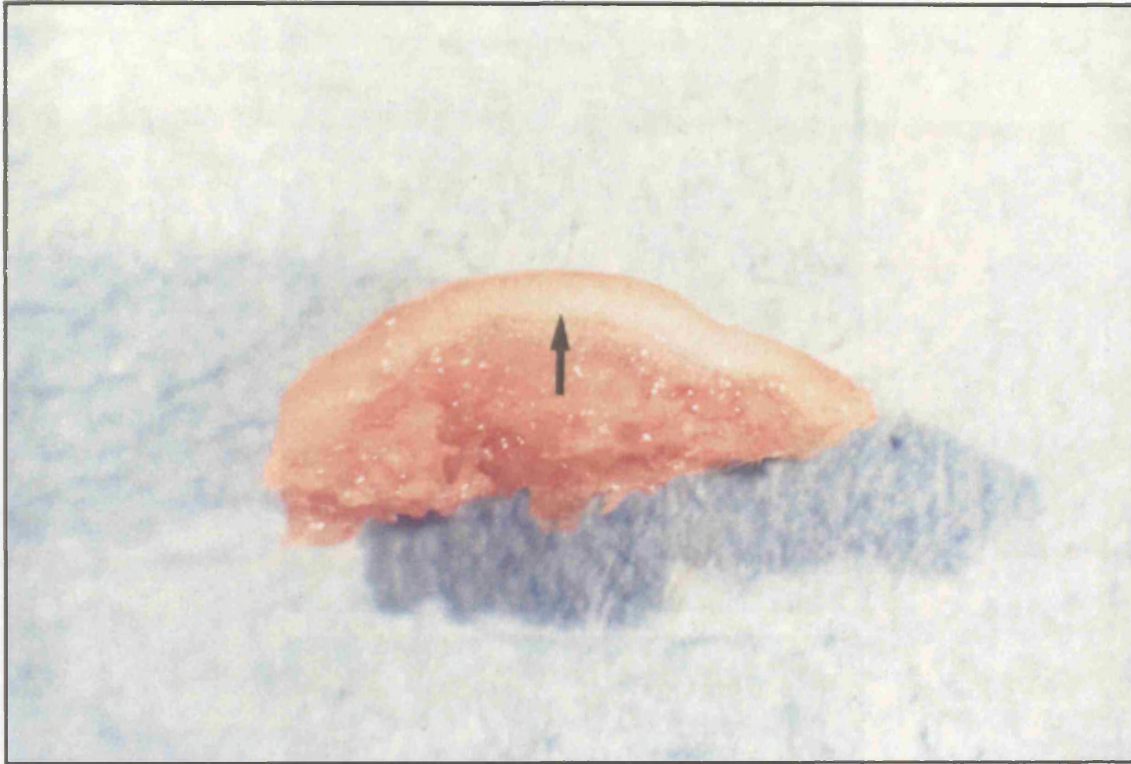
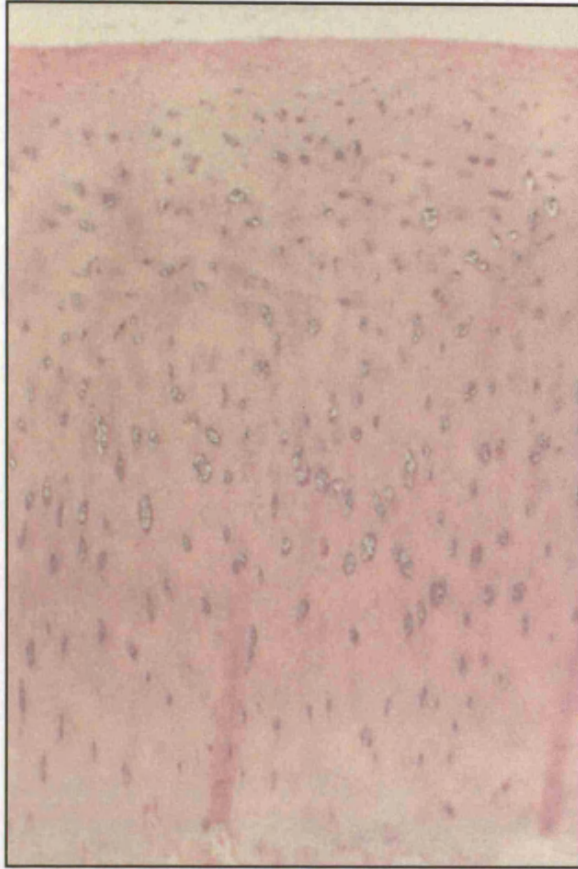


Figure 26 - Photograph of a cross section of marginal osteophyte. (The cartilage mantle is shown with an arrow).

a



b

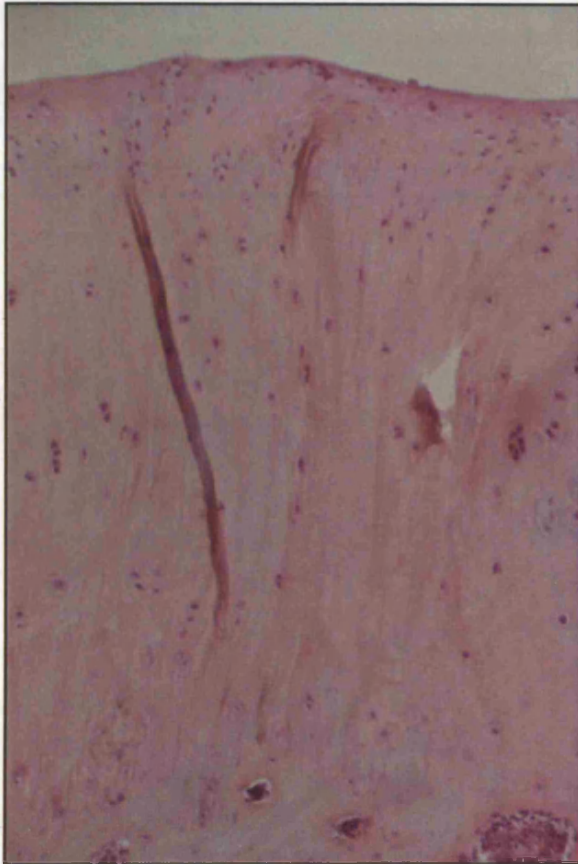
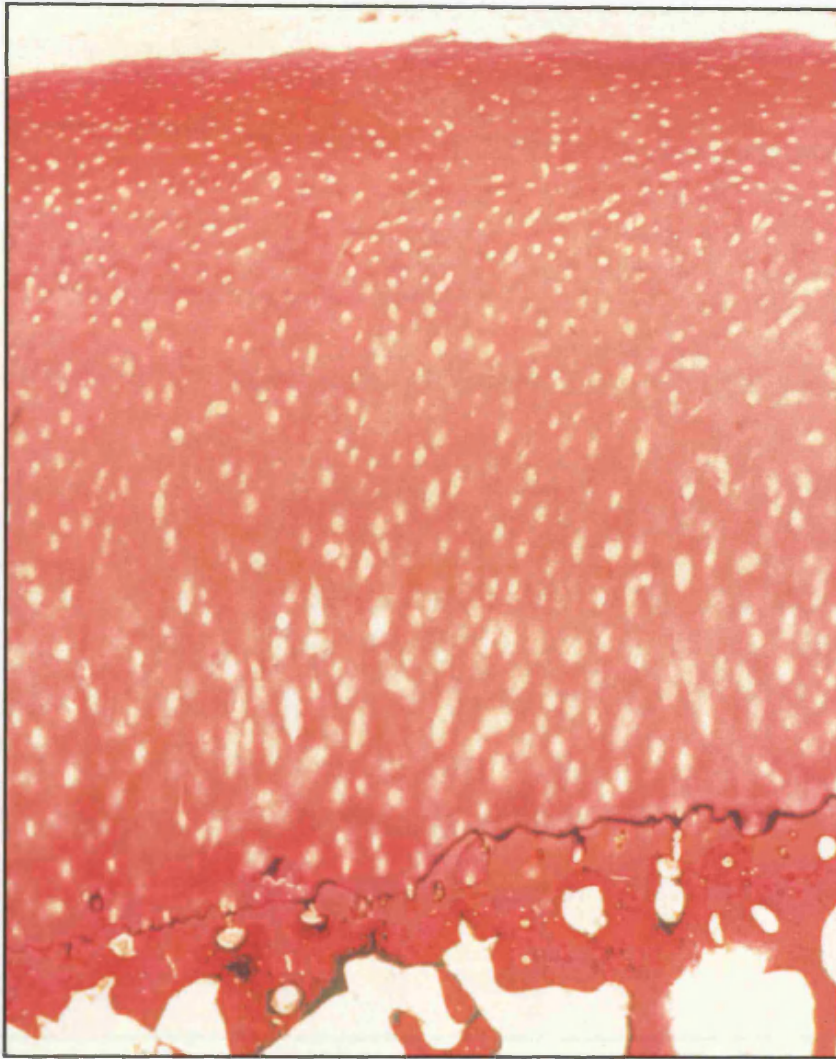


Figure 27a & 27b Sections of normal cartilage (a) and osteophyte (b) stained with Haematoxylin & Eosin.

a



b

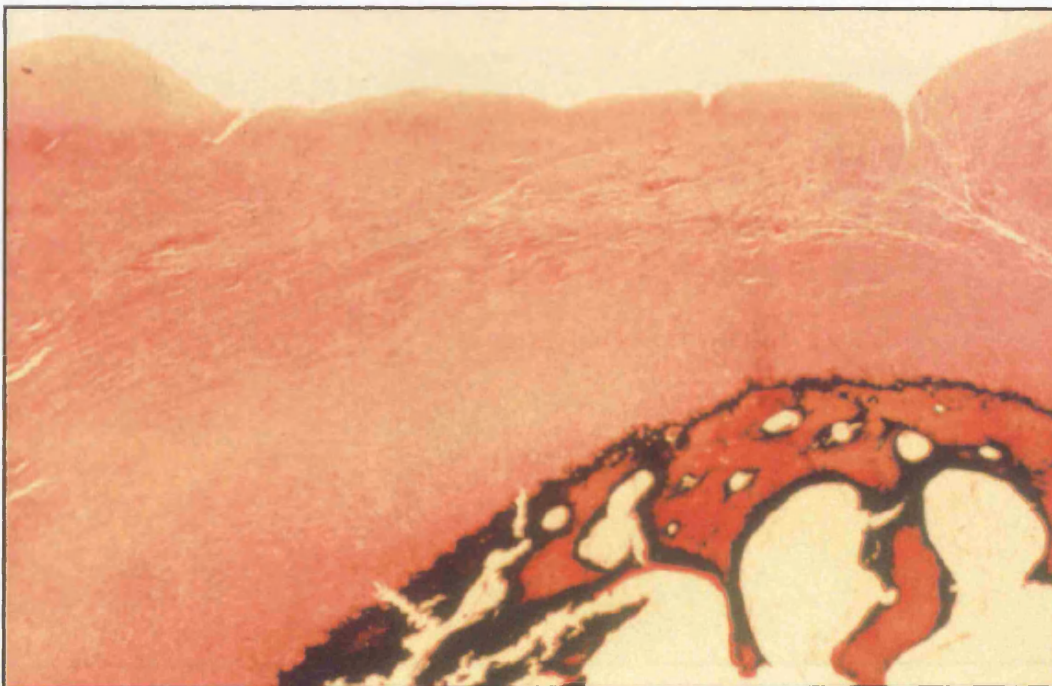


Figure 28a & 28b Sections of normal cartilage (a) and osteophyte (b) stained with Tripp and Mackay.

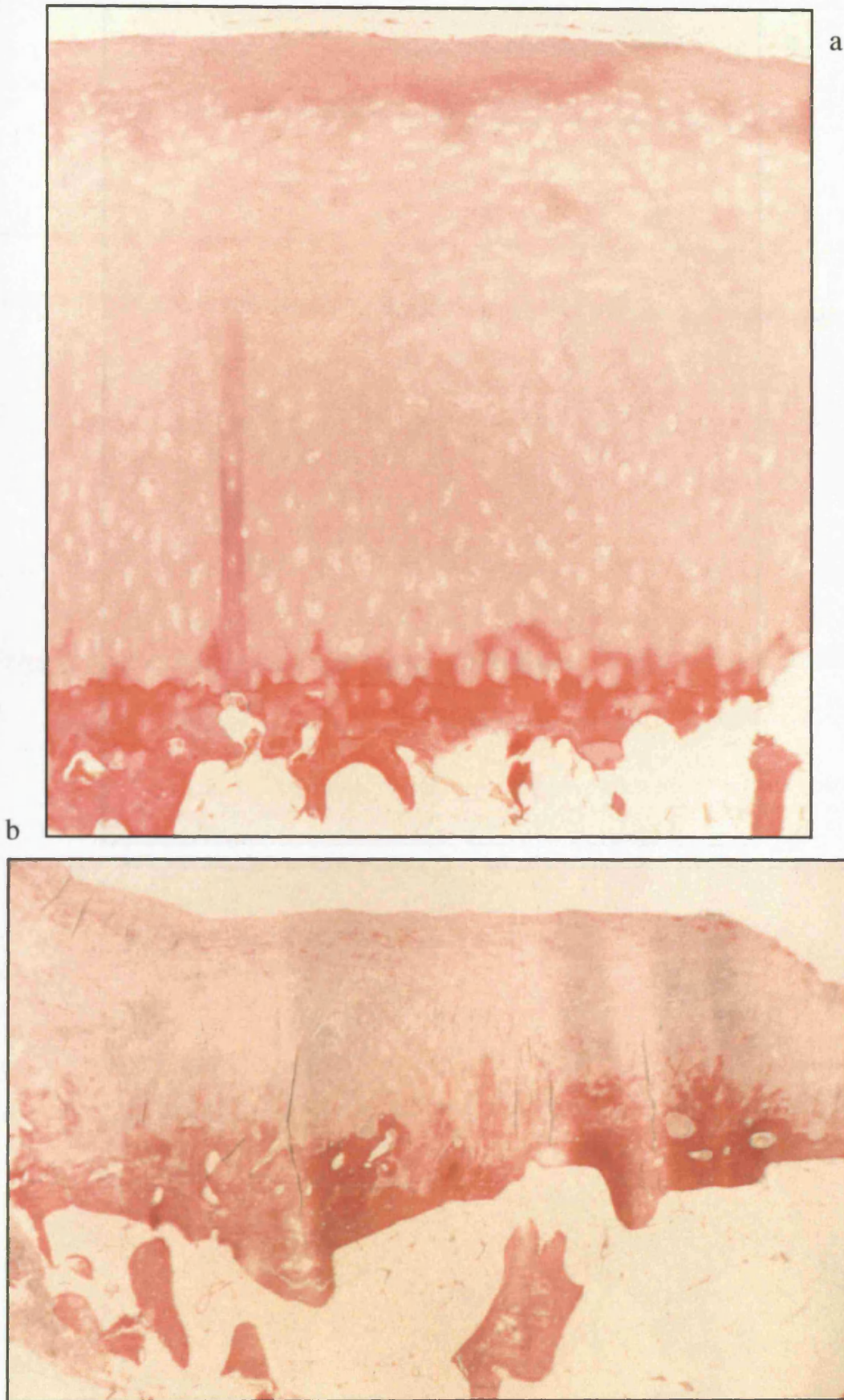
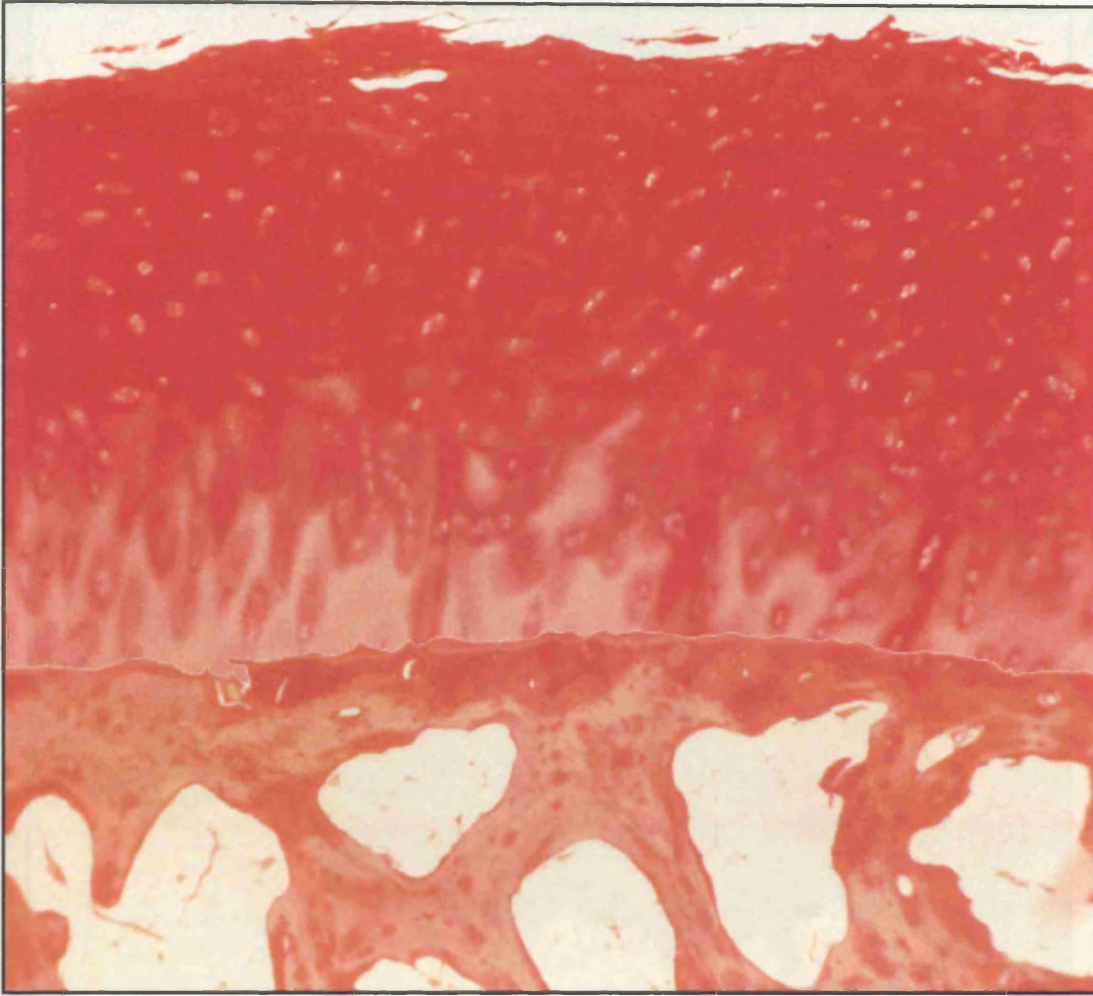


Figure 29a & 29b Sections of normal cartilage (a) and osteophyte (b) stained with Ralis & Ralis.



Figure 30a & 30b Sections of normal cartilage (a) and osteophyte (b) stained with van Gieson.

a



b

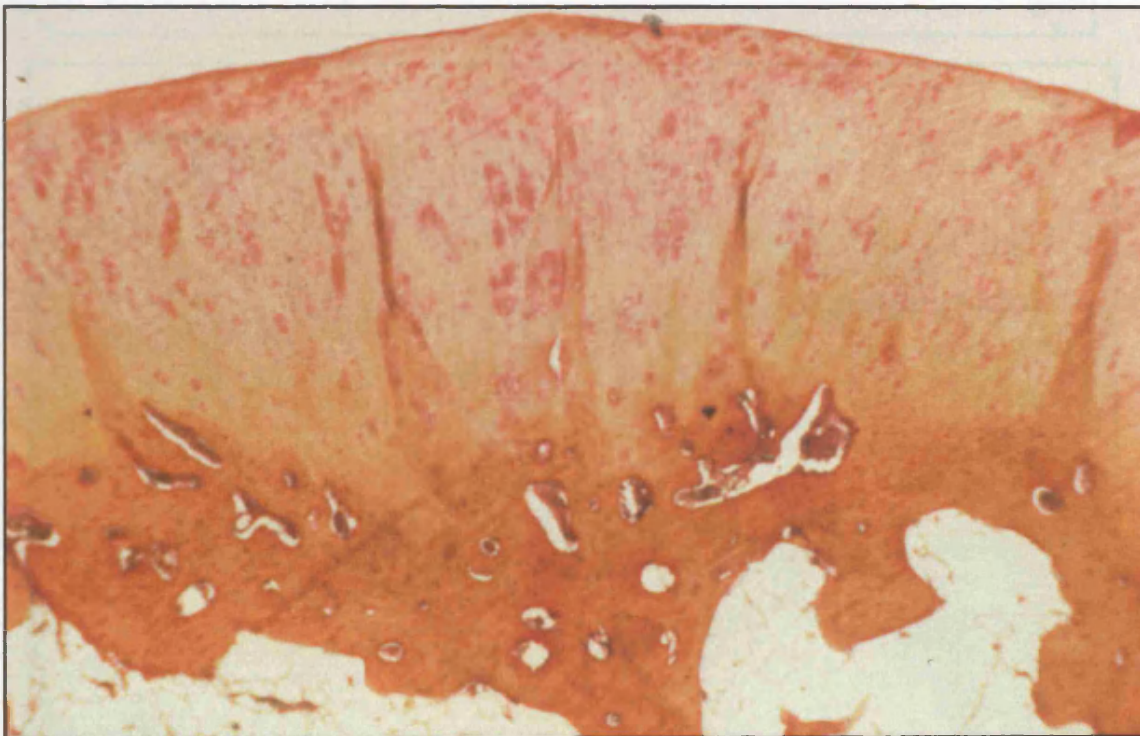
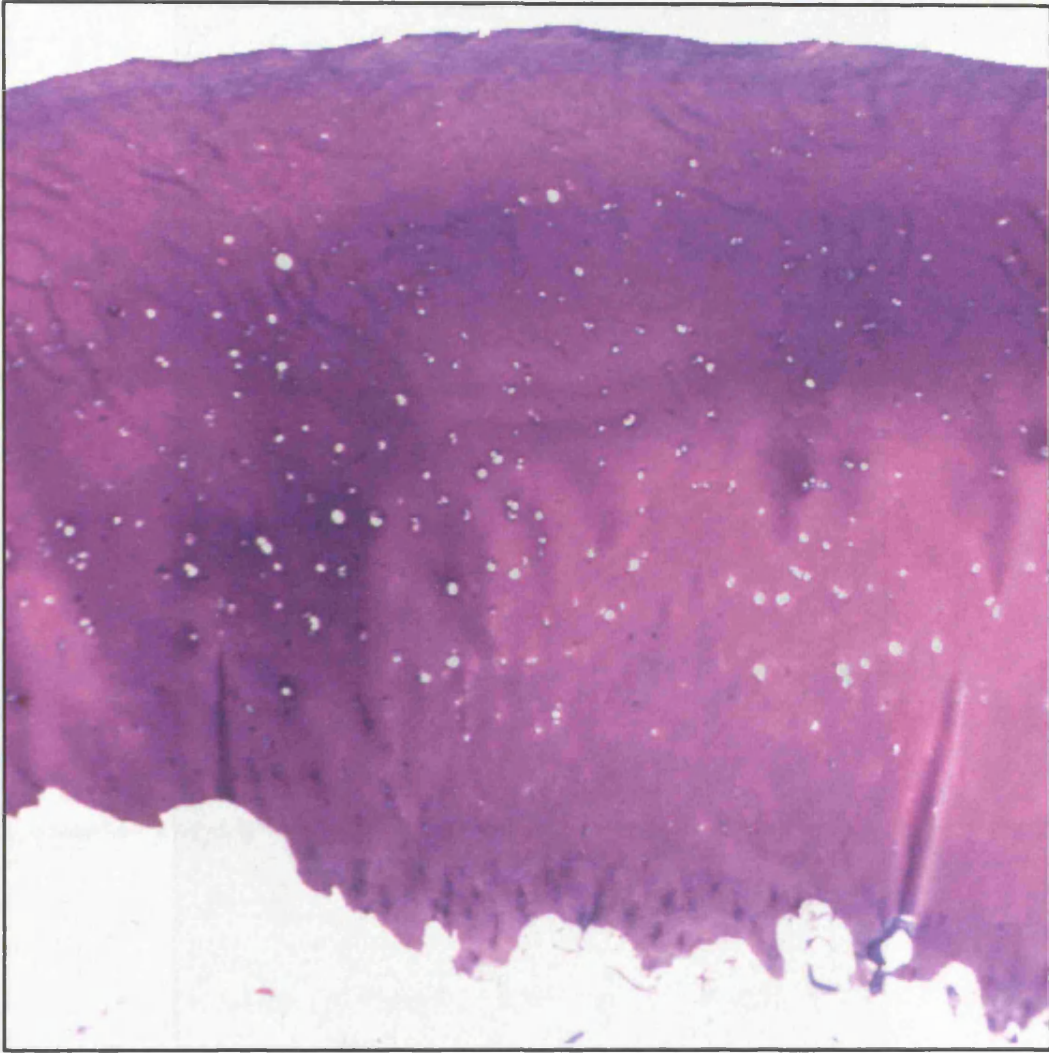


Figure 31a & 31b Sections of normal cartilage (a) and osteophyte (b) stained with Sirius red.

a



b

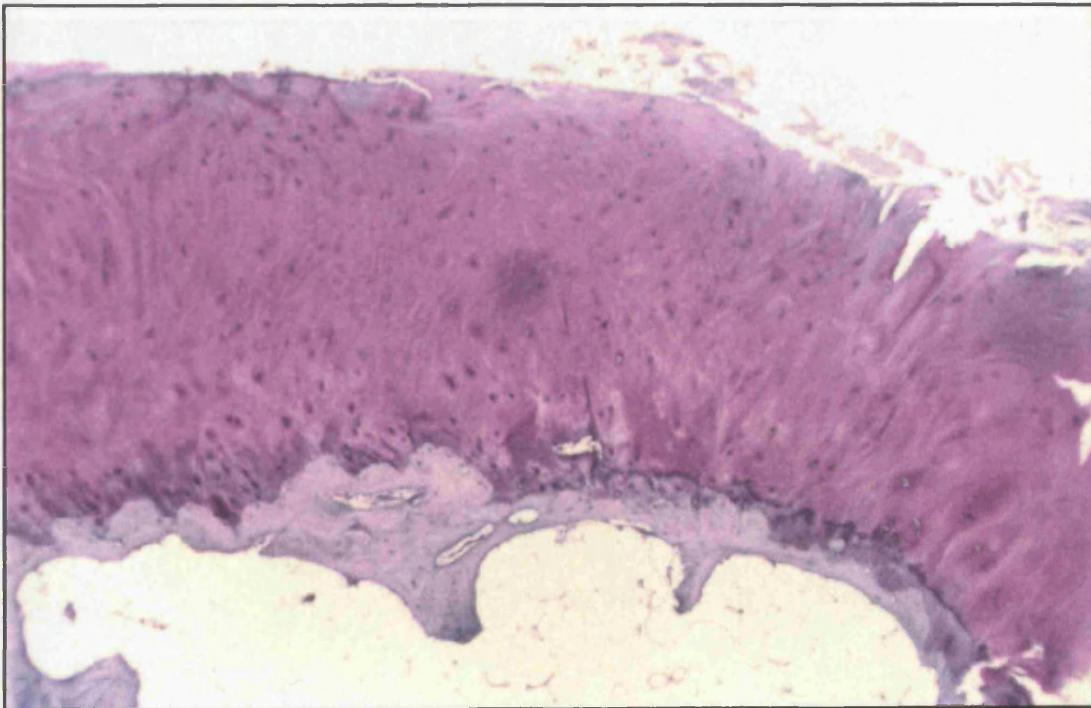
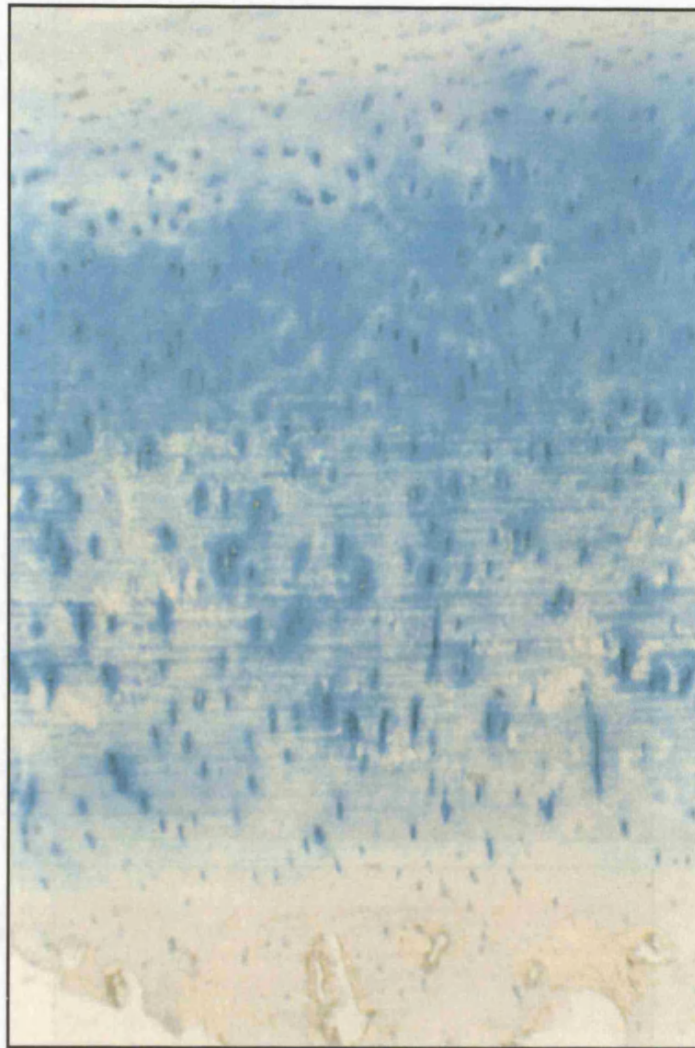


Figure 32a & 32b Sections of normal cartilage (a) and osteophyte (b) stained with Toluidine blue.



a

b

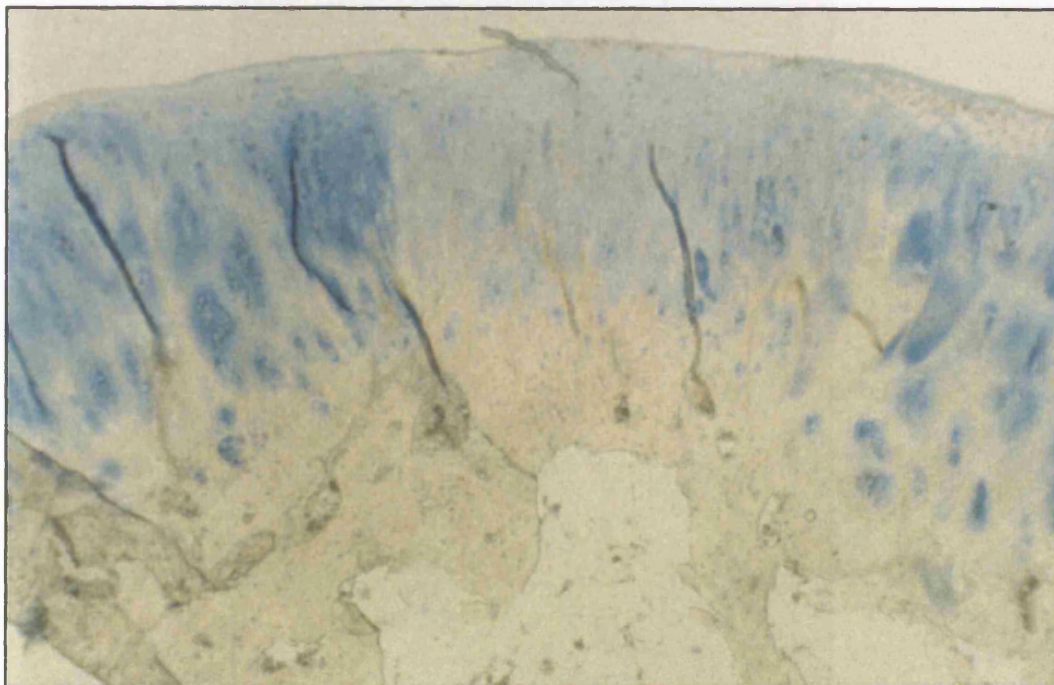
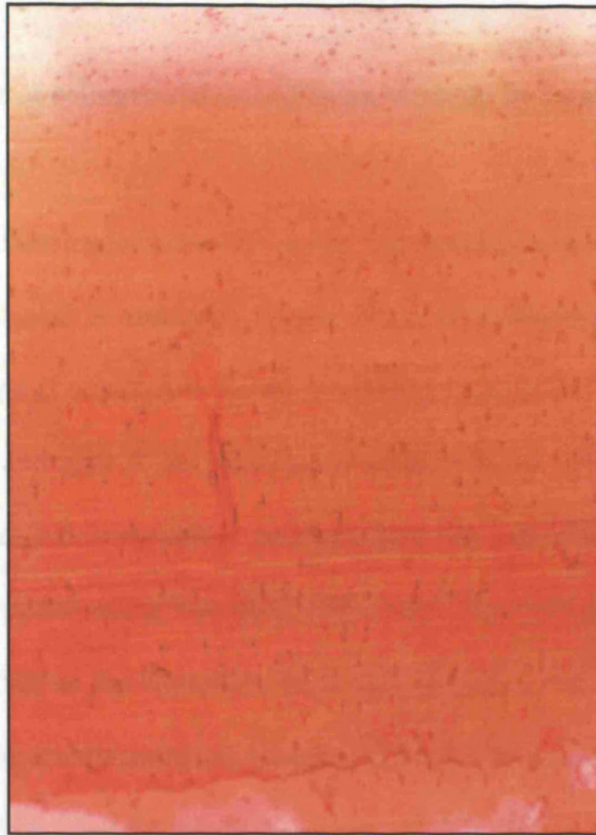


Figure 33a & 33b Sections of normal cartilage (a) and osteophyte (b) stained with Alcian blue.

a



b



Figure 34a & 34b Sections of normal cartilage (a) and osteophyte (b) stained with Safranin O.

The architecture of collagens in osteophytes. A study by immunohistochemistry**Summary.**

The collagen architecture of osteophytes has been investigated by immunolocalisation of types I, II, III and X collagens. Types I and III collagens are observed in the surface layers, type II collagen in the entire cartilage mantle but far more abundant in the middle layer and type X collagen at the cartilage-bone junction. This pattern of distribution suggests a 'maturation' pathway from the precursor cells at the surface layer to the cells transforming into bone at the base. The collagen fibres appear to be oriented horizontally in the superficial layer and vertical in the middle-to-deep layers thus mimicking the architectural arrangement in normal articular cartilage.

Introduction.

Osteophytes are intra-articular osteochondral outgrowths found in association with full thickness defects in the articular cartilage of diarthroidal joints (Key 1931, Fisher 1939, Peterson *et al* 1984, Grande *et al* 1989, Outerbridge *et al* 1995). In these degenerate or degenerating joints, the osteophytes may be found at the margin of the articular cartilage when they are referred to as marginal osteophytes or in the articulating surface of the cartilage when they are known as central osteophytes. Morphologically, they are similar to normal articular cartilage in that they have a cartilage mantle overlying a firm subchondral bone laden with marrow (Figure 26). The cartilage mantle of this heterogeneous tissue may be young, foetal-like, hypertrophic or fibrocartilaginous at various sites or locations. Osteophytes have therefore been suggested as suitable material for grafting full thickness defects in normal articular cartilage (Matyas *et al* 1997, Oni and Morrison 1998). However, it

will be prudent to know the biology of this tissue before its use becomes widespread. The cartilage mantle of osteophytes has been shown to contain a variety of collagens (Aigner *et al* 1995). These extracellular proteins form the polymeric framework, which underlies the tensile strength, elasticity, resistance to friction and pressure and the in-built lubricating mechanism of normal cartilage (Eyre 1991, Aigner *et al* 1992). Although the localisation of these collagen types have been described in osteophytes (Aigner *et al* 1995), the architectural arrangement has not yet been established and this is the object of this study. To aid the interpretation of results, it has been accepted that *chondrocyte precursor* cells such as those observed in limb bud or somite mesenchymal cells, for example, express type I collagen (Linsenmayer *et al* 1973, von der Mark *et al* 1976, von der Mark and von der Mark 1977, Kuijer *et al* 1996); that *differentiated chondrocytes* such as those found in the resting zone of foetal epiphyseal growth plate and articular cartilage, express type II, VI, IX, XII and XIV collagens (Mayne 1989); that *hypertrophic chondrocytes* of the foetal epiphyseal and rib cartilage express type X collagen (Schmid *et al* 1990) , and that “dedifferentiated” chondrocytes which are transforming to fibroblastic cell shape express types I, III and V collagens (von der Mark 1986).

Materials and Methods.

The specimens studied consisted of osteophytes obtained from the margins of the femoral condyles of eighteen patients with osteoarthritis (OA) of the knee joints undergoing total knee replacement.

Paraffin Tissue Embedding

Ten specimens were fixed in 10% neutral buffered formalin for three weeks, decalcified in Kristensen's solution for three weeks, routinely processed and then embedded in paraffin wax. Sections, 7µm thick were mounted on numbered microscope slides and every fourth sample was stained with haematoxylin and eosin (H & E). The remaining slides were prepared for immunohistochemistry by first treating the sections with testicular hyaluronidase (Type 1S, Sigma, Poole, UK) in acetate buffer (1mg/ml) for 1 hour.

Undecalcified (frozen) Tissue Embedding

Eight specimens were cut into smaller pieces and embedded in Cryo-M-Bed (Bright) on a piece of cork, snap frozen in liquid nitrogen and stored at -80°C until use. Sections, 7µm thick were obtained using a Bright cryostat with a blade specifically designed for skeletal tissue and mounted on silane (Sigma, Poole, UK) coated slides, before air-drying at room temperature for 30-60 minutes. The sections were fixed in analytical grade acetone (Sigma Aldrich Co. Ltd. Dorset, UK) for 10 minutes and air-dried before wrapping in aluminium foil for storage at -20°C until use.

Immunohistochemistry of paraffin-embedded sections.

The sections were de-waxed and rinsed with PBS for 10 minutes. Thereafter, the sections were flooded with normal rabbit serum (Serotec Ltd. Oxford, UK) at 1:5 dilution in PBS for 10 minutes. Serum was removed but not rinsed and the primary antibody was added. Goat anti-type I collagen (Southern Biotechnology Associates,

Alabama, USA) primary antibody at 1 : 20 dilution was added to every 1st section, goat anti-type II collagen (Southern Biotechnology Associates, Alabama, USA) primary antibody at 1 : 20 dilution was added to every 2nd section and mouse anti-type X collagen (Kindly donated by A Kwan, University of Cardiff) primary antibody at 1 : 500 dilution was added to every 3rd section. The slides were incubated with the primary antibodies for 1 hour at room temperature and thereafter thoroughly rinsed in PBS.

Rabbit anti-goat FITC conjugated secondary antibody (DAKO Ltd. High Wycombe, UK) at 1 : 200 dilution in PBS was added to the 1st and 2nd sections from each specimen and left to incubate for 1 hour at room temperature. These sections were then rinsed with PBS and then mounted in Aquamount (BDH Lab. Supplies. Poole. UK) before visualisation under ultraviolet light.

The 3rd sections from each specimen were labelled with a Biotin conjugated anti-mouse secondary antibody (DAKO Ltd. High Wycombe, UK) at 1 : 200 dilution for 1 hour and then rinsed with PBS before addition of ABCComplex (DAKO Ltd. High Wycombe, UK) for 20-30 minutes. They were rinsed again and then incubated with DAB Sigma Fast tablets for 10 minutes. The slides were counterstained with haematoxylin and subsequently dehydrated before mounting in DPX mountant (BDH Lab. Supplies. Poole. UK) and examined with a light microscope.

Immunohistochemistry of undecalcified sections.

The sections were brought to room temperature, hydrated in PBS for 10 minutes and then treated as for paraffin embedded sections as outlined above save for the 3rd sections which were stained for type III collagen using goat anti-type III collagen primary antibody (Southern Biotechnology Associates, Alabama, USA) at a dilution of 1 : 20 in PBS.

Controls

Three sets of controls were also evaluated omitting the antibodies as follows: 1) no primary or secondary antibody, 2) primary but no secondary antibody and 3) secondary antibody but no primary antibody.

All dilutions were made with PBS.

Results.

Histology of osteophyte.

In the H & E sections, the osteophytes consisted of a cancellous bony base and a cartilage mantle. For the purposes of this study, descriptively, the cartilage mantle of osteophytes will be divided into superficial, middle and deep layers. The demarcation between these layers is ill defined but the cellular arrangements seem to be unique to each layer. The thickness of the superficial layer was not uniform in the same specimen in all the sections, but the cells were oriented transversely in this layer in all sections. In the middle layer, the chondrocytes are arranged haphazardly and are mainly rounded in appearance whereas in the deep layer, the chondrocytes are arranged in longitudinal columns (Figures 27b). Vascular channels are visualised

below the columnarly arranged chondrocytes and these vessels seem to demarcate the cartilage mantle from the subchondral bone.

Distribution of type I collagen ;

Type I collagen was expressed in the superficial layers in eight of the ten paraffin embedded sections and in all the undecalcified tissue sections. The expression was both pericellular and territorial (Figure 35). The fibres were mainly transverse in orientation. The intensity of expression appeared to be related to the fibrous component of the superficial layer examined.

Distribution of type II collagen;

Five out of ten paraffin embedded sections and all the undecalcified sections expressed type II collagen. The expression spanned the entire depth of the cartilage mantle of the osteophytes but more in the middle and deep zones and the expression was pericellular, territorial and inter-territorial. In the paraffin embedded and frozen sections, fluorescent lines are seen extending from the deep layer to the middle layer (vertical fibres) with a change in orientation from vertical in the deep layer, to tangential and then transverse in the superficial layer (transverse fibres) (Figure 36). In all the sections, the transverse fibres in the superficial layer had an undulating or scalloped configuration (Figure 37).

Distribution of type III collagen;

Type III collagen was observed as transverse fluorescence fibres in the superficial layer. Some isolated cells in the upper part of the middle layer also revealed pericellular expression of type III collagen (Figure 38).

Distribution of type X collagen;

Type X collagen was observed only in the undecalcified sections and in the lowermost part of the deep layer where often it presents as a band at the cartilage-bone junction (Figure 39). Where the superficial layer showed fibrillation, the chondrocytes in this layer were immunoreactive positive for type X collagen.

Controls.

There was negative staining if either or both antibodies were omitted in both the paraffin embedded and undecalcified sections.

Discussion.

This study confirms the complex pattern of collagen expression in osteophytes as previously noted by others (Aigner *et al* 1995, Hoyland *et al* 1991, Aigner *et al* 1993, Nerlich *et al* 1993, Ronziere *et al* 1990, Goldwasser *et al* 1982) and this presumably reflects the heterogeneity of osteophytes. The overlap in the expression of type III collagen (in the superficial and part of the middle layer) is similar to what was observed by Nerlich *et al* (1993) and Aigner *et al* (1993) in articular cartilage of patients with minor osteoarthritis. This may be a pointer to the reparative nature of osteophytes as previously proposed by Aigner *et al* (1995). From this study, two important inferences can be made. First, there appears to be a maturation pathway in

osteophytes with chondrocytes in the superficial layer expressing collagen types I and III, which suggests the presence of primitive cells in this layer. The expression of type II collagen mainly in the middle to deep layers of the cartilage mantle of osteophytes implies that there are predominantly differentiated chondrocytes in these layers. Type X collagen expression is also most intense at the cartilage-bone interface marking the area of endochondral ossification (Aigner *et al* 1992). Thus it will appear that “precursor” cells occupy the surface layers of osteophytes, mature chondrocytes the middle layers while ‘transforming’ or hypertrophic chondrocytes occupy the cartilage-bone interface.

Second, the collagens of osteophytes probably perform a structural function just as they do in normal cartilage since type I, II and III collagens form substantial fibrillary structures. In the superficial layer, these are horizontally aligned which suggests a gliding function with the fibres sliding over one another during motion and/or loading. In the middle layers, the collagen fibres are vertically oriented and are composed of the longer and larger type II collagen bundles, which may suggest a matrix reinforcing function. Thus the collagen organisation in osteophytes appear to be similar to that of normal cartilage and this may explain why in one of my earlier study (Alonge *et al* 1998), the stiffness of osteophyte (particularly ‘white’ osteophyte) compared favourably with that of normal articular cartilage. The overall impression is that the Bennihoff’s (Benninghoff 1925, Jeffrey 1994), collagen arcade has been recreated in osteophytes.

The structural and mechanical integrity of connective tissues including cartilage depends upon the collagens. Their abundance in osteophyte and their arrangement,

which appears to be similar to that of normal articular cartilage, suggest that osteophytes could be a reliable graft material for full thickness defects in articular cartilage. The presence of precursor cells in the superficial layer of osteophytes also raises the possibility that osteophytes may be capable of growth and adaptation. Thus when used as an osteochondral graft, osteophyte could grow (hypertrophy) into, and fill a defect and that the graft may be capable of undergoing maturity into 'normal' articular cartilage in its new 'environment' over time.

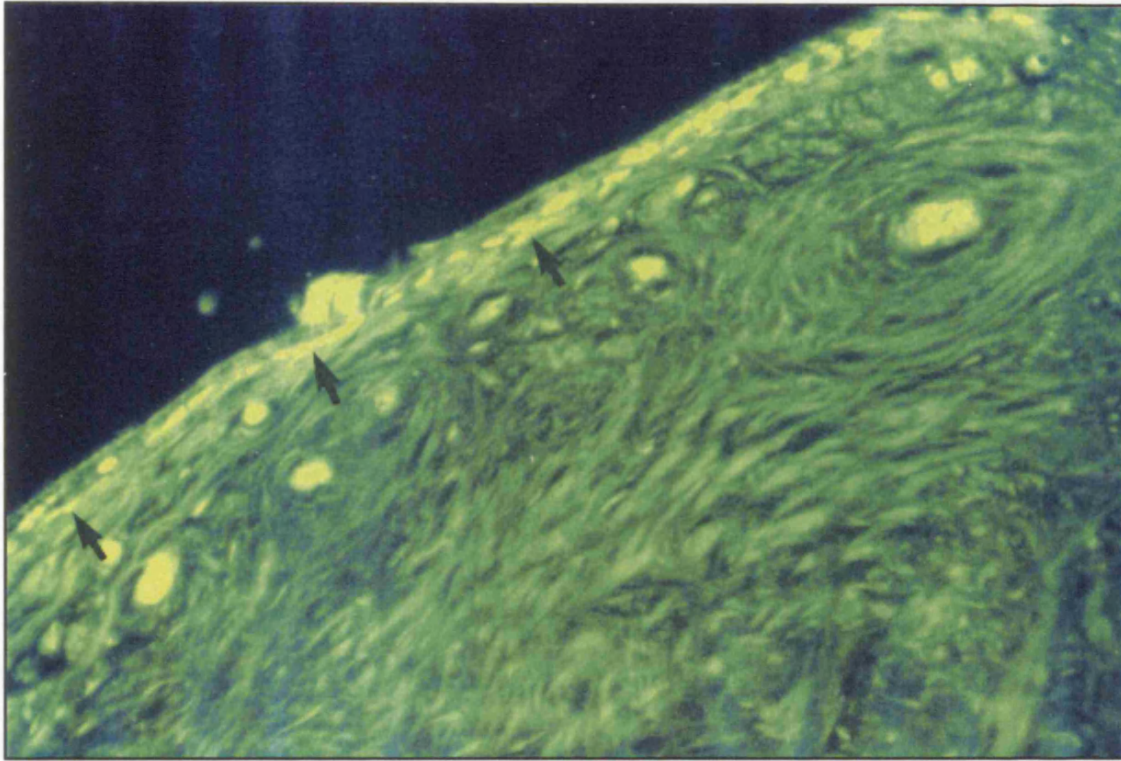


Figure 35 - Photomicrograph of a decalcified section of an osteophyte showing positive immunoreactivity for Type I collagen in the surface layer (arrows). Mag x 200.

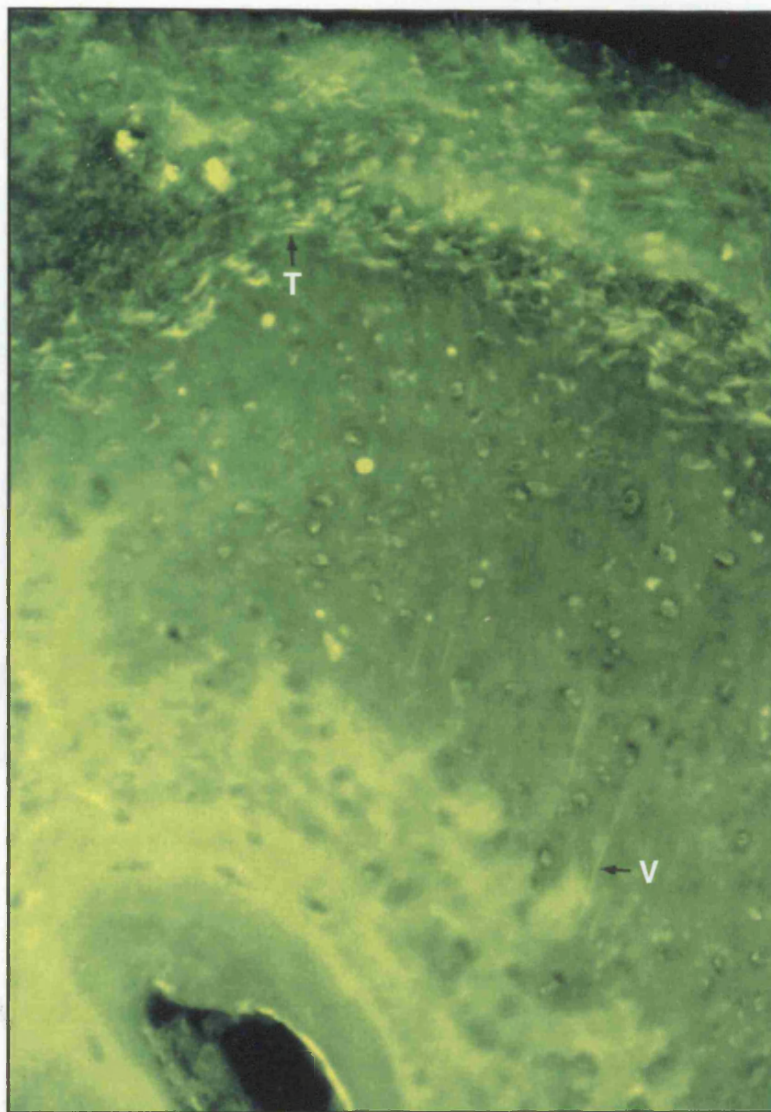


Figure 36 - Photomicrograph of a decalcified section of an osteophyte showing positive immunoreactivity for Type II collagen. The fibres are vertical (V) in the deep and most of the middle layers and transverse (T) in the superficial layer. Mag x 100.

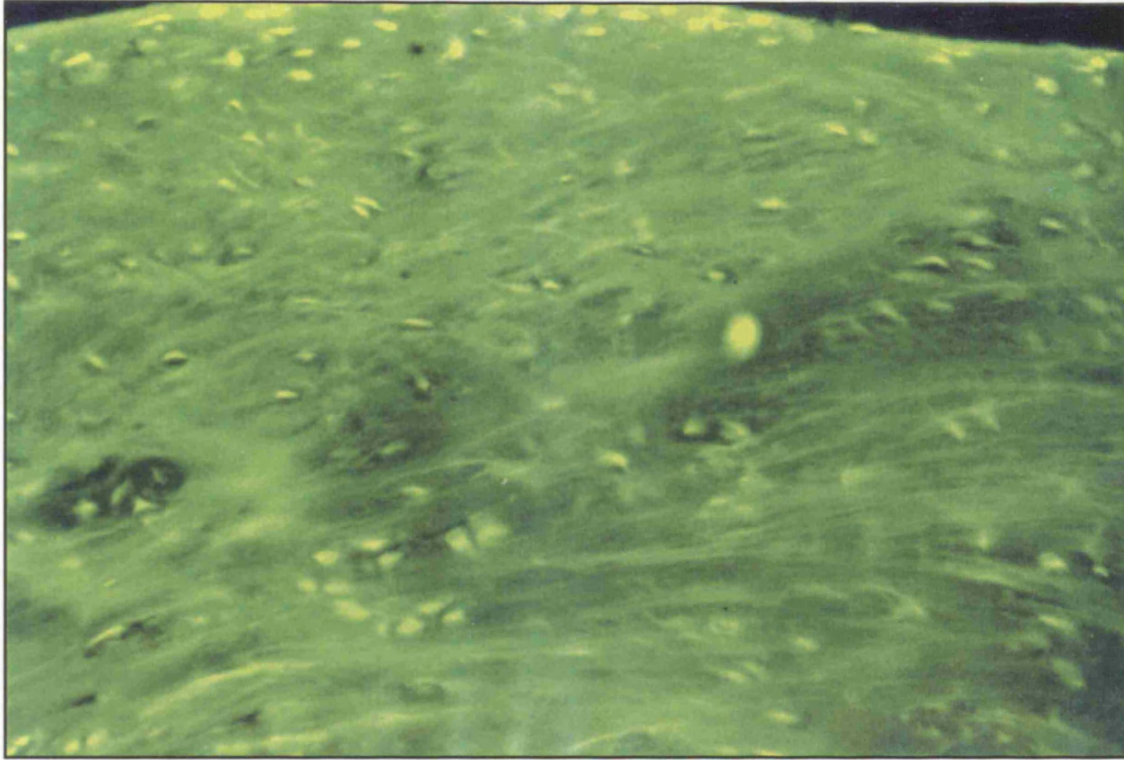


Figure 37 - Photomicrograph of an undecalcified section of an osteophyte showing positive immunoreactivity for Type II collagen. The transverse fibres in the superficial layer shows a scalloped or undulating configuration in some areas. Mag x 200.

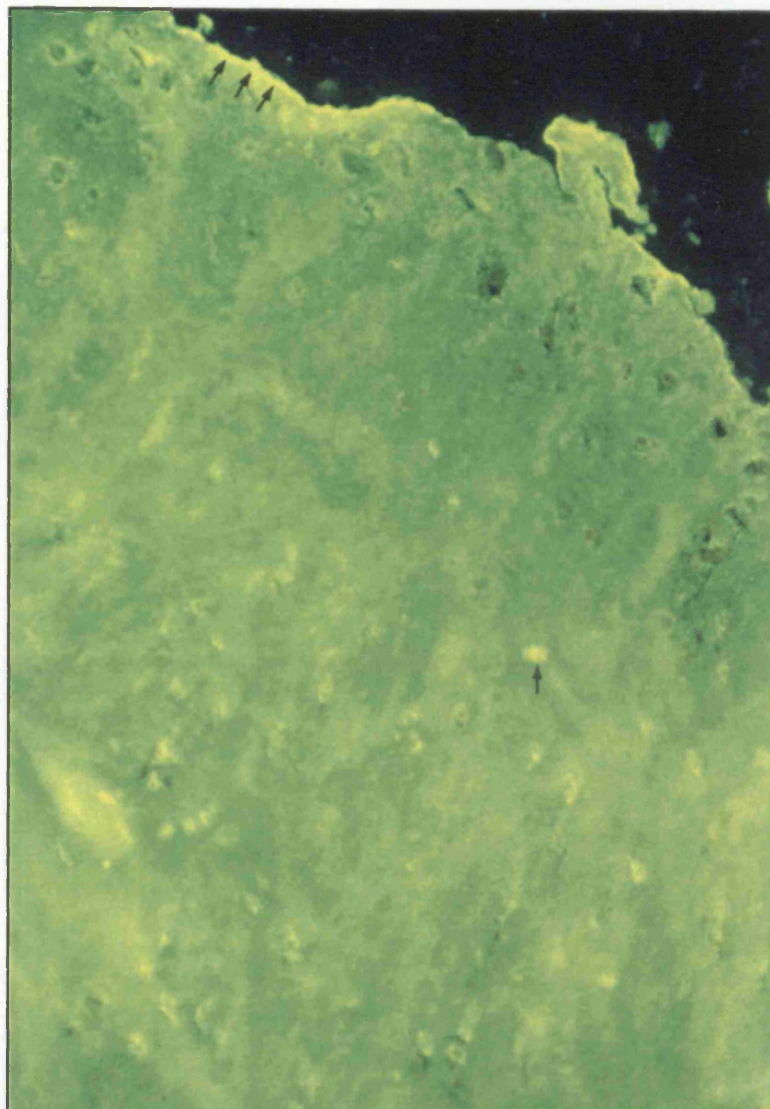


Figure 38 - Photomicrograph of an undecalcified section of an osteophyte showing Type III collagen in the superficial layer and in some isolated cells in the upper part of the middle layer (arrows). Mag x 200.



Figure 39 - Photomicrograph of an undecalcified section of an osteophyte showing positive immunoreactive band for Type X collagen at the cartilage-bone junction (arrows). Mag x 40.

A study of the fibrillary collagen of the cartilage mantle of osteophyte using polarising light.

Summary.

Polarized light microscopy has found numerous applications among which is the evaluation of the birefringes of the collagen structures in cartilage. In this study, the structural arrangement of the collagen fibres in the cartilage mantle of osteophytes has been shown to be arranged in two configurations viz; 'squashed' arch and 'racquet' configurations using the polarized light microscopy. These configurations may confer substantial mechanical advantages and may account for the similarities in the compressive stiffness values of 'white' osteophytes and normal articular cartilage (Alonge *et al* 1998).

Introduction.

The extracellular matrix of normal articular cartilage is reinforced by collagen fibres of high tensile strength (Jeffery *et al* 1991). These collagen fibres are relatively stable materials, characterised by a very slow turn over (Repo and Mitchell 1971) and their main function is to enhance the tensile strength of articular cartilage (Olsen 1996, Miller 1996). The collagen fibres in articular cartilage are also cross-linked together in a co-polymeric network that forms the extracellular framework of the cartilage. They are arranged in such a way as to assist in low-friction movement and transmission of load and they also act as a template upon which hyaluronic acid and proteoglycans (PG) bind either directly or indirectly (Eyre 1991, Mayne 1989, Wu *et al* 1992). This association (i.e. the collagen-PG network) regulates or controls the mobility of water into the cartilage mantle. This effect ultimately restricts the swelling pressure of the hydrophilic

proteoglycans and confers on articular cartilage its tensile strength, normal mechanical function and ultimately its shape (Weckmann and Cabral 1996, Maroudas 1976, Jeffery 1994). Most importantly, collagen fibres have also been shown to anchor the cartilage matrix to the subchondral bone (Speer and Dahners 1979).

There is a unique variation in the organisation of the collagen fibres within different zones of the normal articular cartilage. Benninghoff (Benninghoff 1925) proposed a collagen arcade in which the fibres rose vertically from the subchondral bone, pass towards the surface and arch with a wide loop and return to the bone (Figure 40). This arcade-like orientation is believed to be critical for normal cartilage function (Eyre 1991). MacConaill (MacConaill 1951) on the other hand described a dense network of fibres extending obliquely from the subchondral bone to the superficial region of the cartilage. Most recently, Jeffrey *et al* (1991) described a three-dimensional leaf-like architecture for the collagen fibres of the articular cartilage. In this model, the collagens in the intermediate and superficial zones of the cartilage mantle of the articular cartilage are arranged in a series of closely packed layers of leaves.

Osteophytes are intra-articular osteochondral tissues commonly found at the margins of synovial joints in association with degeneration of the articular cartilage more centrally (Fisher 1939). Although marginal osteophytes are located away from the articulating surface, they have been shown to contribute to some form of joint stability (Pottenger *et al* 1990). The cartilage mantle of osteophytes have also been shown to contain identical genetic types of collagen as normal articular cartilage (Aigner *et al* 1995) and it is therefore to be expected that the organisation of the collagen fibres will be similar to that

of normal articular cartilage. The aim of this study was to examine the arrangement of the fibrillary collagen in the cartilage mantle of osteophytes using polarising light.

Materials and methods.

Osteophytes were obtained from the margins of the femoral condyles in 6 patients undergoing total knee replacement for osteoarthritis (OA) and extra care being taken to avoid contamination by normal articular cartilage. The specimens were formalin-fixed, decalcified and paraffin embedded. Thereafter, 6µm sections through cartilage and bone were obtained, stained with haematoxylin and eosin and then examined using polarising light.

Results

For the purpose of this study, the cartilage mantle of the osteophyte has been divided into superficial, middle and deep layers.

The superficial layer had transversely oriented collagen fibres. The thickness of these fibres was not uniform both within a section and between sections. In the upper part of the middle layer, two types of arrangement of the collagen fibres were observed; (a) 'racquet' shaped arcade with a broad 'racquet' arm (Figure 41) and (b) 'horse shoe' shaped or 'squashed' arch arrangement (Figure 42). In the lower part of this layer, the collagen fibres were orientated almost vertically and there were no tidemarks seen. The collagen fibres in the deep layer were arrayed in a vertical orientation (Figure 43) and they terminated abruptly unto the subchondral bone where the collagen fibres had a whorl appearance. No delicate anchorage of collagen fibres into bone or calcified cartilage was observed.

Discussion.

Polarised light microscopy has found numerous diagnostic applications in clinical medicine because various crystals, fibrous structures, pigments, lipids and bones exhibit birefringence. Birefringence is exhibited by a substance whose molecular structure is asymmetrical or laminated so that two rays of light vibrating in perpendicular planes will travel at different velocities through the substance, producing a fast and a slow ray.

Collagen is bi-refrangent and can be studied using polarising light microscopy, which is an indirect method of studying the fine collagen structure of cartilage. It is also able to resolve major groups of collagen fibres thereby outlining their general orientation (Speer and Dahners 1979). The Benninghoff's arcade (Benninghoff 1925) or the Jeffery's (Jeffery 1991) leaf-like arrangement of the collagen fibres in normal articular cartilage are both similar to an 'open' arch configuration. This configuration does not seem to be mechanically suitable to resist compressive loading. However, the normal articular cartilage is a very resilient tissue and it has the ability to withstand repetitive or cyclical physiological load. This ability has been attributable to the abundant proteoglycans in the extracellular matrix, which form complex networks with the collagen fibres, and these networks in turn confer on the articular cartilage its resilience and viscoelastic properties (Jeffery 1994).

In the osteophyte cartilage, the collagen fibres are arranged in either a 'squashed' arch or a 'racquet' configuration. The 'squashed arch' configuration, which is made up of numerous expanding loops bare some resemblance to, the collagen arcade described by Benninghoff. Mechanically, these configurations ('squashed arch' and 'racquet') appear to be superior to the normal articular cartilage's open arch configuration with respect to their ability to resist compressive load. The multiple 'racquets' and 'squashed' arches and their accompanying cells constitute 'bio-mechanical' units. The large number of

these units and the subsequent volume of the collagen fibres in osteophyte should limit deformation of this tissue when axial load is applied. However, Oni and Morrison in 1998 have shown that the compressive stiffness of osteophytes is far lower compared to that of normal articular cartilage. This could be due to the fact that osteophytes, being a repair tissue (Aigner *et al* 1995) have a relatively high turn over of the collagen contents in its cartilage mantle and thus it is more dynamic than normal articular cartilage. This may therefore alter osteophyte's resistance to deformation or loading. The proteoglycan contents (types and concentration) of the osteophytes cartilage may be different from that of normal articular cartilage. This may affect the collagen-PG interaction alluded to earlier (in normal cartilage) and ultimately the mechanical properties of osteophyte. Although the collagen fibres in the extracellular matrix provides cartilage with its tensile strength, it would appear that this alone does not account for the ultimate mechanical strength of cartilage. Further studies needs to be carried out to ascertain the types and the quantity of the extracellular matrix preoteoglycan in the cartilage mantle of osteophytes.

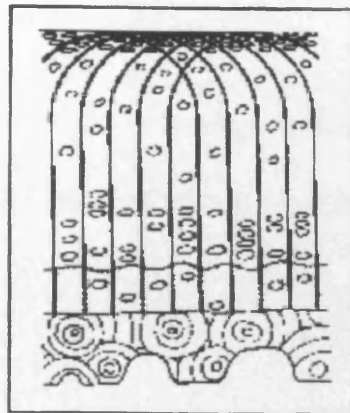


Figure 40 - Bennighoff's collagen arcade in normal articular cartilage

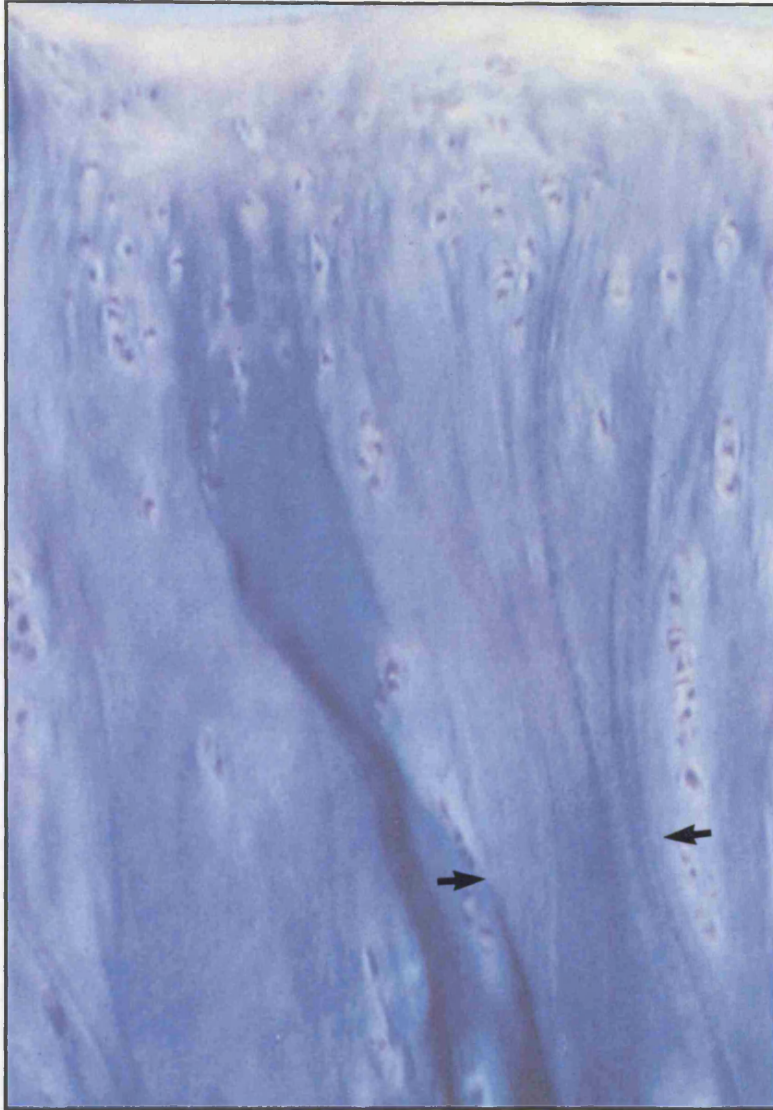


Figure 41 - 'Racquet' shaped collagen architecture in osteophyte. Mag x 200.

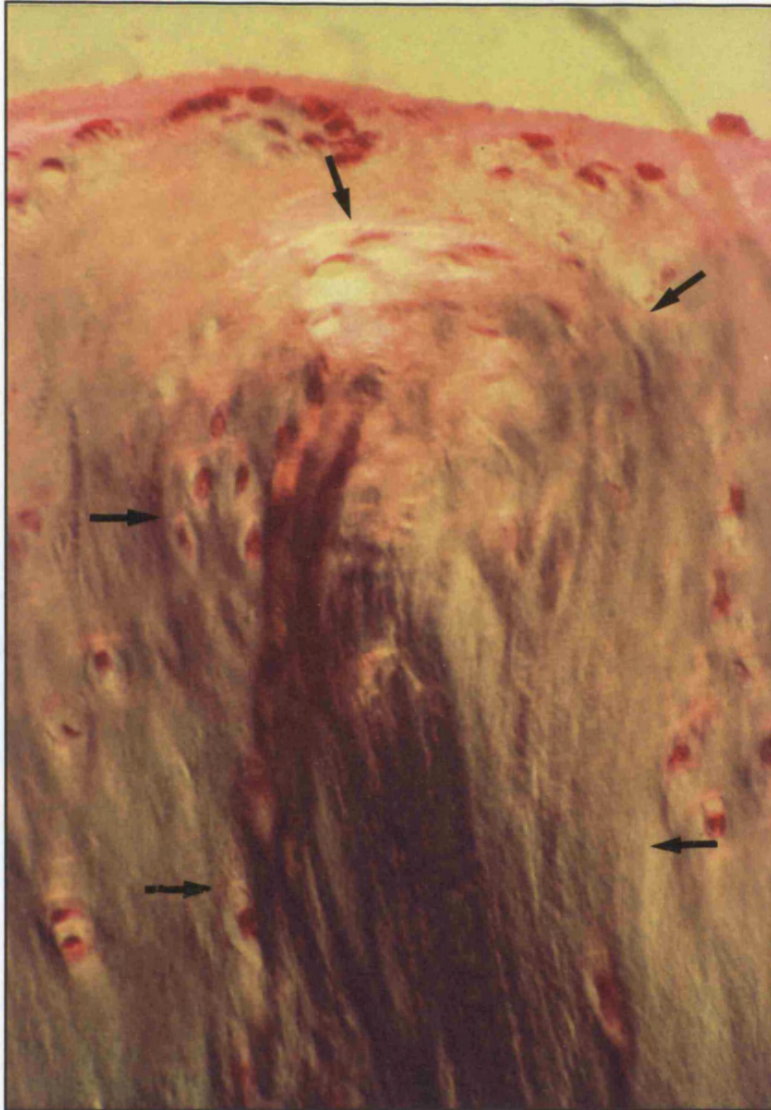


Figure 42 - 'Horse-shoe shape' or 'squashed arch' shaped collagen architecture in osteophyte. Mag x 200.

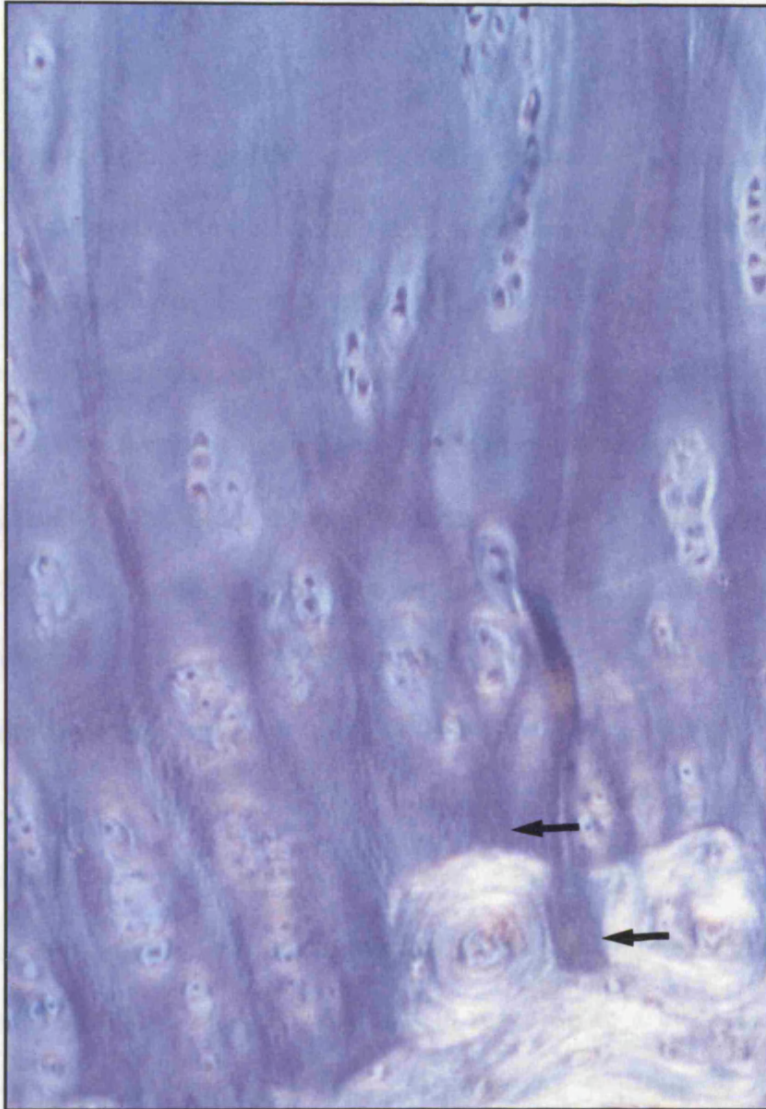


Figure 43 - Collagen fibres in the deep layer of osteophytes oriented vertically.
Mag x 200.

The ultrastructure of the peri-articular osteophytes – an evaluation by scanning electron microscopy.

Summary.

Osteophytes are intra-articular osteochondral tissues, which are usually found at the margins of a degenerating articular cartilage. This study has shown that the cartilage mantle of osteophytes is continuous with that of the adjoining normal articular cartilage and there were criss-crossing of collagen fibres at the point of continuity. However, it would appear that a bar of acellular tissue separates the subchondral bones of both tissues. The cellular arrangements in osteophytic cartilage were also similar to that of the adjoining normal articular cartilage but the fine network of collagen fibres in the cartilage mantle of osteophytes were absent. The calcified zone was also noted to be wider in osteophytes.

Introduction.

Osteophytes are intra-articular outgrowths that are commonly found at the margins of the articular cartilage in synovial joints in response to a more central full thickness damage to the cartilage (Key 1931, Fisher 1939, Peterson *et al* 1984). They are composed of a cartilage mantle and subchondral bone. The native collagen types found in the cartilage mantle of osteophytes have been shown to be similar to those found in normal articular cartilage (Aigner *et al* 1995).

The exact mechanism of formation of osteophytes remains unknown and various mechanisms have been proposed (Moskowitz and Goldberg). These osteochondral tissues have been shown to merge with or even overgrow the adjoining articular

cartilage (Aigner *et al* 1995, Resnick *et al* 1991). The subchondral bone of osteophyte is also presumed to merge with and communicate freely with that of the adjoining articular cartilage subchondral bone (Marshall 1969, Gilbertson 1975). These conclusions will therefore suggest that osteophyte do play a role in load transmission in the degenerate synovial joint.

Scanning electron microscopy has great depth of focus and high degree of resolution and this permits an adequate investigation of relatively large specimens in three planes (Redler and Zimmy 1970, Clark 1985, Jeffery *et al* 1991). The aim of this study was to evaluate the surface architecture of osteophytes, osteophyte-articular cartilage junction and the link between these two intra-articular structures.

Materials and methods.

Osteophytes were obtained from the trochlear margin of the distal femur in 6 patients undergoing total knee replacement for osteoarthritis (OA). Patients were aged 66-88 years. Osteophytes with adjoining articular cartilage were also obtained from the same patients.

Both groups of specimens were fixed in 2% cacodylate buffer for 3 weeks. Thereafter, the cacodylate buffer was replaced with 50% ethanol (30 minutes), 70% ethanol (30 minutes), 90% ethanol (30 minutes), 100% ethanol (1 hour) and stored in 100% acetone until ready for critical point drying. The specimens were transferred into a gelatin capsule and the capsule transferred into the boat of the critical point dryer chamber. The boat was flushed with liquid CO₂ about three times over a period of about 10 minutes to remove all the acetone and the specimens left for one hour. Thereafter, the chamber was heated with hot water until the temperature reached 35⁰C

and then the pressure inside the chamber was slowly reduced to complete the critical point drying. The specimens were removed from the chamber, glued unto specimen stubs, coated with 15 nanometre of gold using a Polaron coating unit and viewed with the DS 130 scanning electron microscope (Hall *et al* 1978).

Results.

The surface of the cartilage mantle of osteophyte had numerous undulations and was covered with materials resembling cellular debris and deposits of inspissated synovial fluid (Redler and Zimmy 1970) (Figure 44). The surface of the articular cartilage adjoining the osteophyte also had numerous debris and cracks on the surface (Figure 45a) and a groove clearly demarcating it from the osteophyte cartilage (Figure 45b).

The sagittal sections of osteophytes revealed a rough granular surface with fewer lacunae in the cartilage mantle, but the collagen fibre in the deep layer was orientated longitudinally. In addition, a thick layer of tissue (calcified cartilage) was observed between the cartilage mantle and the subchondral bone of the osteophytes (Figure 46a).

The middle or intermediate layer of the osteophytic cartilage had fewer lacunae and the fine criss-cross mesh of collagen fibres commonly found in normal articular cartilage was absent (Figure 46b). The chondrocytes in the deeper layer of the osteophytic cartilage were arranged in a columnar fashion (Figure 46c).

The sagittal section of the adjoining articular cartilage showed all the characteristic features of a normal articular cartilage with regards to cellular arrangement and orientation of the collagen fibres (Figures 47a-47c). In addition, there was a thin layer

of transversely oriented fibres studded with debris overlying the superficial layer of this adjoining normal articular cartilage (Figure 48).

Sagittal section of the osteophyte-articular cartilage composite tissue revealed that the cartilage mantle of osteophyte was continuous with that of the adjoining articular cartilage. However, the subchondral bone of both tissue were clearly demarcated by a continuous ridge of acellular tissue which originated from the apex of the cartilage merger down to the subchondral bone proper (Figure 49). In these sections, bridging collagen fibres were observed connecting the cartilage mantles of the osteophyte and the adjoining articular cartilage both in the superficial and deep cartilage layers. In the deep cartilage layer, some of the bridging collagen fibres were 'feather' shaped (Figure 50 & 51). In the superficial layer, the collagen fibres in the articular cartilage were observed to form loops and meshes with a few of the fibres terminating in rounded knobs. On the other hand, most of the collagen fibres in the superficial layer of the osteophytes segment terminated in knobs without forming loops and their meshes were fewer (Figure 50).

The subchondral bone of osteophytes on sagittal section reveals a cancellous pattern mainly (Figure 46c).

Discussion.

The findings from this study clearly show that the cartilage mantle of osteophyte was morphologically similar and was continuous with that of the adjoining articular cartilage, which was relatively normal. This similarity probably explains why osteophytes, particularly 'white' osteophytes have similar compressive and stress-stiffness values with normal articular cartilage (Alonge *et al* 1998).

Osteophytes have been thought to provide no benefit to the joint in which they occur and they are generally implicated as important causes of local joint pain, nerve compression and restriction of joint movement (Marshall, 1969, Anderson 1985, Fisher 1922, Jeffery 1975). Pottenger *et al* (1990) however, found that excision of osteophytes in valgus and varus knees led to instability of the joints. The ability of osteophyte to assist in joint stabilisation in these situations is probably due to buttressing of the adjoining articular cartilage by the osteophytic cartilage during load transmission since their cartilage mantles are continuous.

From this study, it would also appear that in the pathogenesis of osteophytes, the tissue(s) of origin must bear close proximity to the adjoining articular cartilage. The cross-linking of collagen fibres between the osteophytic and normal articular cartilage may also suggest that the adjoining cartilage contribute to the formation of osteophytes.

Fisher (1939) has shown that the more lateral or marginal area of the normal articular cartilage is covered by synovial reflection, which will be referred to as 'cartilage synovium' for the purposes of this discussion. This 'cartilage synovium' meets with the synovial lining of the joint or 'capsular synovium' and the periosteum, in an area which for the purpose of this discussion will be referred to as the tri-furcation. At the tri-furcation, the normal articular cartilage also terminates and it becomes continuous with bone. One can therefore propose that following a full thickness defect more centrally in the articular cartilage, chemical, humoral and other factors are released by the damaged chondrocytes as well as an alteration in the pH of the tissue. The factors released are then transmitted by the 'cartilage synovium' to the tri-furcation. These chemical agents or factors acting at the tri-furcation initiate the proliferation and subsequent differentiation of mesenchymal tissue(s) in this area. The chondrocytes of

the normal cartilage adjoining the tri-furcation may direct or influence the differentiation of the proliferating tissue along a cartilaginous pathway. The resultant cartilaginous tissue is then secondarily invaded by blood vessels probably from the periosteum and this in turn leads to ossification of the basal region of the neocartilage. Although the cartilage mantle of the resulting osteophyte is continuous with the adjoining articular cartilage, the newly formed subchondral bone in this tissue is completely separated from that of the adjoining normal articular cartilage as found in this study (Figure 52).



Figure 44 Scanning electron micrograph of the surface of the cartilage mantle of osteophytes showing numerous cellular debris and undulations.

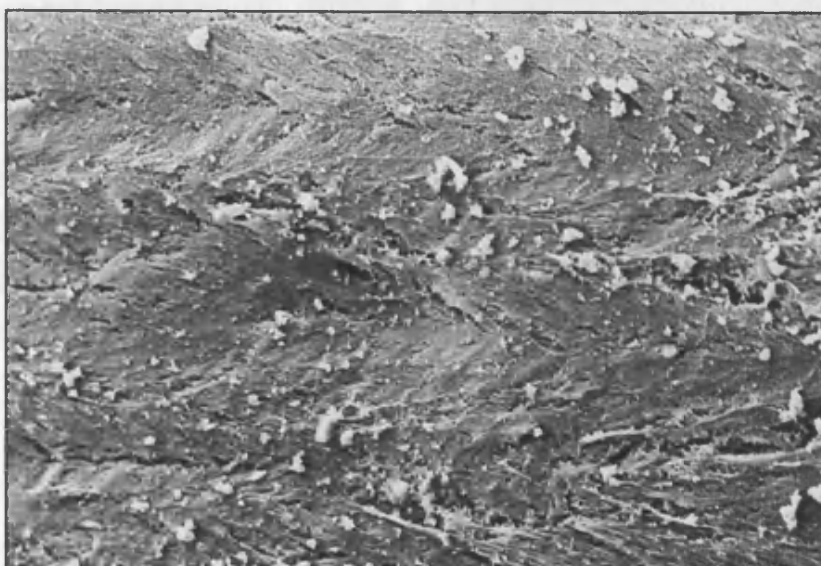


Figure 45a - Scanning electron micrograph of the surface of the adjoining normal articular cartilage showing cracks and debris on the surface.



Figure 45b -Scanning electron micrograph of the surface of the adjoining normal articular cartilage showing a groove demarcating it from the osteophyte cartilage (arrowheads)

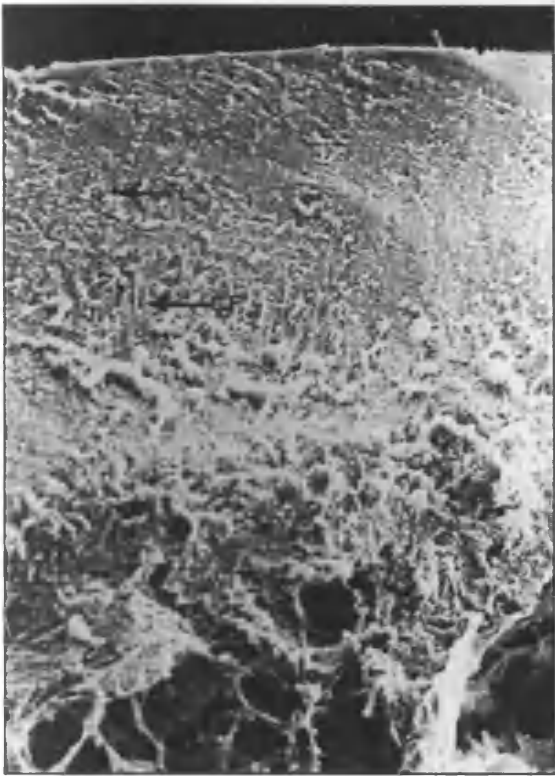


Figure 46a
Sagittal section of the osteophyte showing empty lacunae (L), longitudinal collagen fibres in the deep layer (CF) and a thick layer of calcified tissue at the cartilage/bone interface.

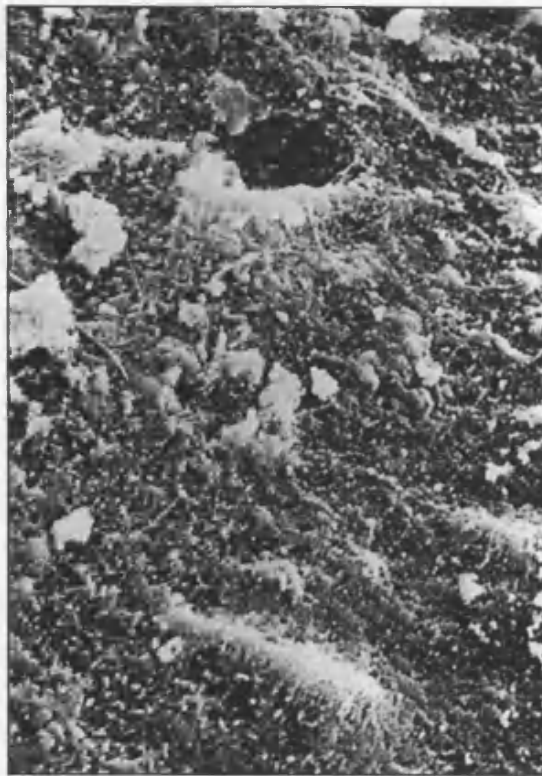


Figure 46b
Sagittal section of the middle layer of the osteophytic cartilage showing an empty lacuna and a rough granular surface. The fine criss-cross mesh of collagen fibres is lacking.

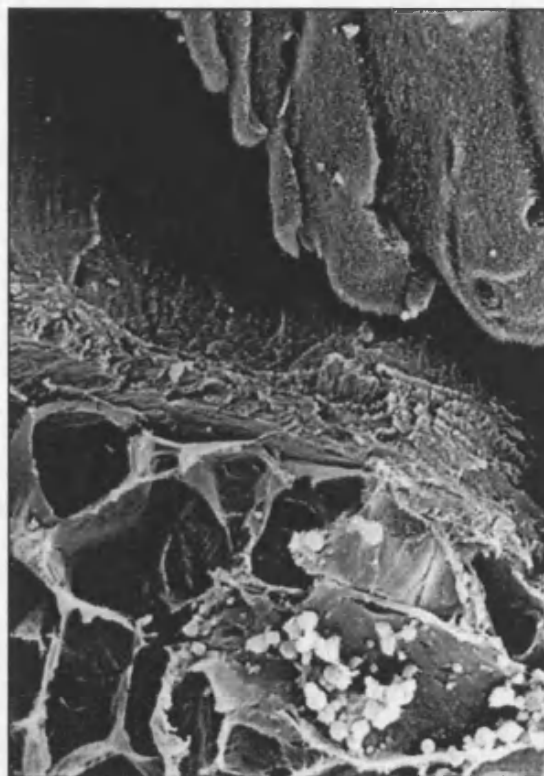


Figure 46c -Sagittal section of the deep layer of the osteophytic cartilage showing columnar arrangement of the chondrocytes.

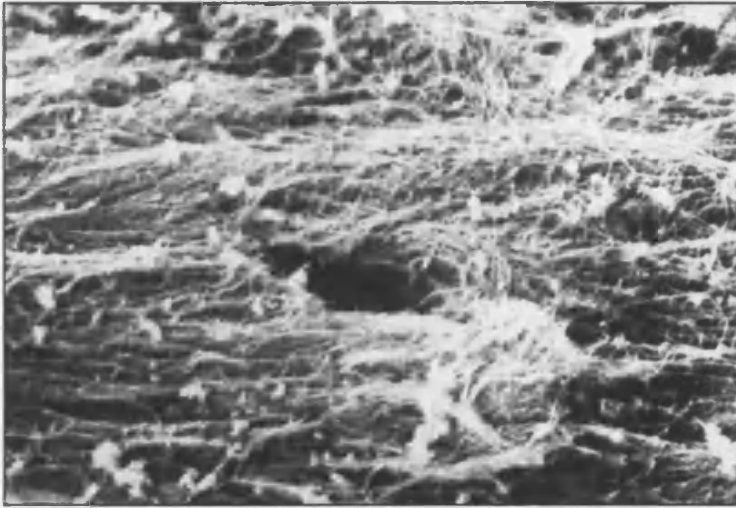


Figure 47a Sagittal section of the superficial layer of the adjoining articular cartilage showing transversely oriented lacuna and collagen fibres.

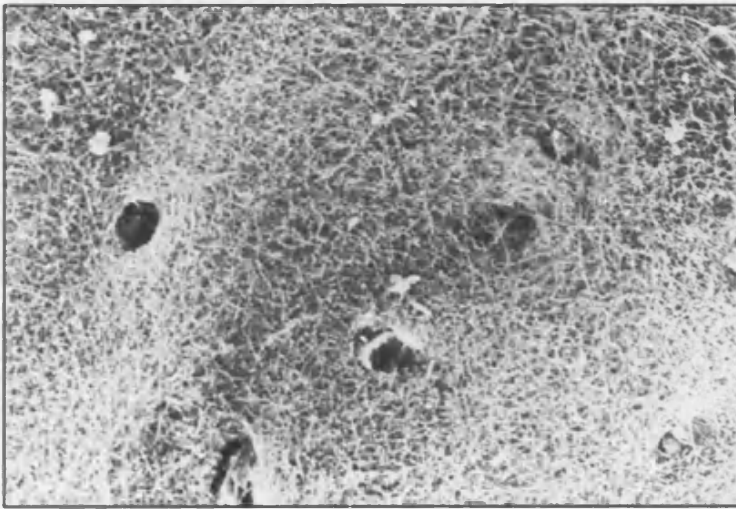


Figure 47b Sagittal section of the middle layer of the adjoining articular cartilage showing a criss-cross mesh of collagen fibres and haphazardly arranged lacunae and chondrocytes.



Figure 47c Sagittal section of the deep layer of the adjoining articular cartilage showing longitudinally disposed collagen fibres and lacunae.



Figure 48 - Sagittal section of the superficial layer of the adjoining articular cartilage showing a thin layer of transversely oriented fibres devoid of cells overlying the superficial layer.

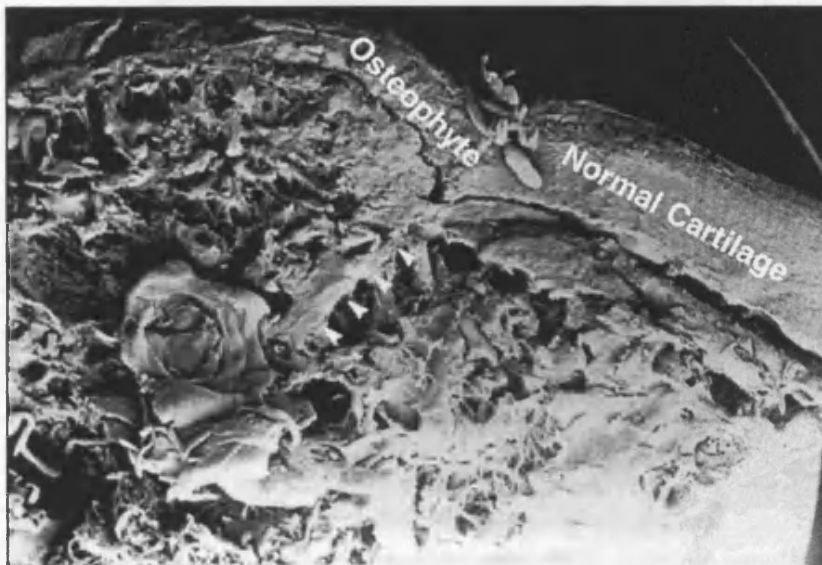


Figure 49 - Sagittal section of the osteophyte/articular cartilage junction showing an acellular bar (arrowheads), separating the subchondral bone of the osteophytes and that of the adjoining articular cartilage.

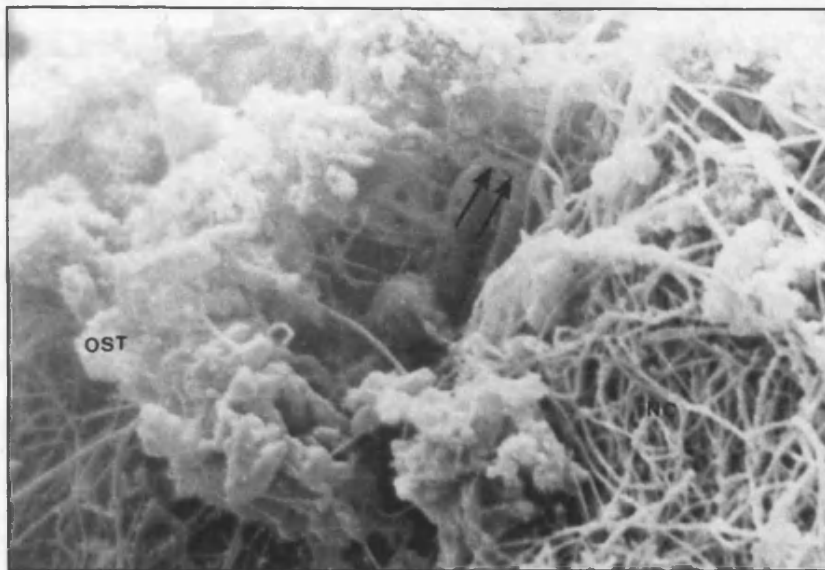


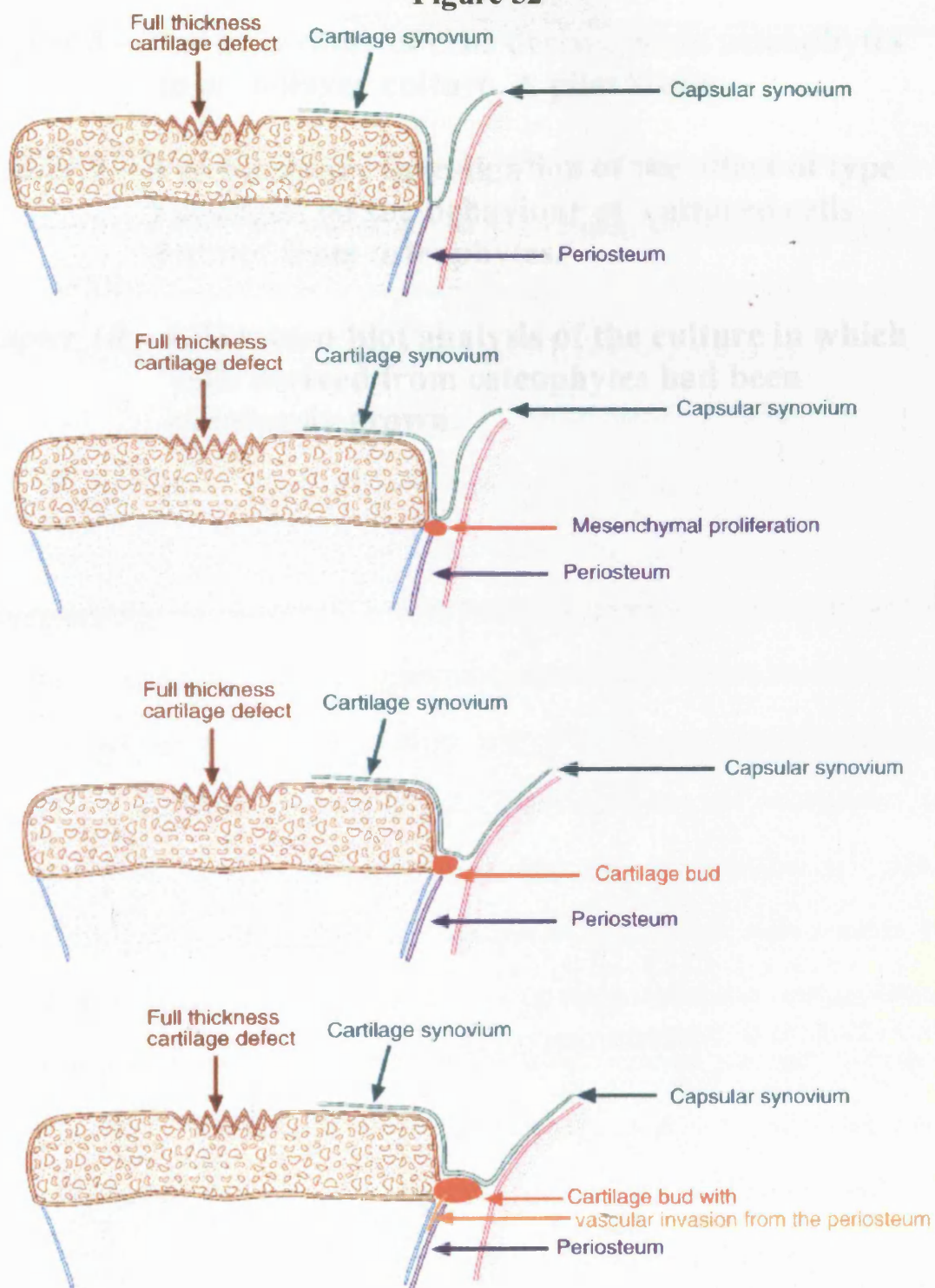
Figure 50 - Sagittal section of the osteophyte/articular cartilage junction showing bridging collagen fibres in the superficial layer (arrows). (NC = Normal Cartilage, OST = Osteophyte).



Figure 51 - Sagittal section of the osteophyte/articular cartilage junction showing bridging collagen fibres in the deep layer. Some of these bridging fibres are 'feather' shaped, (arrowheads). (NC = Normal Cartilage, OST = Osteophyte).

MECHANISM OF OSTEOPHYTE FORMATION

Figure 52



PART 3 – OSTEOPHYTES AS CELLULAR GRAFTS?

***Chapter 8* – The behaviour of cells derived from osteophytes in monolayer culture. A pilot study.**

***Chapter 9* – A preliminary investigation of the effect of type I collagen on the behaviour of cultured cells derived from osteophytes.**

***Chapter 10* – A Western blot analysis of the culture in which cells derived from osteophytes had been previously grown.**

The behavior of cells derived from osteophytes in monolayer cell culture. A pilot study**Summary**

Cells from the cartilage mantle of osteophytes obtained from the margins of osteoarthritic knee joints have been successfully grown in a monolayer culture. Tinctorial staining with von Kossa and Toluidine blue techniques reveal that the cells elaborate cartilage and calcifiable matrix. Protein immunocytochemistry reveal that the cells synthesize types I, II and III collagens as well as 'link' protein.

Introduction

The purpose of this study was to examine the behavior of cells (chondrocytes) of osteophyte origin in a monolayer culture. Such an investigation could be useful for a variety of reasons. First, it could provide an opportunity for investigating the phenotype of osteophyte-derived chondrocyte and its response to various manipulations. An understanding of the properties of these cells could result in the development of new treatment modalities for full thickness articular cartilage defects such as been suggested in gene therapy. Second, it could generate information regarding the fundamental processes in degenerative arthritis since osteophytes, which are osteo-cartilaginous new growths developing at the margins of such joints, may represent an attempt at repair (Aigner *et al* 1995). Third, it may allow the examination of an alternative source of chondrocytes for autologous transplantation. Hitherto,

obtaining chondrocytes for this procedure has involved traumatizing normal articular cartilage (Brittberg *et al* 1994) whereas osteophytes are expendable tissues almost universally present in the margins of joints with full thickness cartilage defects (Peterson *et al* 1994) and advanced osteoarthritis (OA). Osteophytes appear to be an intrinsic feature of osteoarthritis and this may suggest that their formation may be due to the same factor(s), which lead to the imbalance between mechanical demands of the cartilage and its resilience resulting in osteoarthritis. However, osteophytes are not consistently commonly found in association with severe osteoarthritis.

The co-existence of osteophytes and full thickness damage of the articular cartilage, gives an impression that osteophyte-derived chondrocytes may be abnormal in some unspecified way. Changes in the metabolic activity of chondrocytes have been cited as a factor in the genesis of OA (Hammerman 1989) and a reduction in matrix synthesis could, in theory, cause deterioration in the mechanical properties of articular cartilage. On the other hand, increased matrix synthesis in a diseased cartilage could result in its repair and regeneration. Osteophytes are composed of cartilage and bone; they grow and remodel (Marshall 1969, Gilbertson 1975, Resnick *et al* 1991). These activities involve the stimulation of dormant cells to proliferate and differentiate and, to synthesize and turnover various matrix macromolecules. It would seem logical therefore, that osteophytic cells would retain the abilities of their cell(s) of origin.

Following the transplantation of cultured normal articular chondrocytes into full thickness articular cartilage defects, a fibrocartilaginous repair tissue is formed (Brittberg *et al* 1994, Chesterman and Smith 1968). The tissue architecture differs from normal articular cartilage in several respects. In particular, the calcified zone is widened and the chondrocytes are not arranged in columns. By contrast, osteophytes are histologically similar to articular cartilage. In osteophytes, the chondrocytes appear to be isogenic and are arranged in similar fashion as chondrocytes in normal articular cartilage. Therefore, osteophytes particularly 'white' have been suggested as a potential tissue for osteochondral grafting for full thickness articular cartilage defects (Alonge *et al* 1998). The ability of osteophytic cartilage to retain a chondrocytic phenotype during cell culture is investigated in this report.

Materials and methods

The specimens consisted of (a) osteophytes obtained from the margins of the femoral condyles in 6 patients (age: 65-88) undergoing a total knee replacement for osteoarthritis (OA) and (b) visually normal articular cartilage obtained from apparently unaffected areas of the joint.

Cell preparation

The cartilage covering each specimen was scraped off under aseptic conditions and washed in sterile tissue culture medium (α -MEM) supplemented with 10% foetal

bovine serum, L-glutamine, penicillin and streptomycin (Gibco, Life Technologies, Paisley, UK). The specimens were then transferred into sterile universal containers and enzymatically dissociated in 2% w/v collagenase (Type Ia, Sigma, Poole, UK) at 37°C for 3 - 4 hours. Each universal container was vigorously agitated every 30 minutes using a Whirlmixer.

At the end of the dissociation period, each sample was shaken and left for 30 seconds to allow sedimentation of bone pieces and undigested fragments. The digested cell suspension was removed, transferred to a new sterile universal and centrifuged at 2000rpm for 5 minutes. Supernatants were discarded and the pellet was re-suspended in α -MEM.

Culture technique (Figure 53)

Cells were counted using a haemocytometer and thereafter they were plated out into 35mm tissue culture dishes (Nunc, Life Technologies, Paisley, UK) at two cell densities - **Medium** (3×10^5) and **High** (8×10^5) cells/dish respectively. At the end of three weeks, some dishes from both the **Medium** and **High** density original cultures were passaged and re-plated at 3×10^5 and 8×10^5 cells/ 35mm dish to encourage new cell growth (Passage 1 cells: **M-P1** and **H-P1**). Passage 1 cells were grown for 7 days when samples were re-passaged and plated out at 3×10^5 and 8×10^5 cells/ 35mm dish (Passage 2 cells: **M-P2** and **H-P2**). At the end of a two week culture period, Passage

2 cells were once again passaged and plated out either at 1×10^5 cells/dish (low density = **M-P3L** and **H-P3L**) or at 1×10^5 cells in a $10\mu\text{l}$ drop (1×10^7 cells/ml), (high density = **M-P3MM** and **H-P3MM**) using the "micromass" technique of Ahrens et al. (1977) (Passage 3 cells). All passaged and un-passaged cultures were examined for infection, viability and morphology every day using an Olympus phase contrast inverted microscope. They were fed every third day and maintained for a total 10-week culture period. Thereafter, all the cultures were fixed in 10% buffered formalin for 48 hours and processed for histology and immunocytochemistry.

Passage technique

Cells were first incubated in a trypsin/EDTA solution (Gibco, Life Technologies, Paisley, UK) for 15 minutes at 37°C . Next, loose cells were transferred into a sterile universal and an equal volume of α -MEM containing serum was added to inactivate the trypsin. Loosely attached cells were detached using a rubber policeman and added to the universal. Thereafter, the cells were centrifuged at 2000 rpm, the supernatant was discarded and the pellet re-suspended in α -MEM. Finally, they were counted using a haemocytometer and then plated out at the densities outlined above.

Histology

Samples of culture dishes at each time interval (P1, P2 and P3) were stained.

1. Toluidine blue staining.

First, formalin was drained from the culture dish and the culture washed several times with phosphate buffered saline [PBS]. Next, Toluidine blue (TB) was added and the dish was left to stand for 3-5 minutes. Excess dye was removed and the culture washed again thoroughly with PBS. The culture was periodically examined under a light microscope until the desirable intensity of staining was obtained. Finally, the culture was mounted and examined under the light microscope.

2. von Kossa staining(Bancroft and Stevens 1990).

First, formalin was drained from the culture dish and the culture was washed several times with distilled water. Next, 1% silver nitrate solution was added and the culture was then exposed to ultra-violet lamp for 10-20 minutes. The culture was washed in 10 changes of distilled water and then 2.5% sodium thiosulphite was added and the dish was left to stand for 5 minutes. Thereafter, the culture was washed with tap water and counterstained with the von Gieson stain ¹¹ for 5 minutes. Finally, after further several washings with distilled water, the culture was mounted and examined using the light microscope.

Immunocytochemistry

Samples of culture dishes at each time interval (P1, P2 and P3) were stained.

The culture dishes were rinsed in PBS several times to remove the fixative. 1 mg/ml testicular hyaluronidase in sodium-acetate buffer (Sigma, Poole, UK) was added to

the dishes and allowed to stand for 1 hour at room temperature. After rinsing again in PBS, the culture dishes were divided into four groups:

group A dishes were treated with goat anti-type I collagen primary antibody (Southern Biotechnology Associates, Alabama, USA) at 1:20 dilution;

group B dishes were treated with mouse anti-type II collagen primary antibody raised in chicken (Southern Biotechnology Associates, Alabama, USA) at 1:10 dilution;

group C dishes were treated with goat anti-type III primary antibody (Southern Biotechnology Associates, Alabama, USA) at 1:20 dilution; and,

group D dishes were treated with mouse anti-Link protein primary antibody (Developmental Studies Hybridoma Bank, Iowa, USA) at 1:20 dilution.

All dilutions were made in 0.1% bovine serum albumin (BSA) and PBSTween.

Following treatment, each culture dish was incubated with the primary antibody for 90 minutes with gentle sustained rhythmic movement on a rocker machine at room temperature and then washed several times with PBSTween. Next, groups A and C cultures were treated with rabbit anti-goat horse radish peroxidase (HRP) conjugated secondary antibody (DAKO Ltd. High Wycombe, UK) in PBSTween at 1:1000 dilution; groups B and D cultures were treated with anti-mouse HRP conjugated secondary antibody (DAKO Ltd. High Wycombe, UK) in PBSTween at 1:150 dilution, and were again incubated for 90 minutes with gentle sustained rhythmic movement on a rocker machine at room temperature. Thereafter, the dishes were

washed several times in PBSTween and counterstained with DAB Sigma Fast tablets (Sigma, Poole, UK) solution until brown staining appeared.

Results

In general, cultures from osteophytic cartilage appeared to have similar characteristics as cultures of normal articular cartilage.

Phase contrast microscopy

Primary cultures - Cells derived from osteophytes reached confluence before cells of normal articular cartilage. At medium cell densities, osteophyte-derived cells reached confluence by 7 days whilst normal articular cartilage-derived cells took over 10 days.

At higher cell densities, the primary cultures reached confluence by 3 days for osteophyte and 5 days for articular cartilage. Once confluence was reached, both medium and high-density cultures maintained a similar morphology, which was mainly fibroblastic (Figure 54).

At the end of the 10-week culture period, fibroblasts were still the main cell type observed. Some osteophyte cultures contained small areas of polygonal shaped cells embedded in a refractile extracellular matrix (Figure 55). In addition, there were fragments of bone-like tissue (Figure 56), islands of unidentified crystals (Figure 57) and several multinucleated giant cells observed (Figures 58).

Passage 1- **M-P1** cells were consistently fibroblastic during the entire culture period. At the end of the 10 weeks, there were still regions with refractile matrix, very few bone-like clumps and few multinucleated giant cells.

Passage 2. - At the end of the culture period, both **M-P2** and **H-P2** cultures were primarily fibroblastic with scanty bone debris or giant cells. Islands of unidentified crystals were more numerous and there were clusters of polygonal cells within refractile matrix.

Passage 3- **P3L** cultures were fibroblastic regardless of the original source of the cells. Both **M-P3MM** and **H-P3MM** consisted of colonies of tightly packed polygonal cells with adjoining refractile matrix and surrounding these colonies are more loosely packed fibroblastic cells (Figure 59). In the center of the culture were islands of calcification some of which were attached to the unidentifiable crystals.

Histology

All cultures stained positive with Toluidine blue (Figure 60) although there were variations in the intensity depending on the age of the culture. The **M-P3MM** and **H-P3MM** cultures were more intensely stained than the primary, **P1** and **P2** cultures.

All cultures stained positive with the Von Kossa stain (Figure 61) and the trend of staining intensity were very similar to that of Toluidine blue.

Immunocytochemistry

All cultures were immunoreactive positive for types I (Figure 62), II (Figure 63) and III collagens (Figure 64). The intensity of staining was more pronounced for types I and III collagens and positive cells were found throughout the dishes. In contrast, type II collagen staining was more restricted to individual cells, usually in the regions of high cell densities although marked territorial staining were observed in some cultures. All cultures were also immunoreactive positive for link protein (Figure 65); the intensity of the staining being stronger with **M-P3MM** and **H-P3MM** cultures.

Discussion

According to these findings, it is possible to grow in ordinary monolayer cultures cells from cartilage harvested from osteophytes. Importantly, the results reveal that these cells may confluence much earlier than cultured normal articular cartilage-derived cells, which suggests that osteophyte-derived cells have a higher proliferative ability. Other workers have shown that the rate at which cultured cells confluence is related to the rate at which they proliferate (Berry *et al* 1992). It is to be expected, therefore, that, if transplanted, osteophyte-derived cells would repair full thickness articular cartilage defects much quicker than normal articular cartilage-derived chondrocytes.

The inability of chondrocytes cultured from normal articular cartilage to reproduce the normal architecture at their transplanted sites (Brittberg *et al* 1994, Peterson *et al*

1984, Resnick *et al* 1991, Grande *et al* 1989) may be due to the low proliferative ability of the cells (Berry *et al* 1992). Cell proliferation rate is inversely proportional to the differentiation rate (Kuettner *et al* 1982, Grundmann *et al* 1980, Gibson *et al* 1982, Rooney 1984) so that a slower rate of cell proliferation would be accompanied by early differentiation. As a consequence of early differentiation, transplanted chondrocytes may stop proliferating before a defect is filled and chondrocytes may be prevented from expressing their whole gamut of differentiation. Osteophyte-derived chondrocytes may have an advantage over normal cartilage cells in this respect.

Histochemical staining and immunocytochemistry has demonstrated that cultured osteophytic chondrocytes are capable of elaborating cartilage and calcifiable matrices as well as type II collagens and link protein, therefore, these cells must be regarded as maintaining a chondrocytic phenotype. However, production of types I and III collagens may indicate that some cells remain de-differentiated as fibroblast. Type II collagen positive cells tended to remain polygonal in morphology. Thus, if osteophyte cells were transplanted into full thickness articular cartilage defects, they can be expected to re-differentiate into cartilage and possibly bone. Whether or not the architecture of normal articular cartilage will be reproduced under these conditions is not entirely clear and this may be a subject of future research.

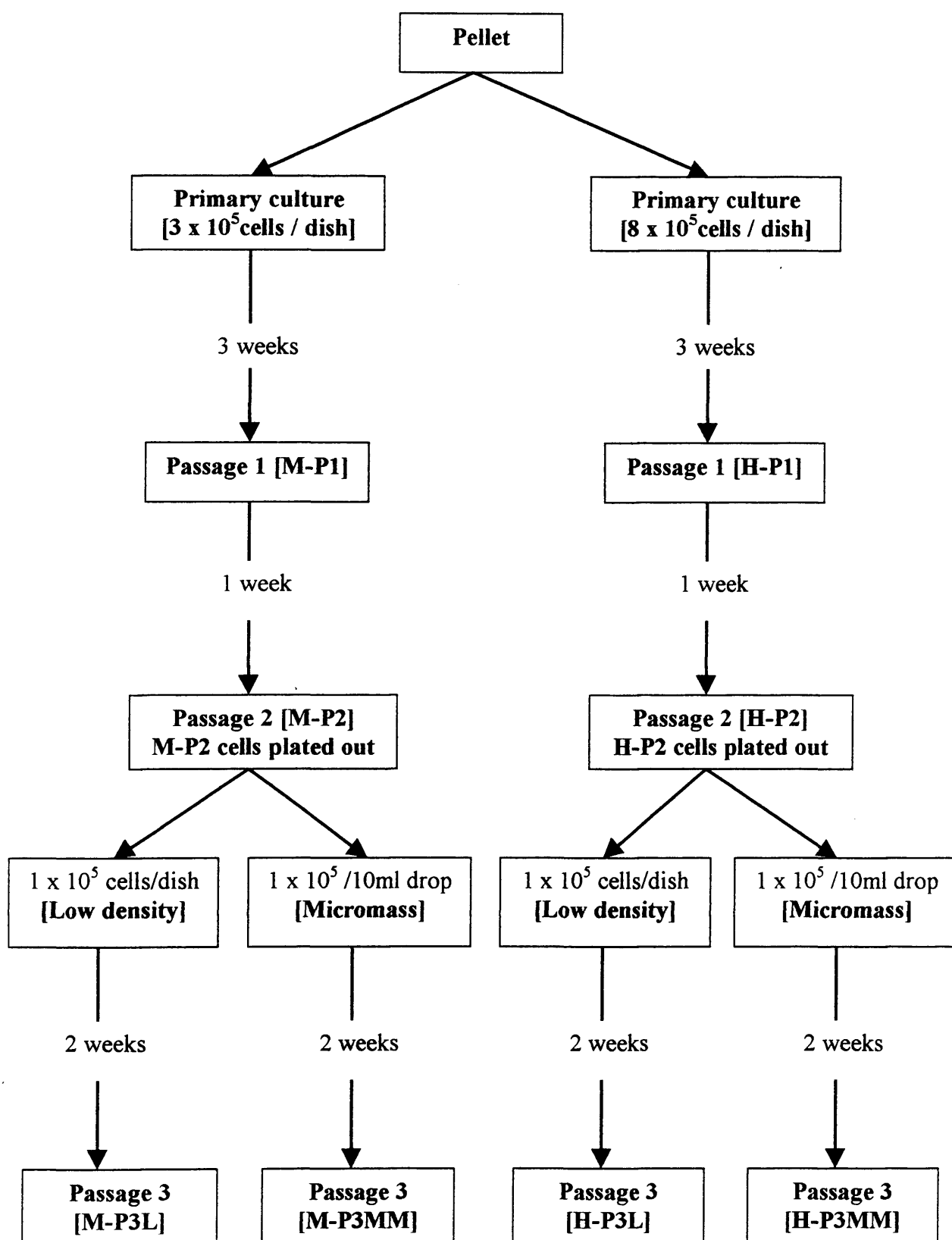


Figure 53 Schema of cell cultures

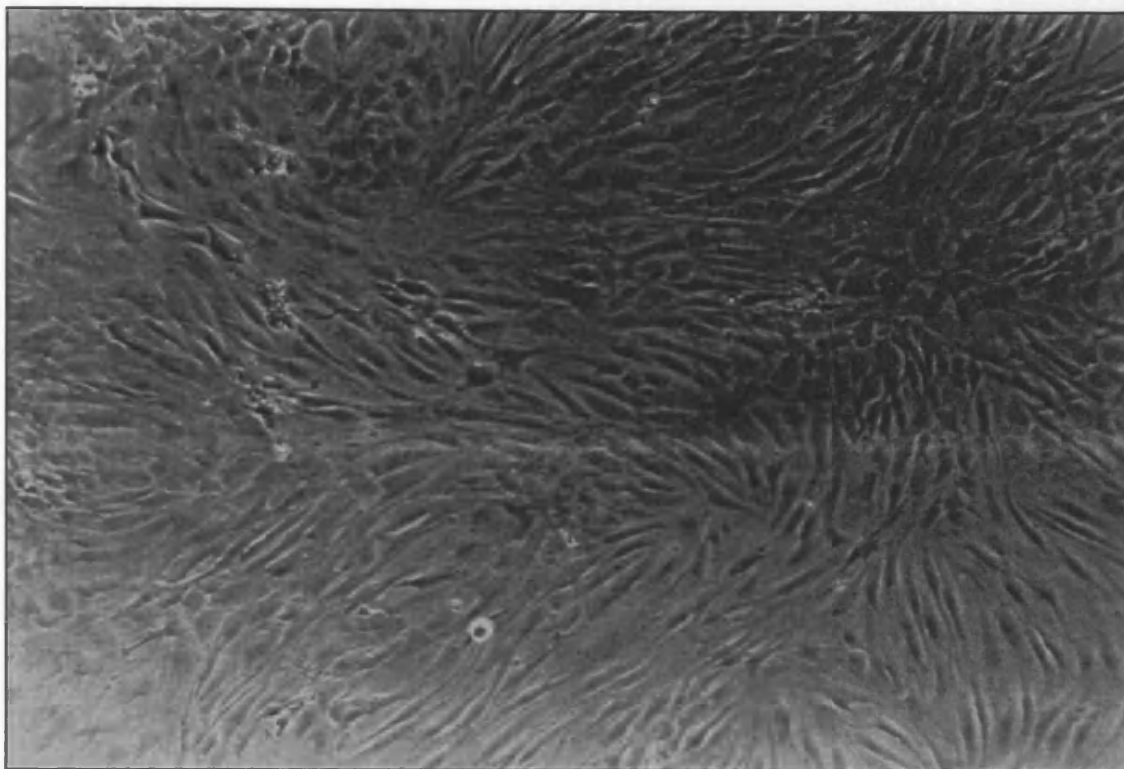


Figure 54 Photomicrograph showing fibroblastic cells (**P1** culture) mag 100 x.

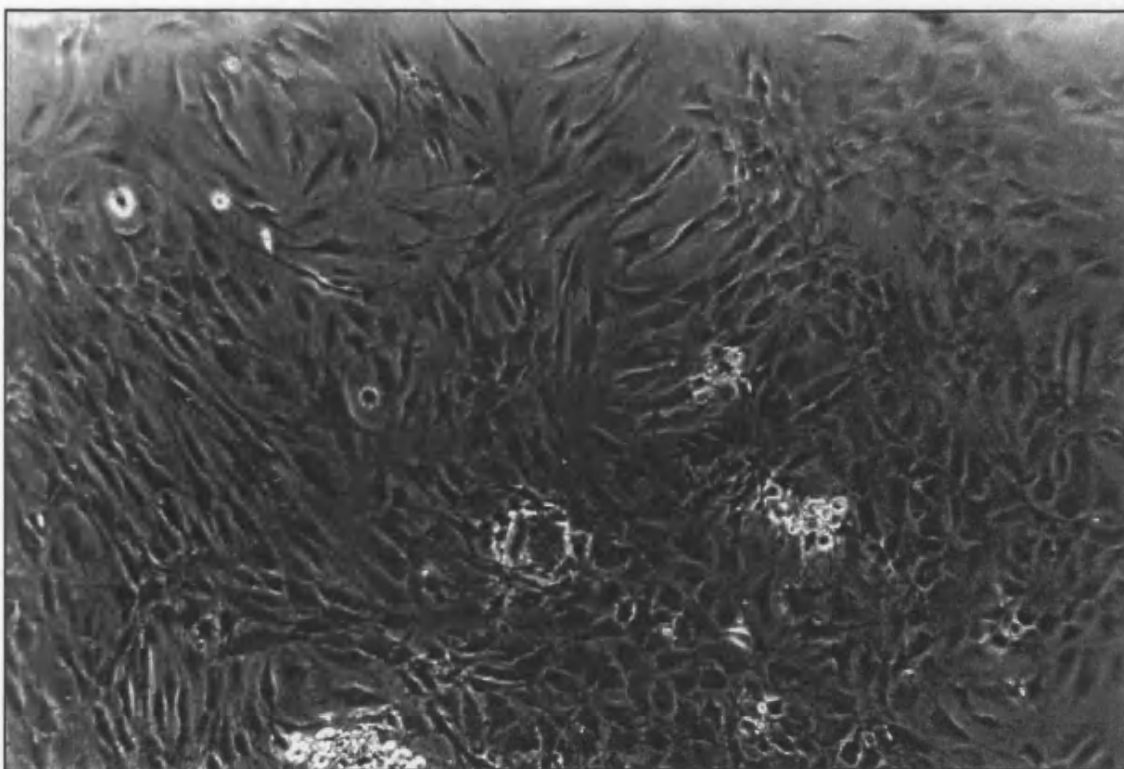


Figure 55 - Photomicrograph showing cells embedded in refractile matrix (**P1** culture) mag 100 x.

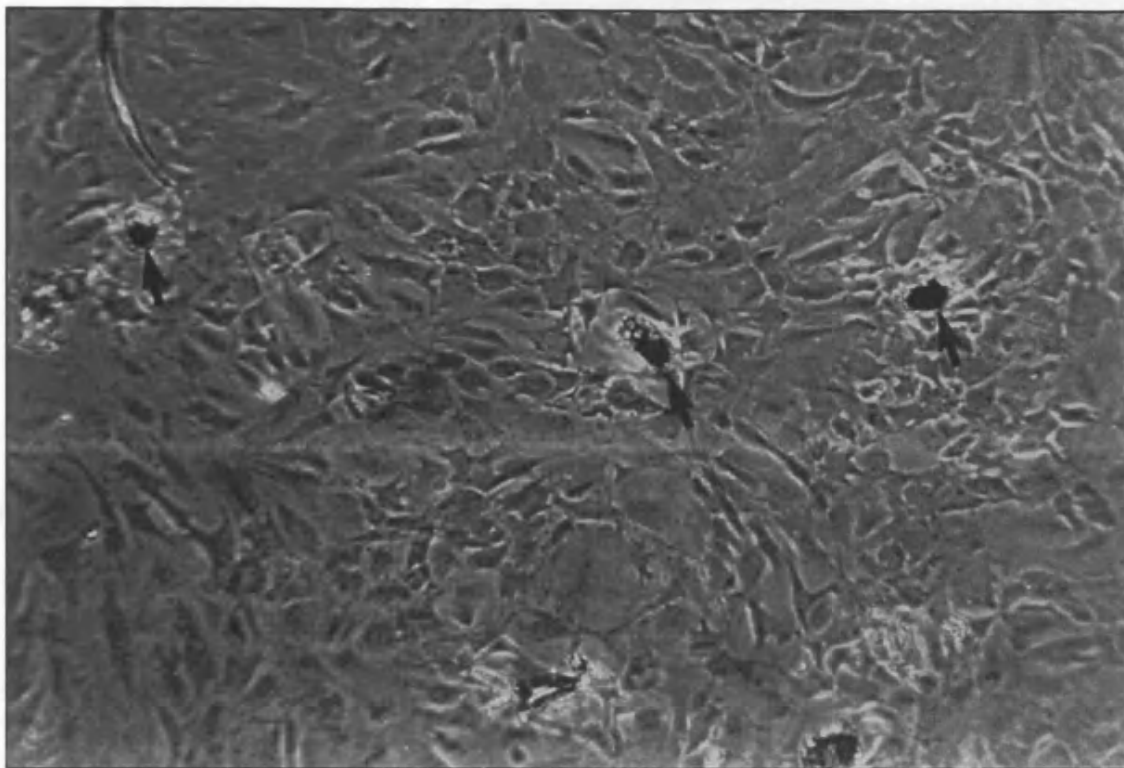


Figure 56 - Photomicrograph showing bone debris (**P1** culture) mag 40 x.

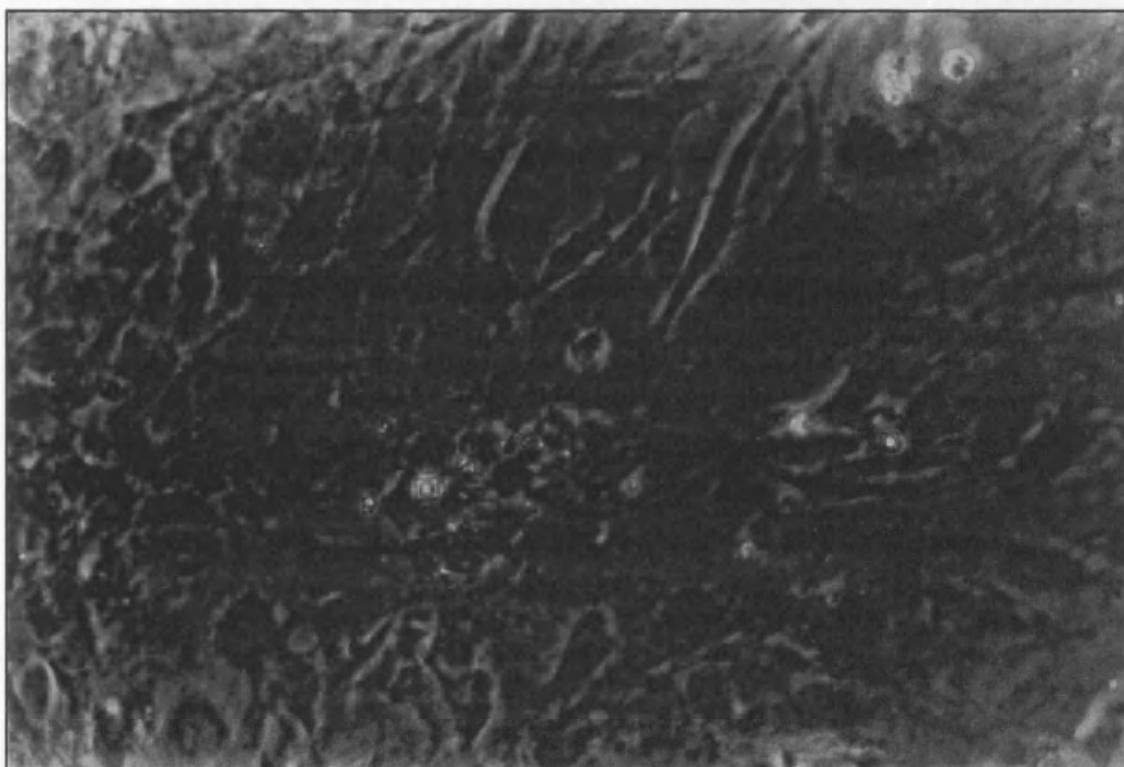


Figure 57 - Photomicrograph showing clumps of unidentified crystals (**P1** culture) mag 100 x.

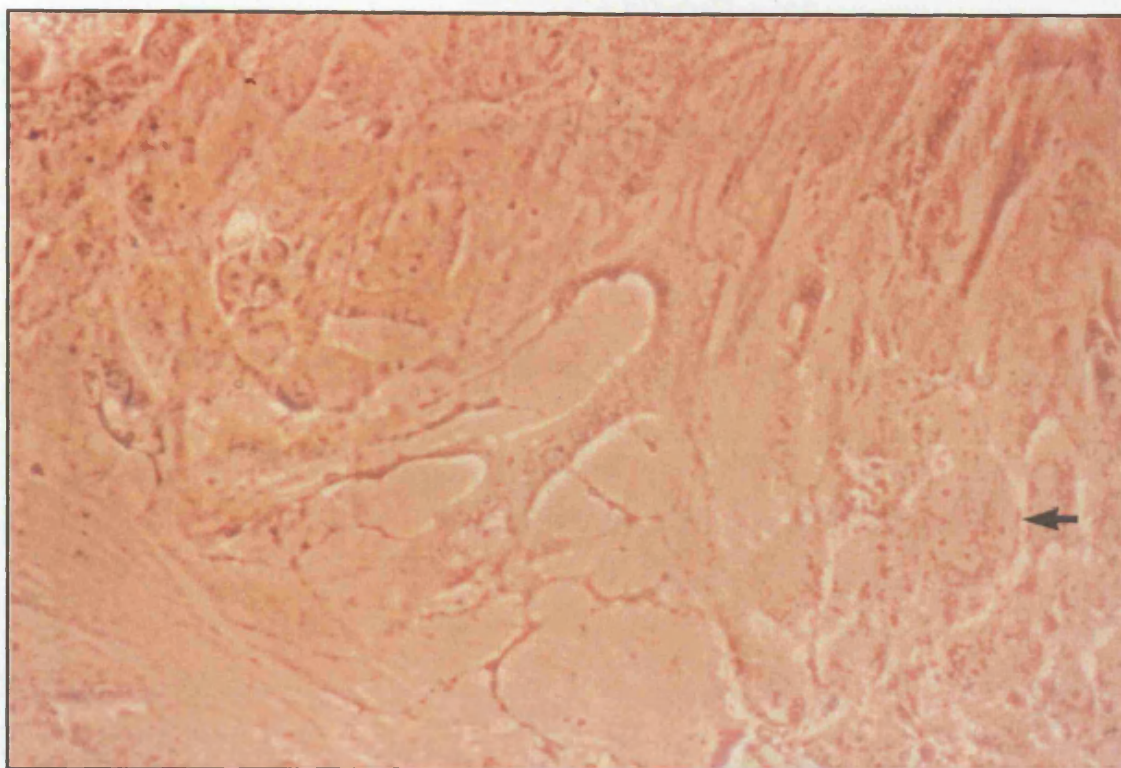


Figure 58 - Photomicrograph showing multinucleate giant cells (**P1** culture) mag 100 x.

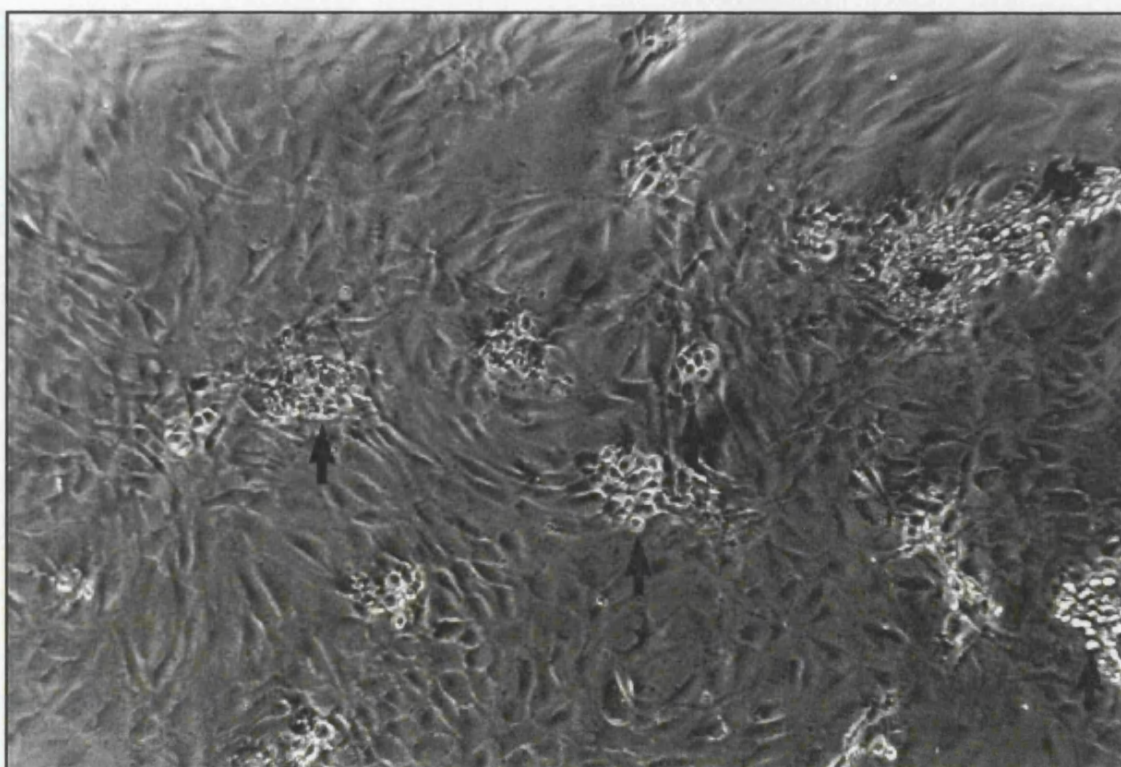


Figure 59 - Photomicrograph showing cells colonies (**P3** culture) mag 40 x.

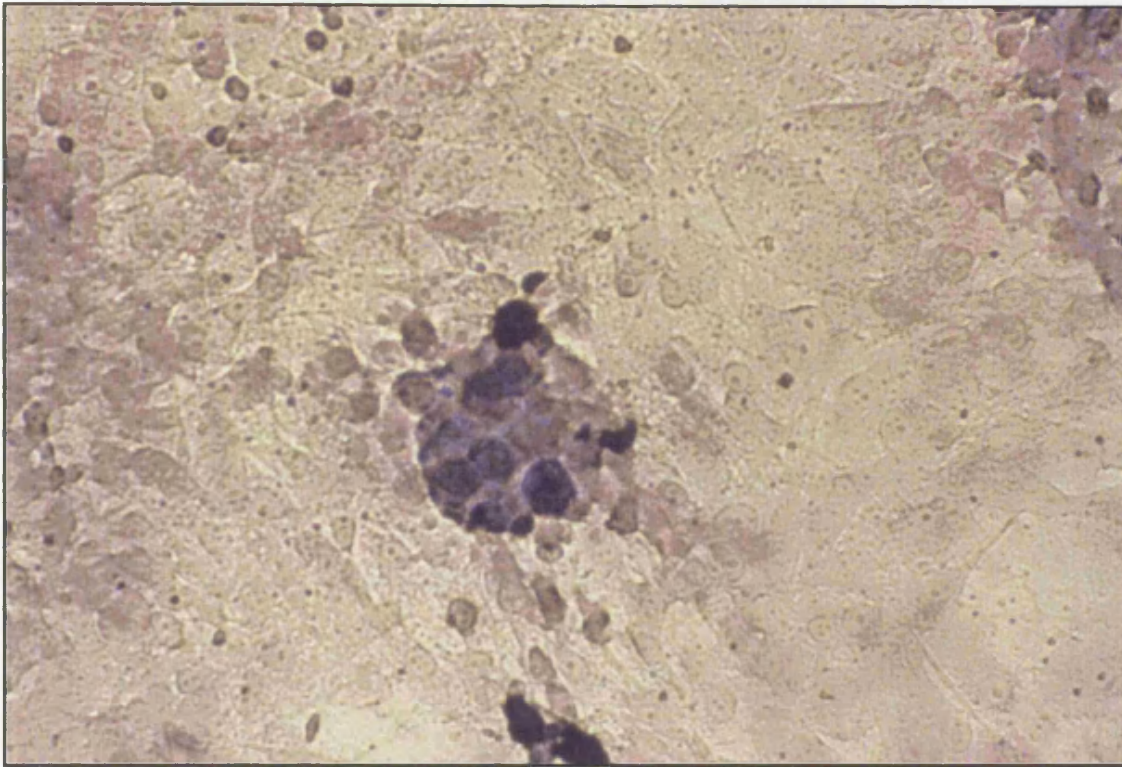


Figure 60 - Photomicrograph of Toluidine blue staining showing cartilage matrix (P3 culture) mag 100 x.

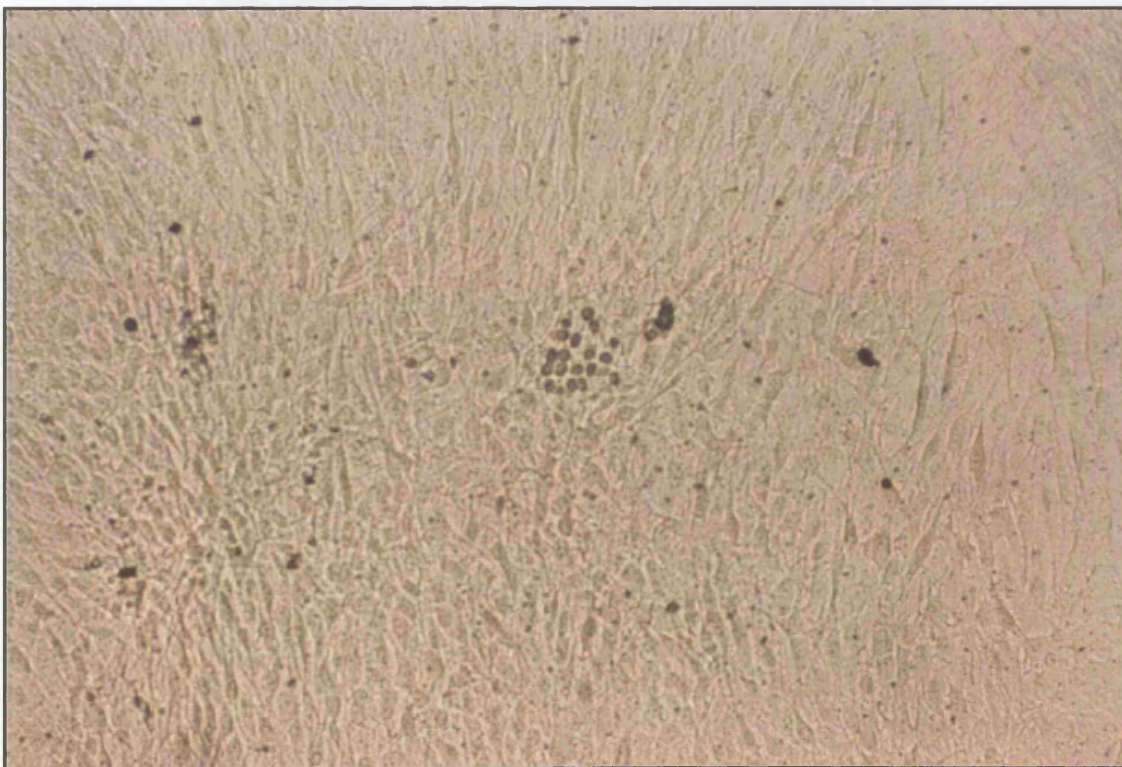


Figure 61 - Photomicrograph of von Kossa staining showing calcified matrix (P3 culture) mag 40 x.

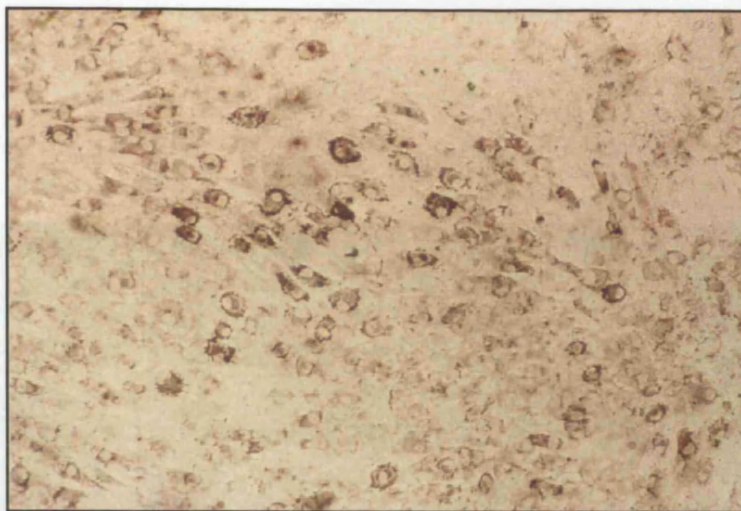


Figure 62 - Photomicrograph showing Type I collagen in monolayer cell culture mag 100 x.

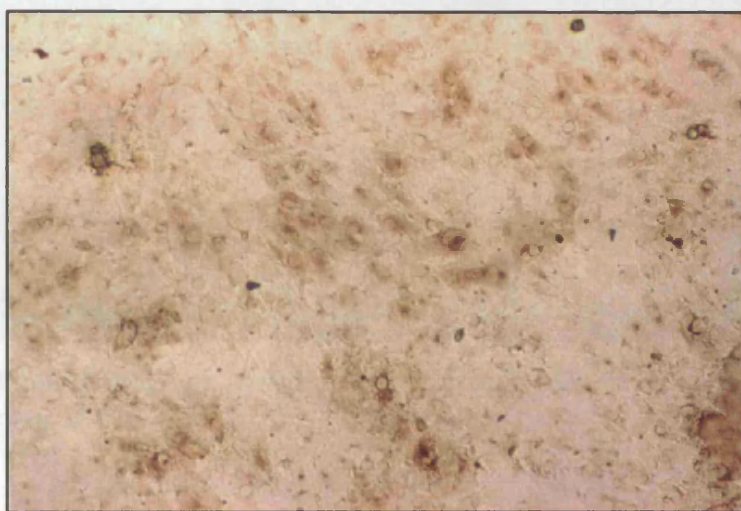


Figure 63 - Photomicrograph showing Type II collagen in monolayer cell culture mag 100 x.

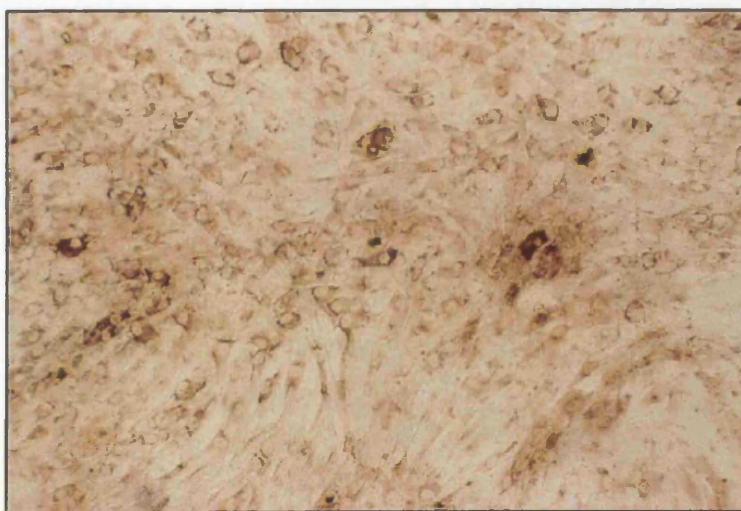


Figure 64 - Photomicrograph showing Type III collagen in monolayer cell culture mag 100 x.



Figure 65 - Photomicrograph showing link protein in monolayer cell culture
mag 100 x.

A preliminary investigation of the effect of type I collagen on the behaviour of cultured cells derived from osteophytes.

Summary.

Osteophyte-derived chondrocytes were grown in monolayer culture and subsequently propagated within and on top of type I collagen gel. The morphological and biochemical characteristics of the cultures were examined. Round and polygonal cells were observed following 'deep' propagation, filamented and polygonal cells were observed following 'surface' propagation and mineralisation of the matrix was only seen following 'deep' propagation. There was an increase in the alkaline phosphatase activity in the culture media and western blotting analysis revealed the production of type II collagen. These results suggest that type I collagen induces chondrocytic re-differentiation in osteophyte-derived cells initially propagated in monolayer culture.

Introduction

In monolayer cultures, chondrocytes display an unstable phenotype and tend to de-differentiate into fibroblast-like cells (Chacko *et al* 1969). The ability of the cultured chondrocytes to elaborate cartilage matrix diminishes (Chacko *et al* 1969) and there is a shift in synthesis from Type II to Type I collagen and from high to low molecular weight proteoglycans (Fronzoza *et al* 1996, Bujia *et al* 1993). In monolayer cultures, cell growth is anchorage-dependent whereas in agarose, suspension, and alginate cultures, there is a shift

from cell proliferation to cell differentiation, phenotypic stability and re-expression of the original chondrocytic phenotype (Benya and Shaffer 1982).

Cultured chondrocytes have been advocated as graft material for healing circumscribed articular cartilage defects using the autologous chondrocyte implantation (ACI) technique (Brittberg *et al* 1994). In practice, a sheet of periosteum is sutured over the defect and the cultured chondrocytes, which in monolayer culture are fibroblastoid-like, are injected underneath it. Over a period of time, the chondrocytes that are retained in the periosteal pouch re-differentiate and form a solid tissue composed of bone as well as cartilage. It is probable that a more rapid and stable repair could be effected if the graft was of a more solid consistency and the transplanted cells were in a re-differentiated state. Following implantation of the cultured cells using the ACI technique, some of the cells can lose their viability or be lost during this procedure and this may explain why in some instances, the implanted cultured cells are not capable of repairing the defects (Brienan *et al* 1997).

Collagens are a family of fibrous proteins secreted mainly by the connective tissues cells. The collagen molecule is a long, stiff and triple-stranded structure in which three polypeptide α -chains are wound tightly around one another to form a right-handed superhelix (Eyre 1991, Jeffery 1994). At least 25 different α -chains have been identified, each encoded for by a separate gene (Ayad *et al* 1994). These molecules aggregate to form long collagen fibrils in the extracellular matrix and these fibrils have a variety of patterns depending upon the tissue

(Eyre 1991). In a tissue culture dish, type I collagen fibrils are randomly oriented but fibroblasts and chondrocytes are still capable of crawling all over them, egressing into the spaces in between and forming a composite 'tissue' of fibre and cells (Stopak and Harris 1982). Yasui *et al* (1982), Wakitani *et al* (1989), Kawamura *et al* (1998) have demonstrated that when articular chondrocytes are cultured in type I collagen gel, the cells maintain their polygonal or rounded morphology and are able to accumulate a metachromatic matrix and also synthesis type II collagen. Whether osteophyte-derived chondrocytes have similar properties is not yet certain and this is the object of this investigation.

Materials and Methods

Osteophytes were obtained from the margins of the femoral condyles in 6 patients (age: 65-88) undergoing total knee replacement for osteoarthritis (OA).

Preparation of cells

The cartilage covering each specimen was scraped off under aseptic conditions and washed in sterile culture medium (α -MEM) supplemented with 10% foetal bovine serum, L-glutamine, penicillin and streptomycin (Gibco, Life Technologies, Paisley, UK). The specimens were placed in sterile universal containers and enzymatically dissociated using 2% w/v collagenase (Type Ia, Sigma, Poole, UK) at 37°C for 3-4 hours. Each universal container was vigorously agitated every 30 minutes using a Whirlmixer. At the end of the dissociation period, each sample was left for 30 seconds to allow sedimentation of bone pieces and undigested

fragments. The digested cell suspension was removed, transferred to a new sterile universal container and centrifuged at 2000 r.p.m. for 5 minutes. Supernatants were discarded and the pellet was re-suspended in α -MEM as above.

Preparation of type I collagen solution. (Schor 1980)

Rat tails were washed in 70% alcohol and the skin removed. Each tail was broken at 2cm intervals and the tendons pulled out each time. The harvested tendons were washed in sterile phosphate buffered saline (PBS), pooled and weighed. Tendons were cut into small pieces and digested in 0.5M acetic acid (2mg tendon /ml) for 2 days at 4⁰C with continuous stirring. Next, the mixture was centrifuged and the supernatant containing extracted collagen was decanted off leaving undigested fragments. The supernatant was mixed with an equal volume of 20% NaCl at 4⁰C for 24 hours to precipitate the collagen. The solution was centrifuged and the pellet was re-suspended in 0.5M acetic acid and dialyzed extensively against distilled water at 4⁰C. An estimation of the concentration of the total collagen obtained was made by freeze-drying and this was adjusted as necessary by dilution with distilled water to obtain approximately 1.5-2mg/ml. The purity of the collagen was assessed by gel electrophoresis.

Culture technique (Figure 66)

The suspended cells were counted using a haemocytometer and then were plated out into 35mm tissue culture dishes (Nunc, Life Technologies, Paisely. UK) at two densities - Medium

(3×10^5) and **High** (8×10^5) cells/dish respectively. Cultures were fed every third day and examined daily for cell morphology, viability and infection using an Olympus phase contrast inverted microscope.

For each density, one half of the cultures were passaged at the end of 3 weeks and plated out at the same density as the original culture [Passage 1 cultures **M-P1** and **H-P1**]. Passage 1 cells were grown for 7 days and thereafter one half of the cultures were re-passaged and plated out again at the same density as the original cultures [Passage 2 cultures **M-P2** and **H-P2**]. Finally after 2 weeks, Passage 2 cells were re-passaged and this time plated out at two densities; 1×10^5 cells/dish [Passage 3 cultures low density = **M-P3L** and **H-P3L**] and 1×10^5 cells in a 10ml drop, equivalent to 1×10^7 cells/dish [Passage 3 cultures high density **M-P3MM** and **H-P3MM**] using the “micromass” technique of Ahrens (Ahrens *et al* 1977). All the cultures were maintained for a further 7 days.

Gel propagation

At the end of the culture period, 2 dishes each of **M-P3L**, **H-P3L**, **M-P3MM** and **H-P3MM** respectively were propagated within the collagen gel (‘deep’ culture) and 2 dishes each on top of the collagen gel (‘surface’ culture) for 4 weeks.

1. ‘Surface’ propagation

A cocktail was made up of: 4.2ml type I collagen solution, 1.0ml 10x DMEM, 0.6ml Na Bicarbonate (7.5%) and 0.1ml HEPES buffer (Gibco, Life Technologies, Paisely. UK). A 1ml

aliquot of this mixture was poured into 35mm tissue culture dishes and incubated at 37°C for 15 minutes to allow the gel to set. Thereafter, the harvested cultured cells were inoculated on top of the gel. Extra media was added as required so that there was about 1ml of media covering the gel. The culture cells were fed every second day.

2. 'Deep' propagation

The concentration of the cells required was calculated, centrifuged at 2000 rpm and re-suspended in an appropriate volume of medium. The collagen mixture was made up as above but the volume of the 10x DMEM was reduced by the same volume as the cell concentration. The collagen mixture and the cells were plated out into 35mm tissue culture dishes and incubated for 15 minutes to allow the gel to set. 1 ml of media was added to each dish and the cells fed every second day.

Assay methods

After propagation, one half of the media obtained from each culture during feeding of the cells was stored at -80°C until analyzed for alkaline phosphatase (ALP) activity using a UV/Spectrophotometer (Ultrospec 2000. Pharmacia. Sweden). The other half was freeze-dried until stained with antibodies to type II collagen using the Western blotting (WB) technique (Hayes *et al* 1989).

a). Alkaline phosphatase (ALP) activity assay.

The MPR 3 kit (Boehringer Mannheim, Germany) was used for the assay. The frozen samples were thawed out by placing their containers in a water bath at 30°C. The reagent solution was prepared as per manufacturer's instructions. Fresh media (3ml), buffer solution (3ml) and a mixture of both (50µl media, 2.5ml buffer solution) respectively were used as negative controls. Measurements were made at 0, 1, 2 and 3 minutes and the mean absorbance change per minute at 405nm was used to calculate ALP activity.

b). Type II collagen assay.

The blotting method consisted of four steps: 1) electrophoretic separation of proteins (SDS-PAGE), 2) transfer 3) immobilization on support membrane and 4) staining and visualization (Hayes *et al* 1989).

Each freeze-dried sample was re-suspended in bromophenol blue to make up a solution equivalent to 1mg/ml. 60µl was transferred into eppendoff's arranged on a metal rack. The rack was lowered into a beaker of boiling water ensuring that the tips of the eppendoff's were immersed in the boiling water for 5 minutes. Two SDS-PAGE gels were prepared for each specimen. 25µl of each sample was emptied into the wells using a Hamilton's syringe. The syringe was thoroughly rinsed after each sample to prevent contamination. The SDS-PAGE was allowed to run for one hour at 15mA per gel. Next, the gels were transferred into sterile dishes. One was flooded with Coomassie blue (CB) and the other with Towbin's buffer solution (Towbin *et al* 1979) in preparation for the Western blotting.

For CB staining, the gel was left for 24 hours in the stain after which it was removed and immersed in a de-stainer. The de-stainer was changed 3 or 4 times until the protein bands became distinctly visible. Thereafter excess CB was washed off with distilled water.

For WB, the gel was blotted onto 7 x 8cm sheets of immobilon P transfer membrane (Millipore, UK) over a period of one hour at 50Volts and $0.8\text{ma}/\text{cm}^2$. After blotting, the membrane was then washed thoroughly in PBSTween for 15 minutes, transferred into a sterile dish and flooded with goat anti-type II collagen primary antibody (Southern Biotechnology Associates, Alabama, USA) in 0.1% bovine serum albumin (BSA) at 1:200 dilution and left to incubate for 90 minutes at room temperature. Gentle rocking of the dish was carried out throughout the incubation period. Thereafter, the membrane was again washed in PBSTween for 15 minutes, flooded with rabbit anti-goat horse radish peroxidase (HRP) conjugated secondary antibody in PBSTween at 1:2000 dilution and incubated for 90 minutes on a rocker. The membrane was washed thoroughly in PBSTween and counterstained with DAB Sigma fast tablet solution (Sigma, Poole, UK) until brownish bands or columns appeared. The efficiency of the transfer of proteins (from the SDS-PAGE gel onto the membrane) was assessed by staining the gel (after blotting) with Coomassie blue.

The non-blotted Coomassie blue stained gel was examined side-by-side with the membrane on a table mounted viewing box to ascertain the presence/location of type II collagen band in the membrane.

Results

“Deep” propagation.

When plated within collagen gel, the majority of cells retained a rounded morphology with a surrounding refractile ‘halo’ suggestive of extracellular matrix. Some of the cells were in a transitional state from fibroblastoid-like cells with long dendrites to polygonal cells with shorter dendrites. Most of these chondroblast-like cells were also surrounded by a refractile halo (Figure 67). Within a few gels, dark fluffy regions suggestive of mineralization were clearly observed adjacent to rounded cells (Figure 68). In some cases, the gels had contracted from the tissue culture wells and in these samples, the rounded cells could be seen within the gel often with a beaded configuration.

“Surface” propagation.

When the cells were plated on top of the collagen gels, cells settled within 24 hours and by the end of the culture period, regions of fibroblastic, rounded and polygonal cells with surrounding ‘halo’ again suggestive of extracellular matrix could be observed. Over time, some of the cells had migrated into the gel and settled within it and these cells retained a rounded morphology (Fig. 69)

At the end of the 10 weeks culture period, the mean alkaline phosphatase activity in the media drained off the cultures was 20.6 U/l (range: 17.36-22.8) compared with the control value of 2.4U/l (range: 0.82-3.56).

Coomasie blue staining of the SDS-PAGE revealed several polypeptide bands of media proteins with a void corresponding to the site of type II collagen expression (Figure 70). Western blotting analysis revealed Type II collagen synthesis in both 'deep' and 'surface' propagated cultures (Figure 71).

Discussion

These results suggest that type I collagen can induce osteophyte-derived chondrocyte in monolayer culture to re-differentiate into phenotypic or matured chondrocytes when these cells are propagated within the collagen gel. The mechanism involved in this phenotypic transformation has been previously proposed by Benya and Shaffer (1982). After colonising the gel, the chondrocytes become immobilized and they then lose cell-to-cell contact leading to a cessation of cell proliferation. Thereafter, the propagated cells switch function and begin to re-differentiate into mature chondrocytes. The expression of type II collagen indicate that the cells retained their normal synthetic ability which implies that the collagen gel was acting as a scaffold which is apparently suitable for this three-dimensional culture system.

In this study, the behaviour of osteophyte-derived chondrocytes in this three-dimensional culture system is similar to that obtained in normal articular cartilage chondrocytes using the same system (Yasui *et al* 1982, Wakatani *et al* 1989, Kawamura *et al* 1998). This finding therefore raise the possibility that osteophytes could provide an alternative source of chondrocytes for the ACI technique or as the cellular component in a three-dimensional

culture technique in the management of full thickness articular cartilage defect as has been used by Wakitani *et al* (1989), Kawamura *et al* (1998). One can also infer that osteophyte-derived chondrocyte behaves like normal articular chondrocytes even in a three-dimensional culture. The mineralization observed in the matrix of some of the ‘deep’ propagated cultures has been reported with the use of type I collagen scaffold (Schor 1980). Therefore, this mineralization observed might not be due to the cell type propagated within the collagen gel but an inherent problem with type I collagen. In future experiments, osteophyte-derived chondrocytes will be propagated in other three-dimensional culture systems that are not complicated by matrix mineralization.

The presence of high alkaline phosphatase (ALP) activity in the culture media may suggest that some of the cells in the ‘deep’ propagated three-dimensional culture systems can also transform into osteogenic cells although ALP is also known to be present in hypertrophic chondrocytes. It could also be that some of the osteophyte-derived chondrocytes do transform into hypertrophic chondrocytes although it has long been established that bone-forming cells may arise directly from cartilage (Bohatirchuk 1969).

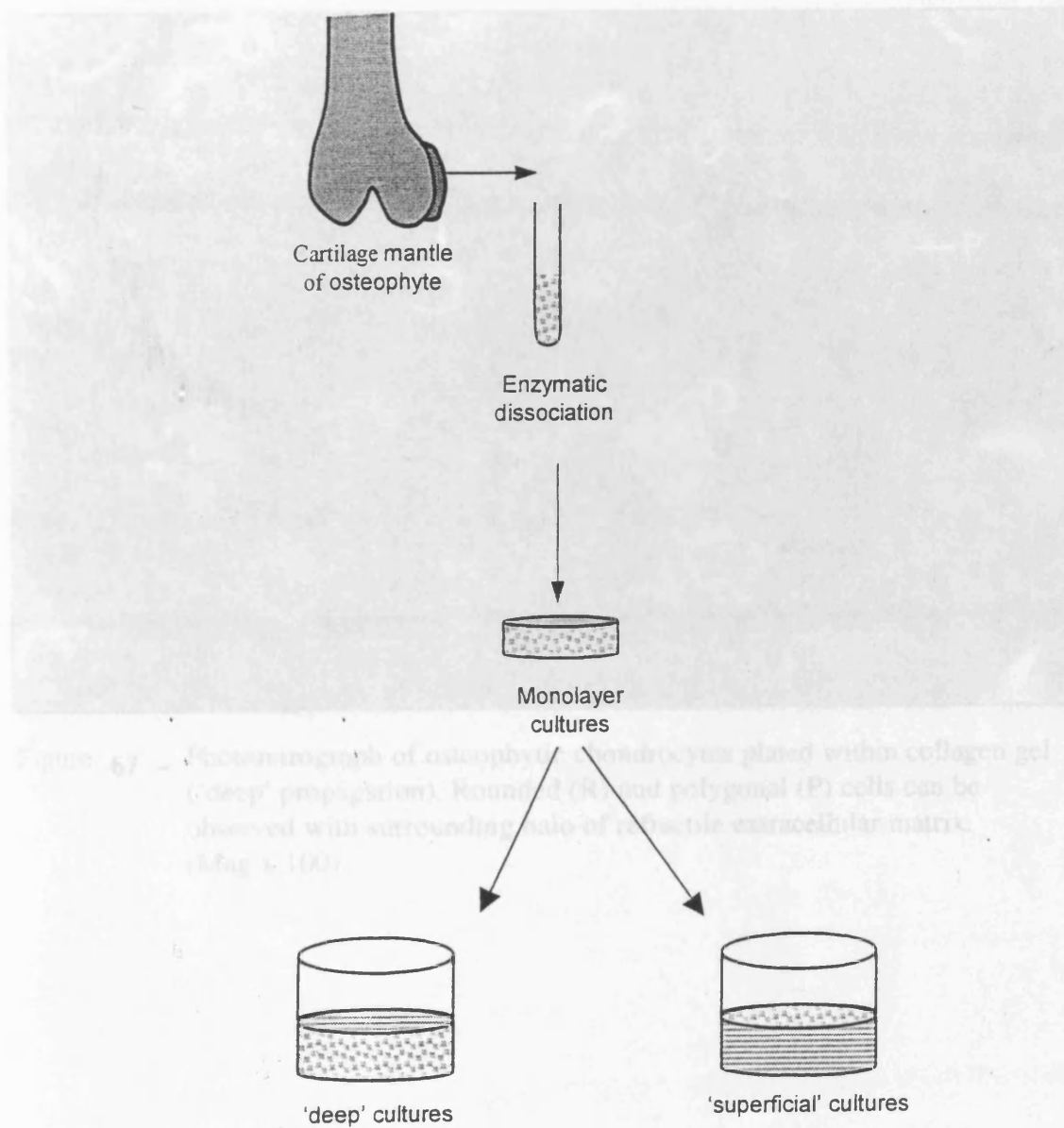


Figure 67 - Photomicrograph of osteophyte chondrocytes plated within collagen gel ('deep' propagation). Rounded (R) and polygonal (P) cells can be observed with surrounding halo of reticulate extracellular matrix (magn. $\times 100$).

Figure 66 Schema of culture technique.

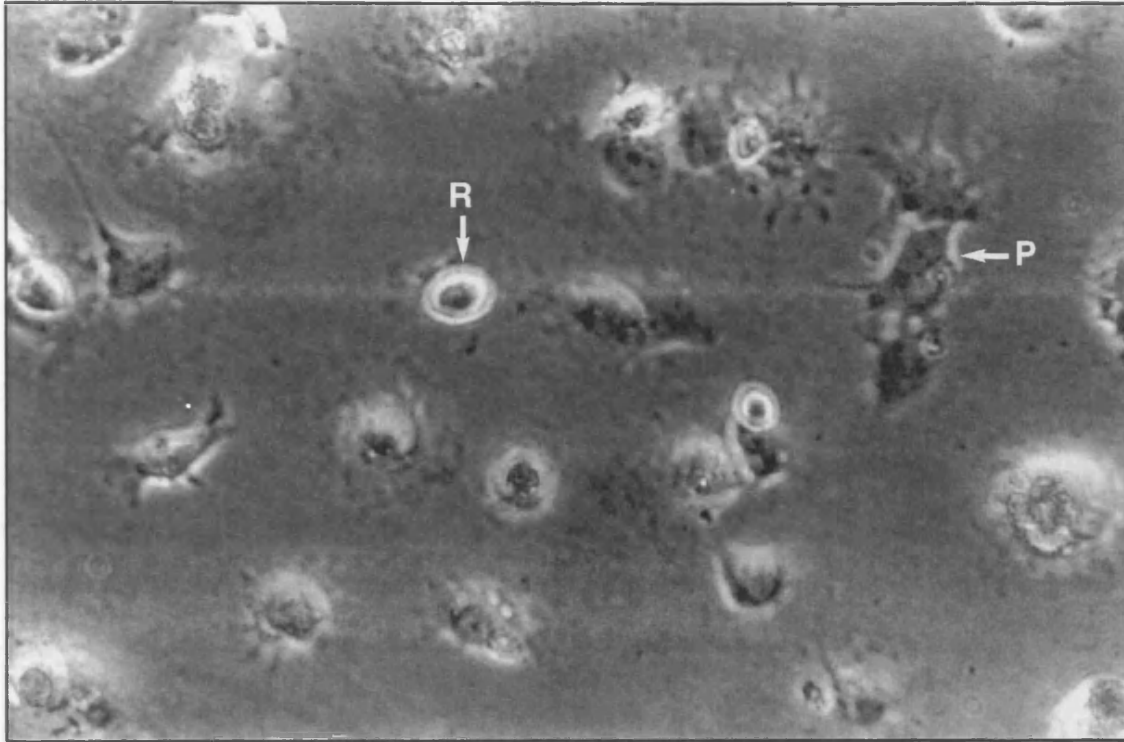


Figure 67 - Photomicrograph of osteophytic chondrocytes plated within collagen gel ('deep' propagation). Rounded (R) and polygonal (P) cells can be observed with surrounding halo of refractile extracellular matrix. (Mag x 100).

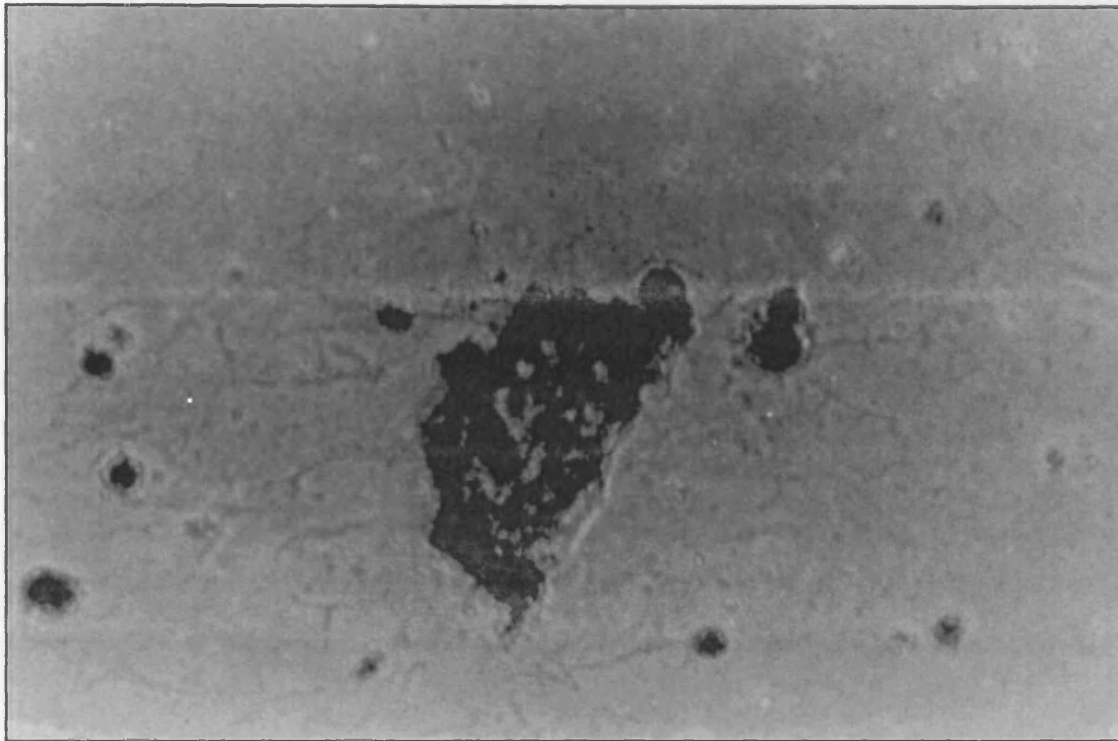


Figure 68 - 'Deep' propagation culture with an area of matrix mineralization.
(Mag x 100).

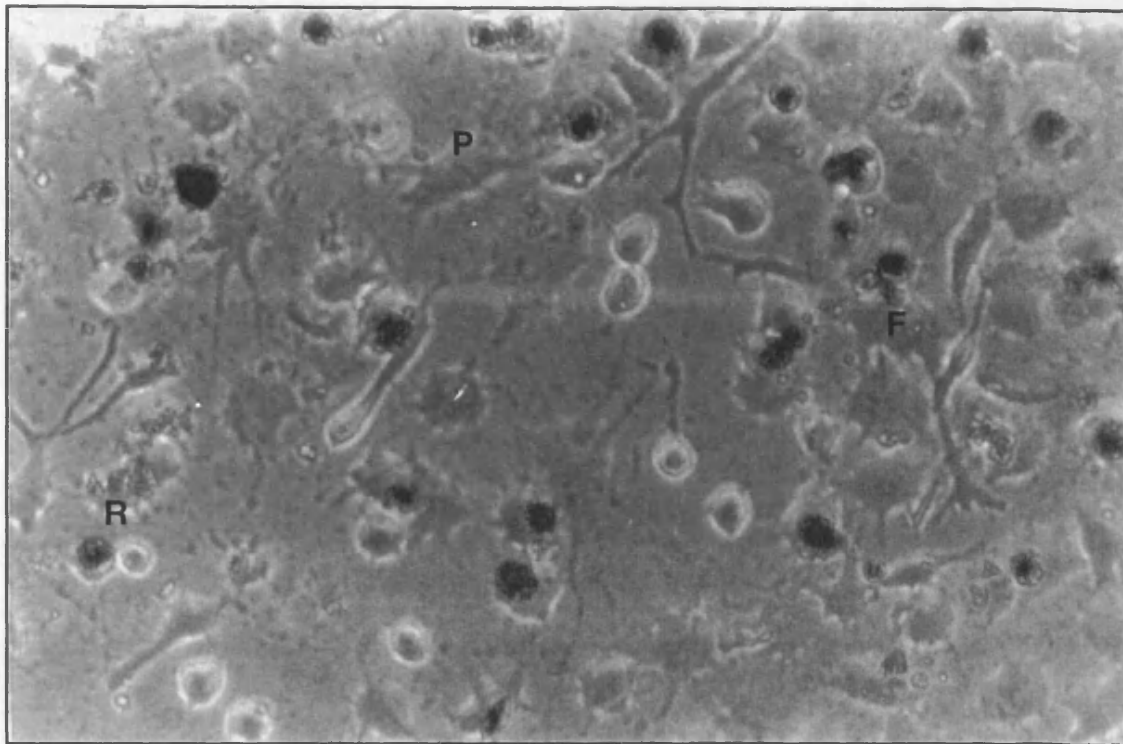


Figure 69 - 'Surface' propagation of osteophytic chondrocytes. A mixture of cell morphologies can be observed: elongated fibroblastic (F) and polygonal (P) cells can be seen growing on top of the collagen gel. Several rounded (R) cells with surrounding refractile matrix are also present at different focal depths suggesting cell migration into the gel.

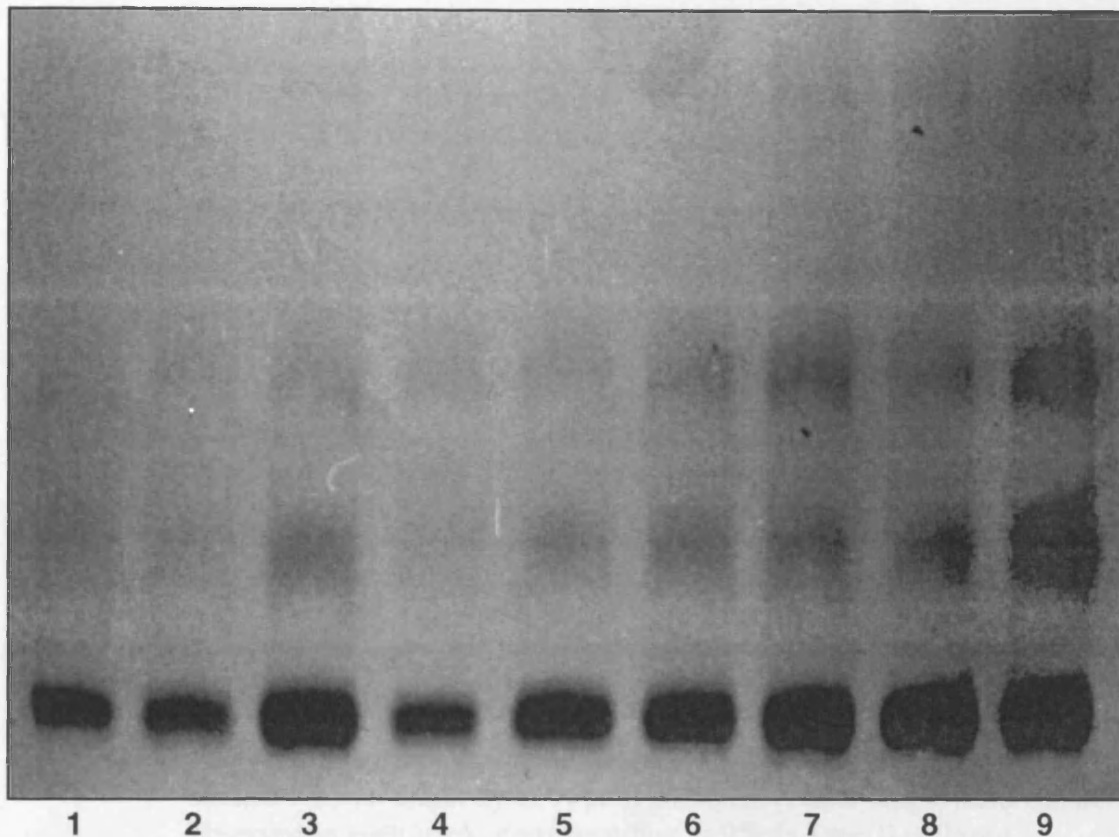


Figure 70 - Photomicrographs of 8% SDS-PAGE gel demonstrating Coomassie blue stained media proteins from various osteophytic cultures. All tracks demonstrate the presence of media proteins with no staining at the site of Type II collagen expression.

- Track 1= Passage 3(2) cells propagated in collagen gel (supernatant);
- Track 2= Passage 3(2) cells propagated on collagen gel (precipitate);
- Track 3= Passage 3(3) cells propagated in collagen gel (precipitate);
- Track 4= Passage 3(3) cells propagated on collagen gel (precipitate);
- Track 5= Passage 3(3) cells propagated in collagen gel (supernatant);
- Track 6= Passage 3(3) cells propagated on collagen gel (supernatant);
- Track 7= Passage 3(2) cells propagated in collagen gel (precipitate);
- Track 8= Passage 3(2) cells propagated on collagen gel (supernatant);
- Track 9= Passage 3(2) cells propagated on collagen gel (precipitate).

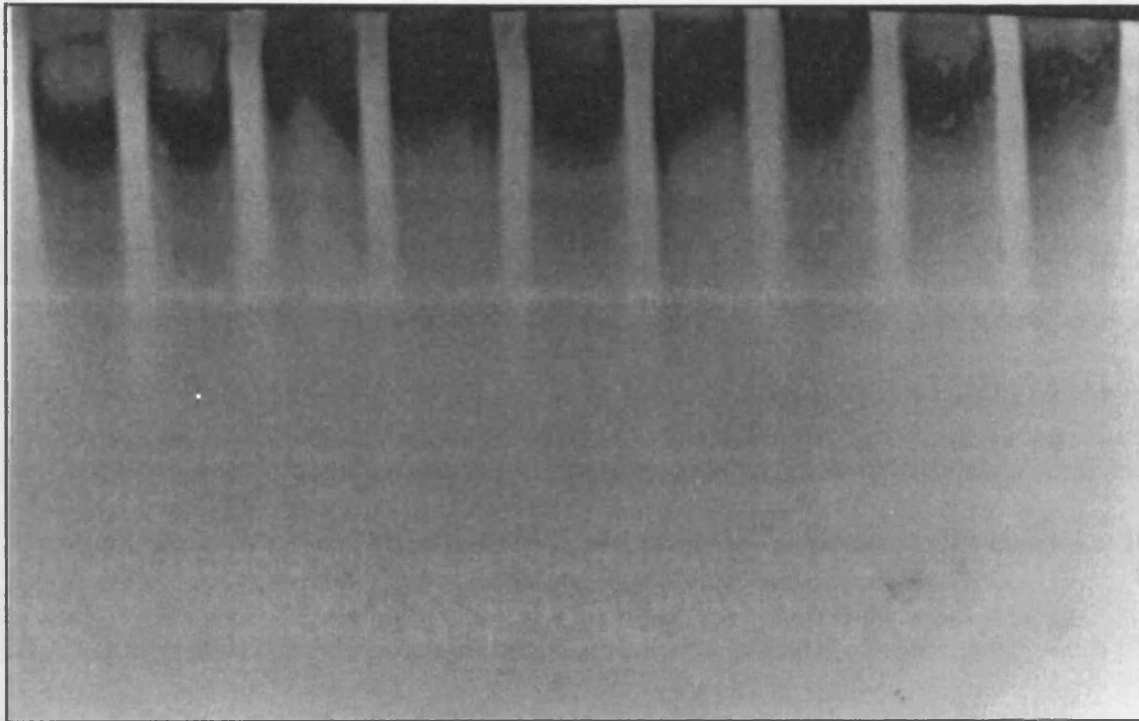


Figure 71 - Photomicrographs of an immunoblot of the SDS gel shown in Figure 70 stained with an antibody to Type II collagen. A clear single band can be observed in each track, corresponding to 95kda Type II collagen.

A Western blot analysis of the culture medium in which cells derived from osteophytes had been previously grown.

Summary

Chondrocytes derived from the cartilage mantle of osteophytes (by enzymatic dissociation) obtained from the margins of osteoarthritic knee joints have been grown in monolayer cultures. The cultures were passaged and repeatedly sub-cultured over a 10-week culture period. Western blotting and protein immunocytochemistry of the culture media in which the cells were grown revealed the presence of types I and II collagens. This raises the possibility of a chondrocytic ancestry for osteophytes.

Introduction

Marginal osteophytes are formed in an area where a number of tissues converge and, hence their tissue(s) of origin is a matter of considerable controversy (Key 1931, Fisher 1939, Calandruccio and Gilmer 1962, Marshall 1969, Gilbertson 1975). Cells may be cultured directly from tissues when they are referred to as **primary cultures**. Cells of **primary cultures** can be used to make a large number of other (i.e. **secondary**) cultures and they may be repeatedly sub-cultured (Alberts *et al* 1983). A characteristic of the resultant cells is that they display many differentiated characteristics of their cells of origin so that fibroblasts, for instance, continue to secrete collagen while epithelial cells form extensive sheets reminiscent of the intact epithelium (Alberts *et al* 1983). Thus, it is to be expected that if the cells of

osteophytes were to be successfully grown in cultures, they will eventually reveal their true tissue(s) of origin.

As a manifestation of the differentiated state, a cell type may produce a protein peculiar to it. It is sometimes possible to detect such a protein in the culture medium. A variety of proteins are secreted into the media by growing cells. In order to analyse these proteins, it is customary to separate the protein cocktail into its constituent proteins usually by the technique of electrophoresis (Laemmli 1970). The separated proteins may then be transferred unto a sheet of nitrocellulose and incubated with antibodies that recognise those proteins individually. The location of the specific proteins on the strip is then revealed by a sensitive staining technique (Hayes *et al* 1989).

In this study, these techniques have been employed to analyse culture media in which chondrocytes derived from osteophytes had been previously grown in an attempt to postulate the ancestral cell(s) of origin of this peculiar tissue.

Materials and methods.

The specimens consisted of osteophytes obtained from the margins of the trochlear of the femur of 6 patients (aged: 65-88) undergoing total knee replacement for osteoarthritis (OA). Extra care was taken to avoid contamination of the specimen by the adjoining normal articular cartilage. The specimens obtained from each patient were cultured separately.

Cell preparation

The cartilage mantle of each specimen was scraped off under aseptic conditions and washed in sterile culture medium (α -MEM), supplemented with 10% foetal bovine serum, L-glutamine, penicillin and streptomycin (Gibco, Life Technologies, Paisley, UK). Next, the specimens were enzymatically dissociated in 2% w/v collagenase (Type Ia, Sigma, Poole, UK) at 37°C for 3-4 hours. Thereafter, each sample was allowed to sediment and the supernatant was centrifuged at 2000 rpm for 5 minutes. Supernatants were discarded and the pelleted cells were re-suspended in α -MEM and counted using a haemocytometer.

Primary culture

Cells were plated out into 35mm tissue culture dishes at two densities - **Medium** (3×10^5) and **High** (8×10^5) cells/dish respectively. Cultures were fed every third day and examined for morphology daily using an Olympus phase contrast inverted microscope.

Secondary culture

Samples from three-week old cultures (**Medium** and **High** density) were passaged and re-plated [**M-P1** and **H-P1**]. After 7 days, passage 1 cultures were re-passaged and re-plated [**M-P2** and **H-P2**]. After 2 weeks, passage 2 cultures were again re-passaged but plated out at either 1×10^5 cells/35mm dish (low density = **M-P3L** and **H-P3L**) or as 1×10^5 cells in a 10 μ l drop. The latter is equivalent to 1×10^7 cells/ml

[high density **M-P3MM** and **H-P3MM**] according to the “micromass” technique of Ahrens *et al* (1977) ⁹. These cultures were maintained for a further 4 weeks. All cultures were fed every third day and examined for morphology daily using an Olympus phase contrast inverted microscope.

Passage technique.

Cells were passaged by incubation in trypsin/EDTA solution (Gibco, Life Technologies, Paisely). UK) for 15 minutes at 37⁰C, loose cells were transferred to a sterile universal and an equal volume of α -MEM containing serum added to inactivate the trypsin. Loosely attached cells were detached using a rubber policeman and added to the universal. Cells were centrifuged at 2000 rpm, the supernatant discarded and the pellets re-suspended in α -MEM. Cells were counted using a haemocytometer and plated out at the densities outlined above.

Western blotting (WB)

At the end of the 10-week culture period, the supernatants from all the cultures were freeze-dried, weighed and then analysed for their collagen contents. Blotting consisted of four steps: 1) electrophoretic separation of proteins (SDS-PAGE), 2) transfer and immobilisation on support membrane 3) staining and 4) visualisation.

1. SDS-gel preparation

The freeze-dried samples were weighed and re-suspended in bromophenol blue to make up a solution equivalent to 1mg/ml. 100µl of sample was transferred into eppendoff's (with lids perforated with an 18G needle) arranged on a metal rack. The rack was lowered into a beaker of boiling water for 5 minutes. Four SDS-PAGE gels were prepared. 25µl of each sample was emptied into the SDS-PAGE gel wells using a Hamilton's syringe. The syringe was thoroughly rinsed after each sample had been taken to prevent contamination. The SDS-PAGE gels were allowed to run for 1 hour at 15mA per gel. Four comparable gels were prepared for each culture and they were transferred into sterile dishes after the separation; two gels were flooded with Coomassie blue (CB) and two with Towbin's buffer in preparation for transfer.

2. Transfer onto support membrane

The two gels flooded with Towbins buffer were transferred unto two 7 x 8cm sheets of immobilin P transfer membrane (Millipore, UK) and were supported by twelve pieces of blotting paper (six for each gel). The transfer was carried out over a period of one hour at 50Volts and $0.8\text{mA}/\text{cm}^2$ per gel.

3. Staining

Coomasie blue staining - The two gels flooded with Coomasie blue had excess stain drained off after 3-4 hours. Next, the gels were flooded with a de-stainer and left overnight. The destainer was changed 3-4 times until the protein bands on the gel became distinctly visible. Thereafter, excess Coomasie blue and de-stainer removed and the gel stored in distilled water until viewed.

Immunocytochemistry - After blotting, the two membranes were thoroughly washed in PBSTween for 15 minutes. One membrane was incubated with goat anti-type I primary antibody (Southern Biotechnology Associates, Alabama, USA) and the other with goat anti-type II primary antibody (Southern Biotechnology Associates, Alabama, USA) in 0.1% BSA at 1: 200 dilution for 90 minutes with gentle continuous rocking. Next, the membranes were again thoroughly washed in PBSTween for 15 minutes and thereafter incubated with rabbit anti-goat horseradish peroxidase (HRP) secondary antibody in PBSTween at 1: 2000 dilution for 90 minutes on a rocker. After further washing, the membranes were counterstained with DAB (Sigma, Poole. UK).

4. Visualisation

To confirm positive blotting or complete transfer, the non-blotted Coomasie blue stained gels were examined side-by-side with the blotted gels and the immunostained transfer membranes on a table mounted viewing box.

Results

The findings were similar for all the osteophyte cultures examined. The Coomassie blue stained gels revealed bands of the normal protein constituents of the culture media (control) (Figure 72). The intensity of staining was different in the bands highlighted by Coomassie blue. In addition, the CB staining of the non-blotted gels revealed negative staining or void at certain locations on the gels. These voids were observed to correspond to the sites where types I and II collagens were expressed when these gels were compared to the blotted immunostained nitrocellulose papers. The blotted gels, which were later stained with Coomassie blue, revealed complete transfer of the proteins unto the nitrocellulose transfer membrane.

Immunostaining of the membranes revealed positive staining for collagen types I (Figure 73) and II (Figure 74).

Discussion

According to these results, cells derived from the cartilage mantle of osteophytes are able to synthesize types I and II collagens when grown in a monolayer culture. Type I collagen is characteristic of the fibroblastic phenotype (Muller *et al* 1975) and type II collagen is characteristic of the chondrocytic phenotype (Mayne 1989). Their co-existence in the culture medium suggests either a heterogeneous cell ancestry, two separate differentiation pathways pursued by the cultured cells or cells in transition from one phenotype to another.

In this study, particular care was taken to ensure that only the cartilage mantle of osteophytes was harvested and contamination with normal articular cartilage mantle was avoided in the osteophytic cultures. In similar culture environment, normal articular cartilage cells also synthesise types I and II collagen and the cells change from a polygonal to a fibroblastic morphology (von der Mark *et al* 1977). von der Mark *et al* (1977) did not observe any relationship between cell shape and collagen type synthesised. They also demonstrated that cells are able to ‘switch’ abruptly from synthesising one type of collagen to another. The inference to be made from this study in the light of the above findings, therefore, is that the chondrocyte of osteophytes in monolayer culture behave like that of normal articular cartilage. This therefore suggests that chondrocytes may be the ancestral cell of the osteophyte.

There is no universal agreement as to the origin of marginal osteophytes. Most workers agree that its formation is preceded by metaplasia of the mesenchymal marginal tissues of articular cartilage in a degenerate or degenerating joint in response to synovial stretching, joint instability and inflammation (Bennett and Bauer 1937, Llyod-Roberts 1953, Resnick *et al* 1991). The marginal mesenchymal metaplasia is followed by hyperplasia and subsequent increase in the vascularity of the resultant fibrocartilaginous bud (Key 1931, Fisher 1939, Calandruccio and Gilmer 1962, Marshall 1969, Gilbertson 1975). Moskowitz and Goldberg (1987) noted that this cartilaginous bud has a hyaline cartilage base and a fibrocartilage cap. As the osteophyte matures, the basal region of the hyaline cartilage becomes hypertrophic

and is partly invaded by blood vessels and subsequently undergoes endochondral ossification.

The findings of collagen type I and II in the culture media suggests that the cells are functionally chondrocytic although phenotypically they almost all appear fibroblastic. This may further buttress the suggestion that osteophytes originates from a mesenchymal proliferation which then differentiates initially to form cartilage. This cartilage may be more epiphyseal than articular in nature and this may explain why the osteophytic cartilaginous bud can be invaded by blood vessels.

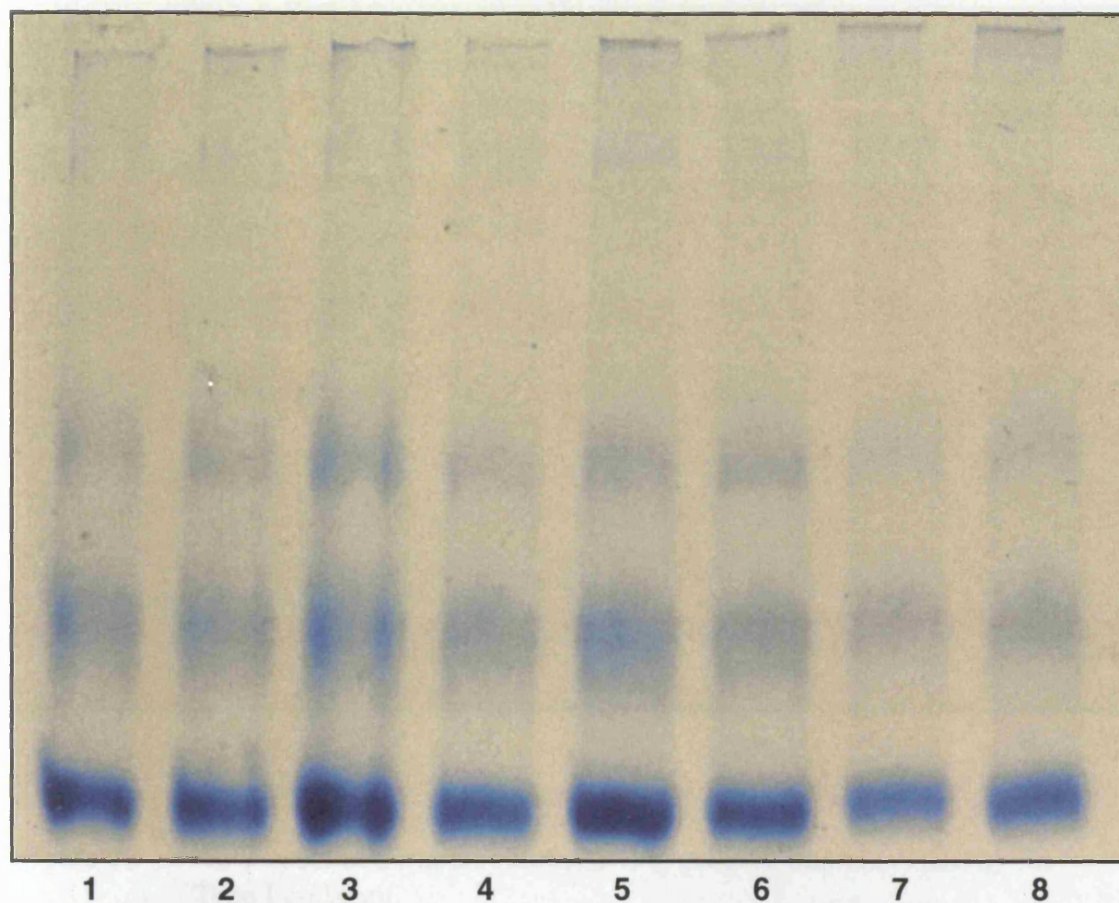


Figure 72 - A Coomassie blue stained gel (8 specimens) showing the normal protein constituents of the culture medium.

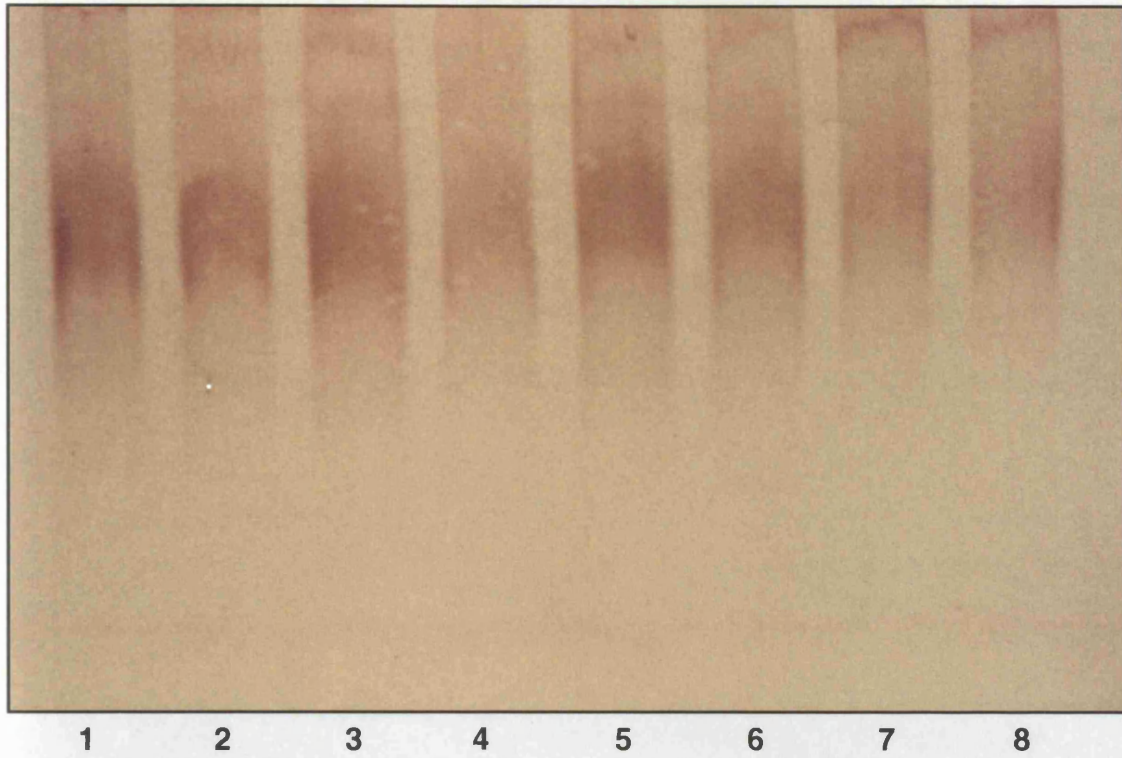


Figure 73 - An 8-specimen immunoblot showing positive immunoreactivity for Type I collagen.

PART I - MOLECULAR BIOLOGY OF OSTEOPHYTES.

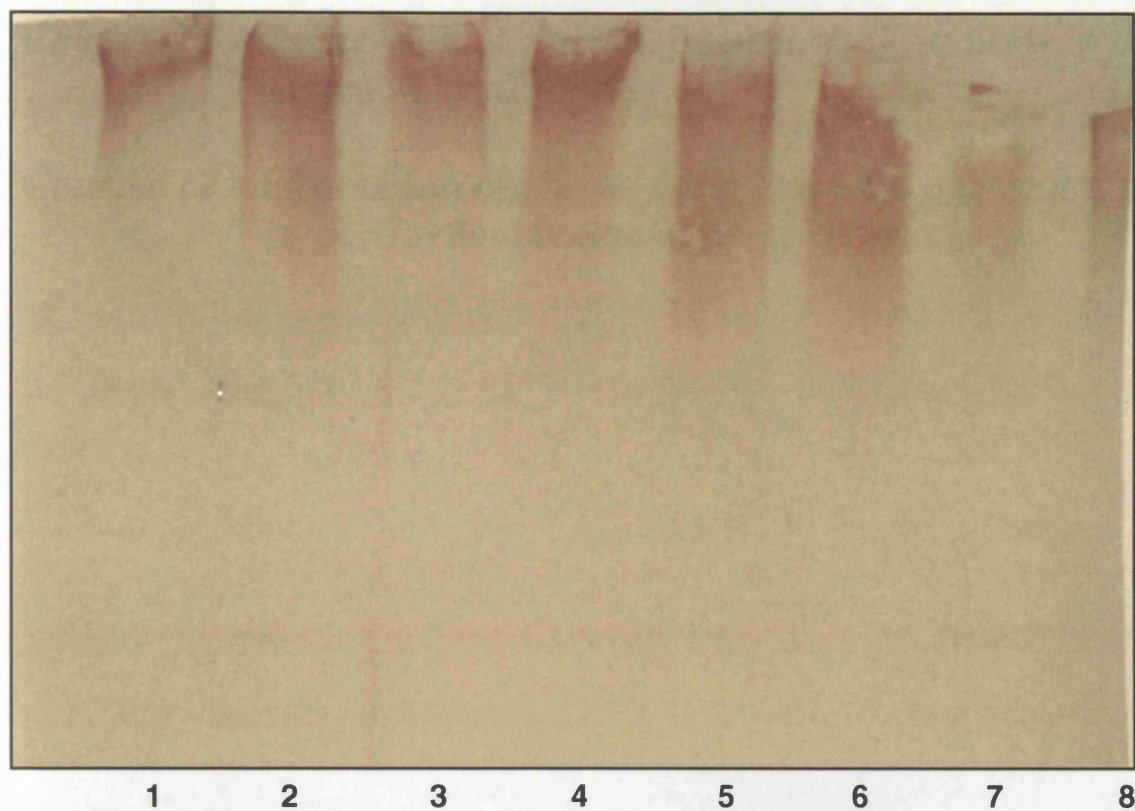


Figure 74 - An 8-specimen immunoblot showing positive immunoreactivity for Type II collagen.

PART 4 – MOLECULAR BIOLOGY OF OSTEOPHYTES.

***Chapter 11* – Expression of osteoblastic phenotype in peri-articular osteophytes.**

***Chapter 12* – Expression of c-myc, c-jun and c-fos oncogenes in peri-articular osteophytes.**

Expression of the osteoblastic phenotype in peri-articular osteophytes.**Summary**

Sections of osteophytes and normal articular cartilage (control) were stained for alkaline phosphatase using histochemical technique and sections of osteophytes alone were stained for alkaline phosphatase and osteocalcin using immunohistochemical technique. There was positive staining for alkaline phosphatase and osteocalcin by the chondrocytes in the deep layer of the osteophytic cartilage but not in the normal cartilage, which suggests that this layer (deep layer) in the peri-articular osteophyte is capable of undergoing ossification.

Introduction

An osteophyte consists of a cartilage mantle and a cancellous bony base. In this respect, it is similar to normal articular cartilage and therefore could be used as an osteochondral graft to repair full thickness articular cartilage defects (FTCD) (Oni and Morrison 1998, Alonge *et al* 1998). The survival of the graft may be predicted if the ultimate fate of the grafted cartilage was known. Differentiation of chondrocytes along an osteoblastic lineage has been previously described in the fracture callus (Stafford *et al* 1992) and in the growth plate (Oni 1997). The osteoblastic phenotype demonstrated by the chondrocytes in these instances was characterised by the production of alkaline phosphatase and osteocalcin (Hauschka *et al* 1985). It is important to determine, for example, whether the chondrocytes of the cartilage mantle of the osteophytic graft are terminal chondrocytes or transitional cells.

The initiation and calcification of cartilage involves the activity of alkaline phosphatase (ALP). The mechanisms involved include the hydrolysis of pyrophosphate with subsequent release of high concentration of phosphate, which then precipitates out along with calcium [booster theory], as well as the suppression of the inhibitors of mineralization by hydrolysis. Alkaline phosphatase acts as a nucleator, which brings together calcium and inorganic pyrophosphate on a hydrophobic membrane (Harrison *et al* 1995). ALP also regulates the phosphate content of the matrix through the release of phosphate from matrix phosphoprotein (Harrison *et al* 1995, Beertsen and van den Bos 1989).

Osteocalcin (BGP) is a gamma-carboxyglutamic acid (GLA) protein exclusively expressed by osteogenic cells (Hauschka *et al* 1985, Salisbury *et al* 1994). It is synthesised by osteoblasts and secreted into the matrix of the osteoid after the initiation of mineralization. It helps to regulate crystal growth and recruitment of osteoblasts. The secretion of BGP is controlled by vitamin D, TGF- β and parathroid hormone (Salisbury *et al* 1994).

The aim of this study was to evaluate the osteoblastic potentials of the chondrocytes in the different layers of the cartilage mantle of osteophytes with a view to predicting the fate of these cells.

Materials and Methods

Osteophytes were obtained from the margins of the femoral condyles of 10 patients undergoing total knee replacement for osteoarthritis (OA). As controls, visually normal articular cartilage samples were also obtained from apparently unaffected areas of the knee of these patients during the femoral cuts. Each

specimen was embedded in Cryo-M-Bed (Bright) on a piece of cork, snap frozen in liquid nitrogen and stored at -80°C . Thereafter, $7\mu\text{m}$ sections were cut using a Bright cryostat with a blade specifically designed for skeletal tissue and mounted on silane-coated slides. The slides were air-dried at room temperature for 30-60 minutes, and fixed in analytical grade acetone (Sigma Dorset. UK) for 10 minutes. The slides were air-dried again before wrapping in aluminium foil for storage at -20°C until ready for staining. Osteophyte and normal articular cartilage sections were used for alkaline phosphatase (ALP) histochemistry while osteophyte sections alone were used for ALP and osteocalcin immunohistochemistry.

Alkaline phosphatase

Histochemistry.

The staining solution was prepared by dissolving 15 mg of sodium alpha-naphthyl phosphate and 20 mg of Fast red Tris salt in 20mls of 0.2M Tris buffer (pH 10) and the mixture was then filtered.

The slides were brought to room temperature, re-hydrated in PBS for 10 minutes and flooded with the staining solution for 30 minutes. Excess stain was drained and the sections rinsed with PBS, mounted in aquamount (BDH Lab. Poole UK) and viewed under the light microscope.

Immunohistochemistry

The sections were brought to room temperature, re-hydrated with PBS for 10 minutes and treated with 1mg/ml testicular hyaluronidase in sodium acetate buffer (Type Is, Sigma, Poole UK) for one hour. After rinsing in PBS, the sections were flooded with B4-50 primary antibody (Developmental Studies

Hybridoma Bank, Iowa, USA) at 1 : 20 dilution and left to incubate for 90 minutes. Next, they were washed repeatedly with PBSTween for 15 minutes and thereafter flooded with rabbit anti-mouse horse radish peroxidase (HRP) conjugated secondary antibody (DAKO Ltd. High Wycombe, UK) at 1 : 150 dilution and allowed to incubate for 90 minutes. The sections were again repeatedly washed with PBSTween for 15 minutes and then counterstained with DAB Sigma Fast tablets (Sigma Poole, UK) solution until brown staining appeared.

Osteocalcin

Immunohistochemistry

The staining technique was as for alkaline phosphatase except for a change in primary antibody to rabbit anti-bovine osteocalcin primary antibody (kindly donated by Dr S Robins, Aberdeen) at 1 : 20 dilution. The secondary antibody used was goat anti-rabbit HRP conjugated secondary antibody (DAKO Ltd. High Wycombe, UK).

All dilutions were made in 0.1% bovine serum albumin (BSA) and PBSTween.

Results

Positive staining for ALP was observed in some chondrocytes in the deep layer of the osteophytes adjacent to the subchondral bone. The cells were more deeply stained following histochemistry (Figure 75) whereas with immunohistochemistry brownish bands and clumps were observed as opposed to cellular staining (Figure 76). Positive staining was also observed in the endothelial vascular lining in the subchondral bone as well as the endosteal lining.

In all cases, the chondrocytes in the middle and superficial layers of the cartilage mantle did not stain for ALP. In the normal articular cartilage, the chondrocytes in all the layers of the cartilage mantle did not stain for ALP (Histochemistry) (Figure 77).

Positive staining for osteocalcin was observed in the chondrocytes in the deep layer of the cartilage mantle of osteophyte i.e. cells close to the subchondral bone (Figure 78). Chondrocytes in the middle and superficial layers did not stain for osteocalcin.

Discussion

According to these findings, the chondrocytes of the deep layer of the cartilage mantle of osteophytes elaborate ALP and osteocalcin. Therefore, it may be assumed that these cells are capable of transforming into bone and this raises the possibility that the cartilage of osteophyte may be susceptible to bony encroachment.

Cartilage exists in two forms depending on the environment: resorbable cartilage and permanent cartilage. Cartilage that is destined to be removed and transformed into bone is referred to as resorbable cartilage. This type of cartilage produce ALP (Robinson 1923) and it may posses a built-in obsolence (Ali 1980). It is commonly found in diaphyseal skeletal cartilage, neocartilage and fibrocartilage. Permanent cartilage by contrast, does not produce ALP and are not endowed with a built-in obsolence. This type of cartilage is found in articular cartilage, the pinna and nasal septal cartilage (Archer 1994). This feature of the articular cartilage presumably is the basis for Mankin's (1982) suggestion that

the articular cartilage may be designed to last the lifetime of an individual differing therefore embryologically and functionally from resorbable cartilage.

Since the chondrocytes in the middle and superficial layers of the cartilage mantle of the osteophytes do not express ALP and osteocalcin, this may imply that there are two types of cartilage in an osteophyte. The middle and superficial layers of the cartilage mantle of the osteophytes may be made of permanent cartilage and the deep layer of resorbable cartilage. This may explain why the chondrocytes in the middle and superficial layers do not produce ALP and osteocalcin. This result supports the finding of Moskowitz and Goldberg (1987) on the composition and the pathogenesis of osteophytes.

From this result, since almost two-thirds of the cartilage mantle of osteophytes is 'permanent' by virtue of their biochemical composition, osteophytes may still be a suitable substitute (as an osteochondral graft) for repairing full thickness articular cartilage defects (FTCD) (when this osteochondral tissue is found in association with FTCD).

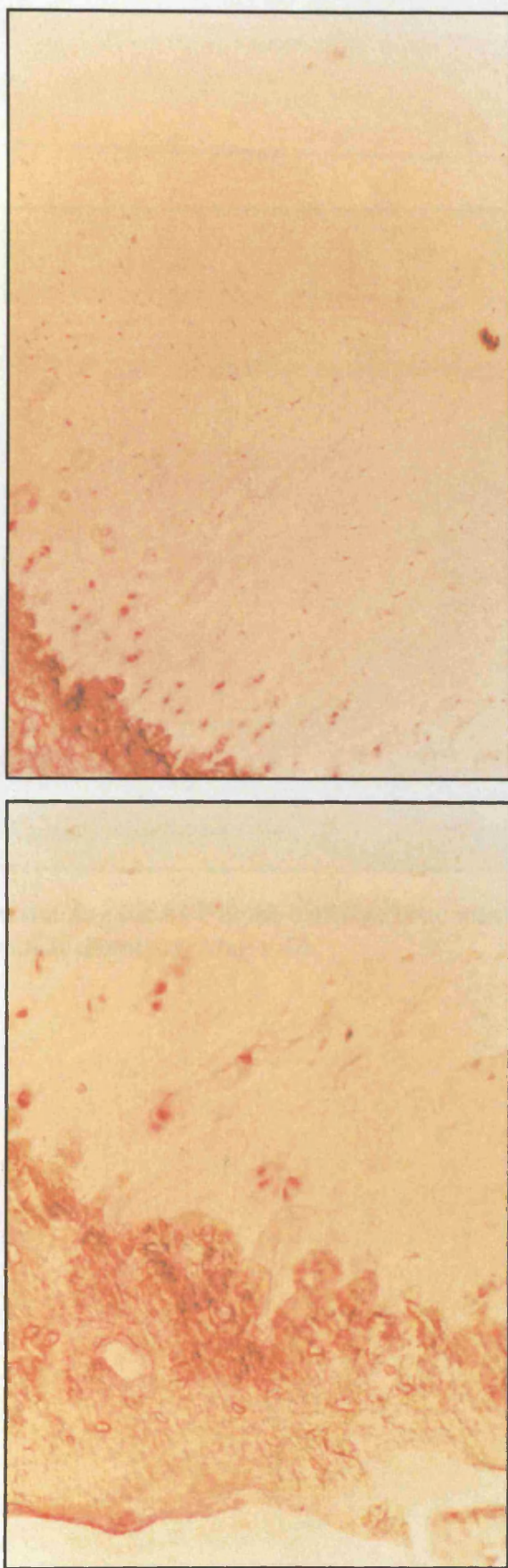


Figure 751b Positive staining for alkaline phosphatase (ALP) by the basal chondrocytes in the deep layer of the cartilage mantle of osteophytes. Histochemistry. Mag 1a (x 40), 1b (x 100).

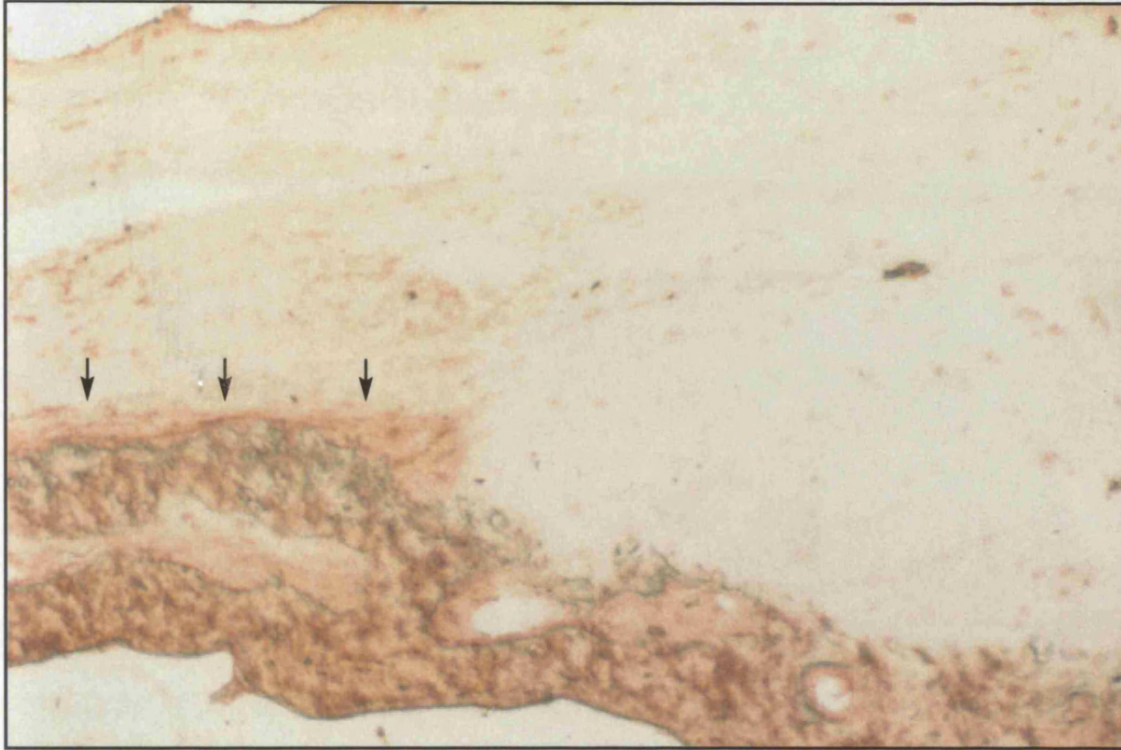


Figure 76 - Positive staining for ALP at the cartilage/bone interface.
Immunohistochemistry. Mag x 40.

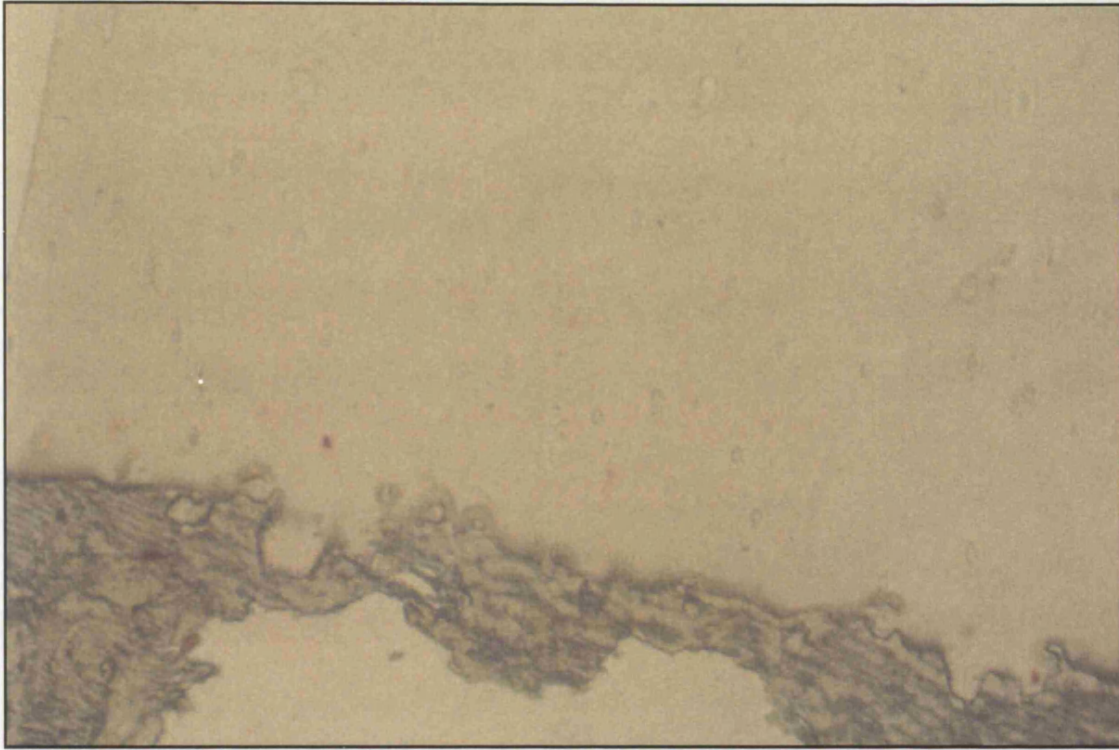


Figure 377 - Negative staining for ALP in the cartilage mantle of normal articular cartilage. Mag x 100.

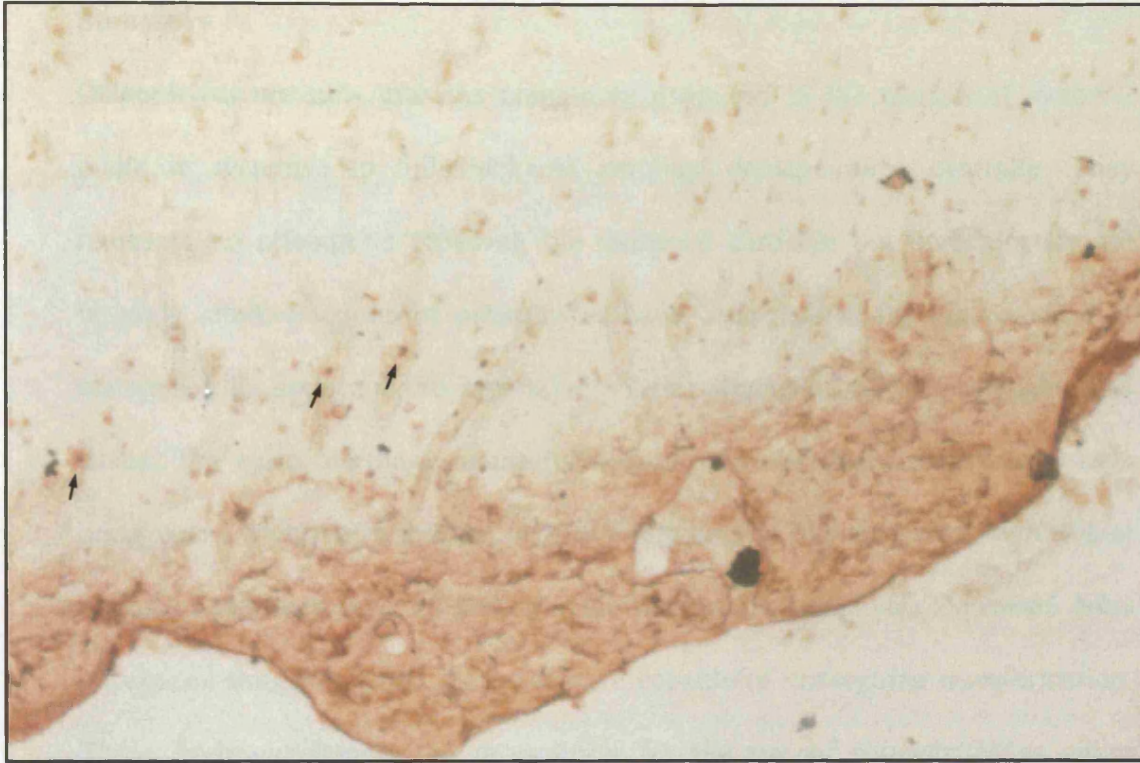


Figure 78 - Positive staining for osteocalcin by chondrocytes in the deep layer of the cartilage mantle of osteophytes. Immunohistochemistry. Max x 100.

Expression of c-myc, c-jun and c-fos oncogenes in peri-articular osteophytes.**Summary**

Osteophytes are new growths commonly produced in the margin of synovial joints in response to full thickness cartilage damage more centrally. They represent an attempt at repairing the damaged cartilage but unfortunately are wrongly sited. Sections of osteophytes have been stained for the presence of oncogenes to ascertain the reparative role or otherwise of this osteochondral tissue. The entire cartilage mantle of osteophytes was found to express c-myc oncogenes, which is a marker of proliferation. The chondrocytes in the basal region of the deep layer of the cartilage mantle of osteophytes expressed c-fos oncogenes suggesting that these cells are capable of undergoing transformation. These findings support the proposition for the use of osteophytes as either sources of chondrocytes for autologous transplantation or as osteochondral grafts for repairing full thickness articular cartilage defect.

Introduction

Osteophytes are neoplastic intra-articular outgrowths that are commonly found at the margins of the articular cartilage in response to a more central full thickness damage to the articular cartilage. Basically, they are osteochondral repair tissues, which are apparently wrongly sited (Aigner *et al* 1995). Osteophyte formation is presumed to be a response to subtle changes in the biochemical composition and stiffness characteristics of the diseased articular cartilage (Buckland-Wright *et al* 1991). The presence, number, size and the rate of growth of osteophytes has been found to be influenced in part by the activity of the predisposing disease process

and certain growth factors (Buckland-Wright *et al* 1991, Knaggs 1932, Anderson 1985, Horn *et al* 1992, Schouten *et al* 1993). The exact mechanism of formation of this repair tissue remains unclear, however, two types of osteophytes 'white' and 'pink' have been described based on the thickness of their cartilage mantles, and this is probably a reflection of the stage of maturation or development of the tissue (Alonge *et al* 1998).

Oncogenes are genes, which are capable of promoting the transformation of normal cells into neoplastic cells. Cellular oncogenes (c-oncs) are derived from proto-oncogenes, which are pre-existing in the cells. Alteration or inappropriate activation of the proto-oncogenes leads to the formation of oncogenes and these oncogenes themselves can be activated by insertion of promoter sequence in their vicinity, point mutation or gene amplification (Davies and Wright 1996). C-myc, C-jun and C-fos are nuclear binding oncogenes and their products, myc, jun and fos proteins are nuclear transcription factors (Kumar *et al* 1992).

The aim of this study was to localise the site(s) of expression of c-myc, c-jun and c-fos oncogenes in peri-articular osteophytes since they (osteophytes) are commonly referred to as repair tissues.

Materials and method.

Osteophytes were obtained from the margins of the trochlear and the femoral condyles in 6 patients aged 66-88years undergoing total knee replacements for osteoarthritis (OA). Visually normal articular cartilage was obtained from the posterior femoral condyles cuts in another 6 patients undergoing total knee replacement for OA in compartments not affected by the disease.

Decalcified specimens.

One set of specimens was formalin fixed, decalcified, routinely processed and embedded in paraffin. 7µm sections were obtained and stained for c-myc and c-jun oncogenes using immunohistological technique.

Undecalcified or Frozen specimens.

Another set of specimens from the same patients was embedded in Cryo-M-Bed (Bright) on a piece of cork and snap frozen in liquid nitrogen. Thereafter, 7µm sections were cut using the Bright cryostat model with a blade specifically designed for skeletal tissue. Sections were stained for c-fos using immunohistological technique.

Staining technique.*Avidin HRP technique for c-myc and c-jun.*

Sections were de-waxed through graded alcohol and xylene, taken to water and dipped in 6% hydrogen peroxide (to block endogenous alkaline phosphatase) for 10 minutes. Thereafter they were washed in water and PBS (pH 7.6). Sections were now flooded with normal goat serum at 1 : 20 dilution in tris buffered saline (TBS) for 10 minutes and excess buffer drained but not washed. Primary antibody was added to labelled slides (100µl/slide): mouse anti-myc (Novocastra Lab. Ltd. Newcastle-upon-Tyne, UK) primary antibody at 1 : 150 dilution in TBS and mouse anti-jun (Novocastra Lab. Ltd. Newcastle-upon-Tyne, UK) primary antibody at 1 : 20 dilution in TBS. The primary antibody was allowed to incubate for 60 minutes before washing the sections in phosphate buffered saline

(PBS) for 20 minutes. Biotinylated goat universal anti-mouse secondary antibody (DAKO Ltd. High Wycombe, UK) at 1 : 200 dilution was then added to the sections and left to incubate for 30 minutes. Thereafter, the sections were washed in PBS for 20 minutes before adding the ABC complex horseradish peroxidase (HRP) (DAKO Ltd. High Wycombe, UK) for 30 minutes. The sections were then washed in PBS for a further 30 minutes before adding 0.05% DAB (BDH) solution (Sigma, Poole. UK) for 5 minutes. Sections were then washed in tap water for 5 minutes the nuclei were counterstained with haematoxylin for 30 seconds and rinsed again in running tap water. The sections were now dehydrated through graded alcohol and xylene, mounted and examined with the light microscope.

Avidin alkaline phosphatase technique for c-fos.

Sections were brought to room temperature, washed in water for 2 minutes and flooded with TBS buffer for 2 minutes. Excess buffer was wiped from the slides and the sections flooded with normal rabbit serum in TBS at 1 : 20 dilution (DAKO Ltd. High Wycombe, UK) for 10 minutes. Excess buffer was drained and mouse anti-fos primary antibody (100µl/slide) was added to the sections at 1 : 20 dilution in TBS (Novocastra Lab. Ltd. Newcastle-upon-Tyne, UK). The primary antibody was allowed to incubate for 60 minutes and thereafter; the sections were washed in TBS for 20 minutes. Biotinylated rabbit anti-mouse secondary antibody was now added to the sections at 1 : 400 dilution in TBS (DAKO Ltd. High Wycombe, UK) for 30 minutes after which the sections were washed in TBS for 20 minutes. Avidin conjugated alkaline phosphatase (ACAP) at 1 : 400 dilution in TBS (DAKO Ltd. High Wycombe, UK) was now added to

the sections and allowed to incubate for 30 minutes. The sections were washed in TBS for a further 20 minutes before immersing the sections in a developer made up of: Levamisole (24mg), Fast Red TR (50mg), Veronal acetate buffer (VAB) pH 9.2 (100ml) and Naphthol asB1 PO₄ (50mg) for 60 minutes. Thereafter, the sections were washed in water for 5 minutes, counterstained with haematoxylin for 30 seconds, washed again in running tap water, mounted and examined with a light microscope

Result

C'-myc.

Sections of breast carcinoma had positive cellular (brown) staining (positive controls) (Figure 79).

In all the osteophyte sections, positive staining of the chondrocytes was observed in the entire cartilage mantle (Figure 80a), the endothelial vascular lining and the endosteal lining of the cancellous subchondral bone (Figure 80b). There was no staining in the chondrocytes (Figure 81a) nor the subchondral bone of the visually normal articular cartilage (negative controls) Figure 81b).

C'-jun.

Sections of breast carcinoma had positive cellular (brown) staining (positive control) (Figure 82).

The chondrocytes of the osteophyte did not stain for c-jun (Figure 83) neither did the chondrocytes of the visually normal articular cartilage (Figure 84). No staining was observed in the subchondral bone of either the osteophyte of the normal articular cartilage.

C-fos.

Sections of human skin had a positive cellular (red) staining for c-fos (positive control) (Figure 85).

The chondrocytes in the upper and middle layers of the cartilage mantle of the osteophyte sections had negative staining for c-fos. The chondrocytes at the basal region of the deep layer of the cartilage mantle had intense positive staining. The endothelial vascular lining and the endosteal lining of the cancellous subchondral bone also had positive staining (Figure 86). The chondrocytes in the entire cartilage mantle of the visually normal articular cartilage (Figure 87a) had a negative staining for c-fos, but there were very scanty, isolated positive endothelial staining in the subchondral bone (Figure 87b).

Discussion.

Myc oncoprotein is required by cells to enter and transit the cell cycle, and hence it is required for cell proliferation (Prendergast 1997). Expression of c-myc has been shown to drive quiescent cells into the cell cycle and at the same time block cell differentiation (Prendergast 1997). From this study, the expression of c-myc oncogenes by the chondrocytes in the entire cartilage mantle of osteophyte therefore suggests that active proliferation is apparent in these cells. The lack of expression of c-myc in the chondrocytes of the visually normal articular cartilage suggests that these cells are propelled towards terminal differentiation (Prendergast 1997). One can then conclude that the metabolic activities of osteophytic chondrocytes are higher than those of normal articular cartilage.

C-jun and c-fos belong to the bZIP (basic/leucine zipper) super family of transcription factors. Like c-fos, c-jun is a cytoplasmic oncogene and it is only active in the nucleus (Bannister and Kouzarides 1997). In contrast to c-fos, however, c-jun is a relatively weak inducers to transformation and in general, normal mammalian cells (including humans) are resistant to transformation by c-jun. In addition, the cellular localisation of c-jun is less regulated compared to c-fos (Bannister and Kouzarides 1997). These may be responsible for the lack of expression of this oncogene in both the osteophytic tissues and the normal articular cartilage save for the malignant cells (breast carcinoma cells).

C-fos has been shown to be involved in the determination of cell differentiation along the osteoclast/macrophage lineage and it is crucial for bone remodelling (Bannister and Kouzarides 1997). The cellular localization of c-fos is well regulated and its expression is found and retained in the nuclei of quiescent or non-active proliferative cells (Bannister and Kouzarides 1997). C-fos has also been shown to aid the transformation of fibroblasts into osteogenic and chondrogenic lineage (Bannister and Kouzarides 1997). The expression of c-fos by the chondrocytes at the basal region of the deep layer of the cartilage mantle of osteophytes where the cartilage is continuous with bone strongly suggests that c-fos oncogene has been switched on and that these cells are capable of transforming into bone. The expression of c-fos by the chondrocytes adjoining the subchodral bone may also suggest that promoter genes in the vicinity of these cells (probably from the blood vessels) is responsible for the expression of this oncogene in this location.

This study confirms that osteophytic tissue is endowed with proliferative potential and therefore may be reparative in nature. Although the cartilage mantle of osteophytes are thinner than that of the normal articular cartilage (Alonge *et al* 1998), the cartilage mantle of osteophytes may be capable of adaptive hypertrophy if osteophytes were to be used as osteochondral grafts for full thickness articular cartilage defect. The ability of the c-fos expressing basal osteophytic cartilage chondrocytes to transform into bone along with the proliferative ability of the remaining chondrocytes may further aid good integration of the osteophytic osteochondral graft into the recipient bed. The proliferative potential of the chondrocytes may also make osteophytes an abundant alternate source of chondrocyte in autologous chondrocyte implantation when osteophyte is found in conjunction with full thickness articular cartilage defect.

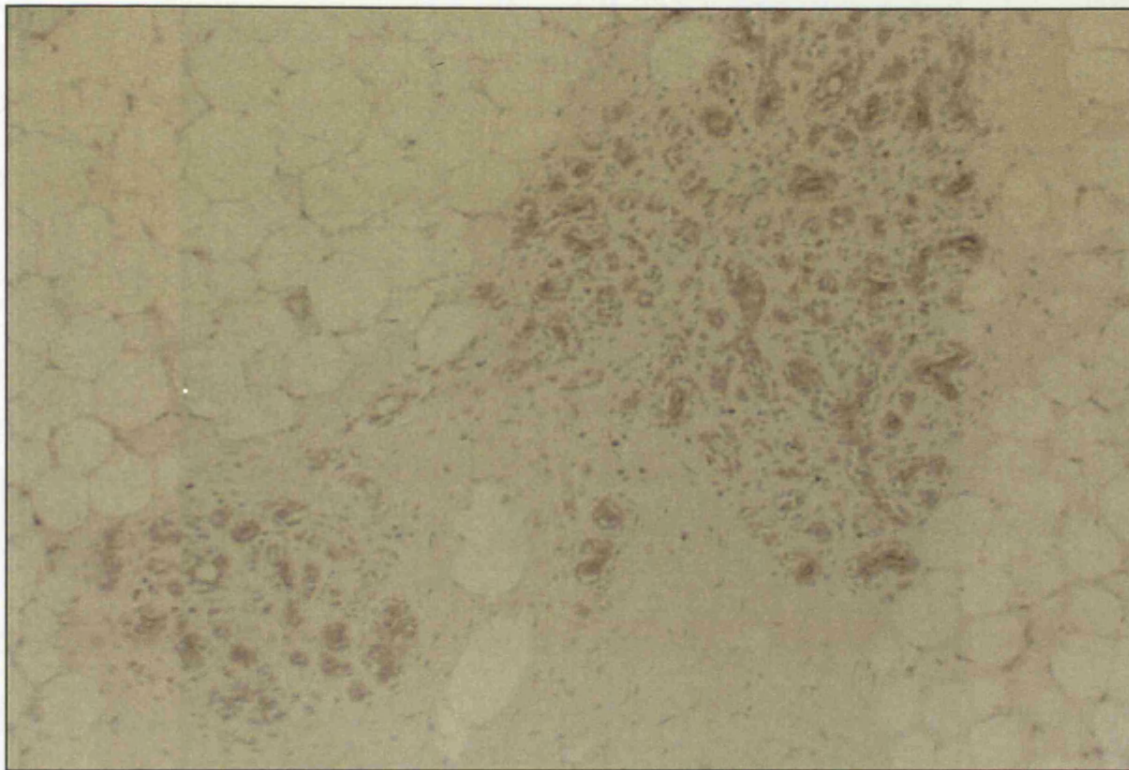


Figure 79 - c-myc expression in breast carcinoma. (positive control). mag x 100.



Figure 80- c-myc expression in the chondrocytes of the cartilage matrix of osteophytes. mag x 100.



Figure 80a- c-myc expression in the chondrocytes of the cartilage mantle of osteophytes mag x 100.

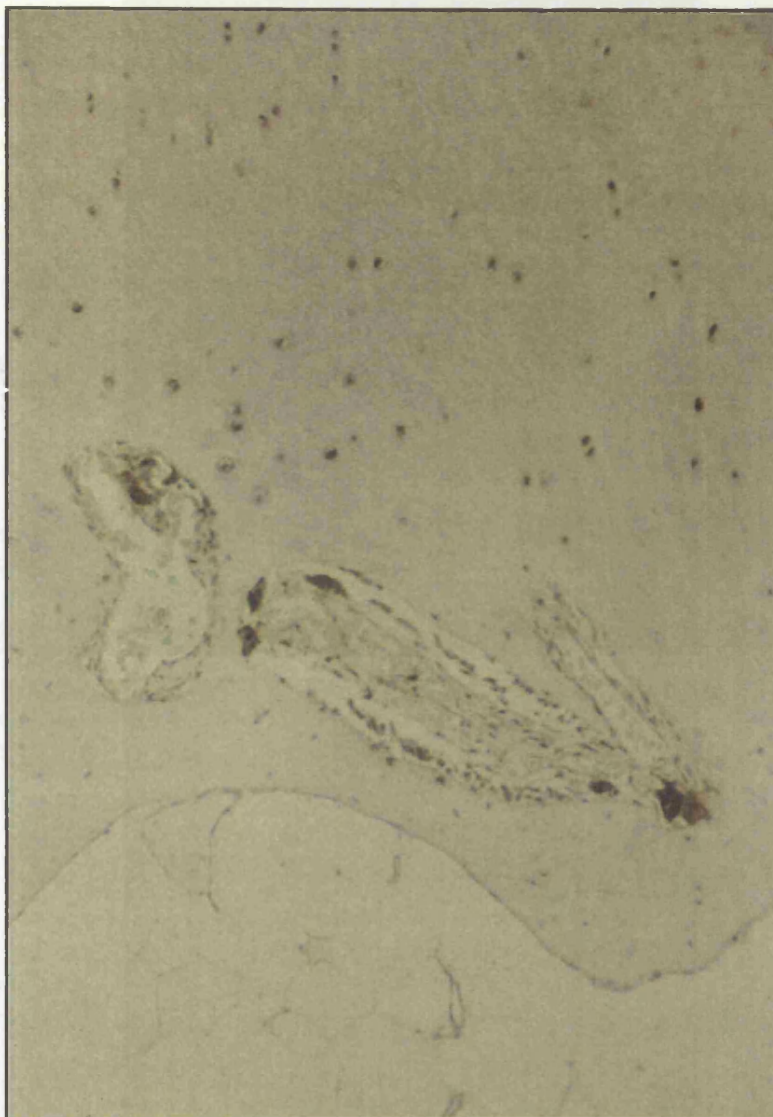


Figure 80b – c-myc expression in the basal chondrocytes of the osteophytic cartilage mantle and vascular endothelial lining of the subchondral bone. mag x 100.

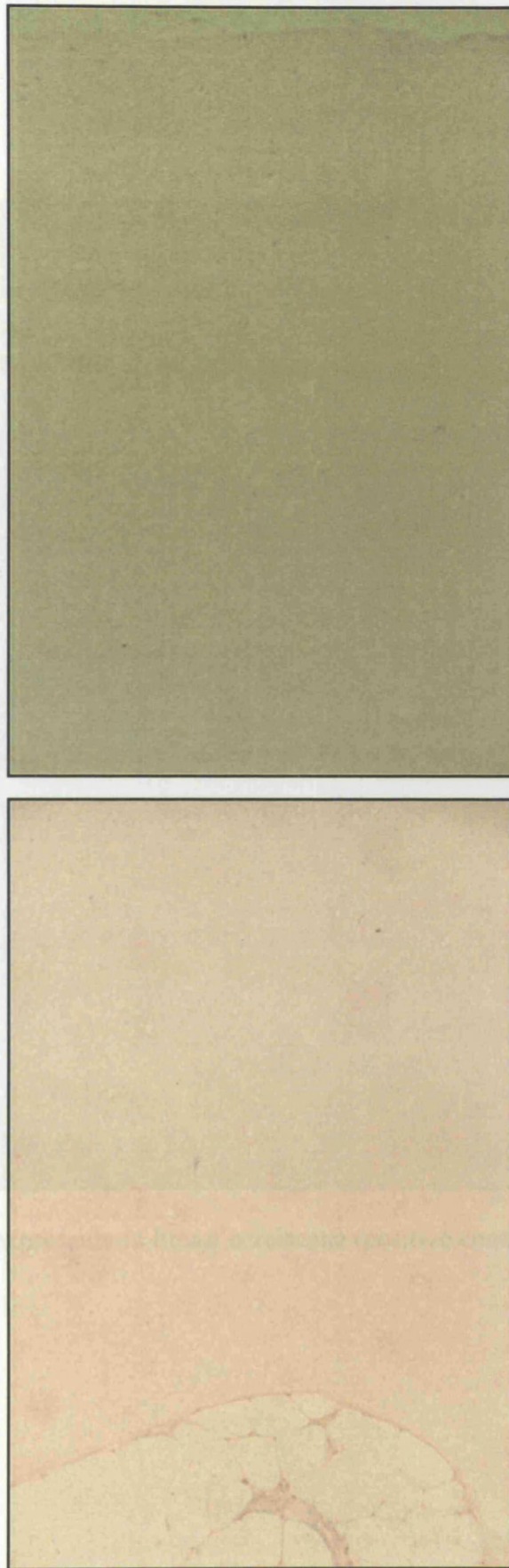


Figure 81a 81b Normal articular cartilage showing no staining for c-myc. The nuclear staining is due to the counterstaining with haematoxylin. mag x 100.

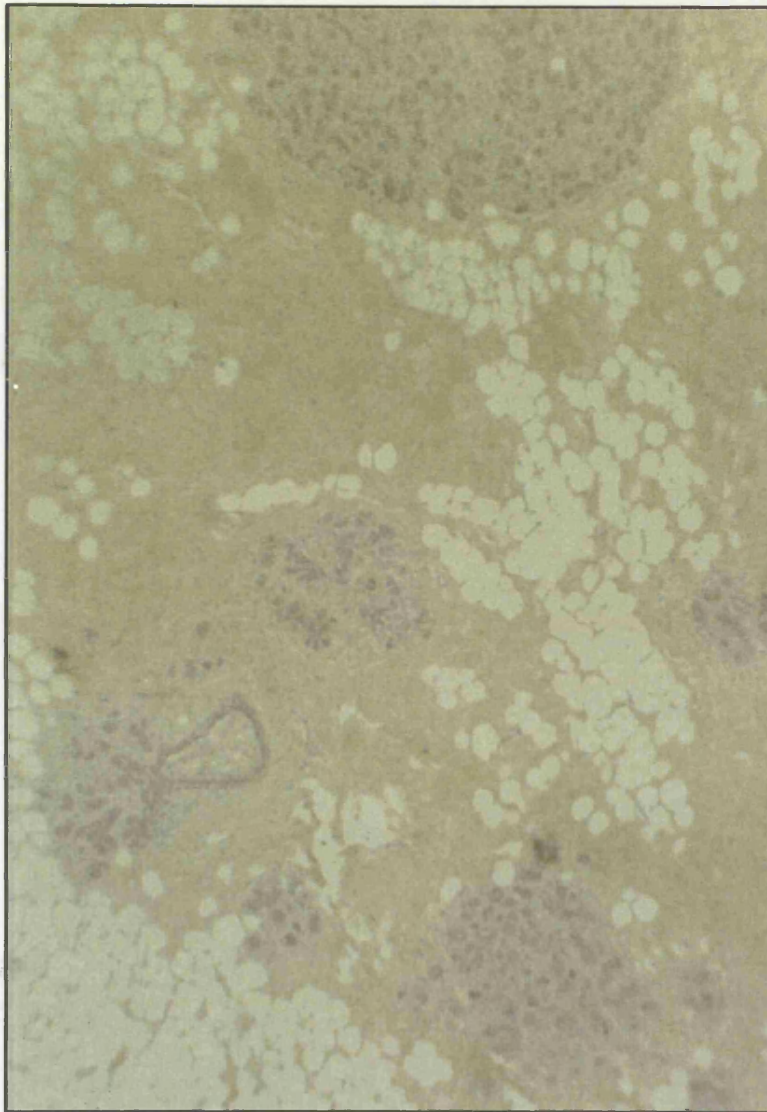


Figure 82 - c-jun expression in breast carcinoma (positive control). mag x 40.



Figure 83 - Osteophyte showing no positive staining for c-jun. The nuclei staining of the chondrocytes is due to the counterstaining with haematoxylin. mag x 40.

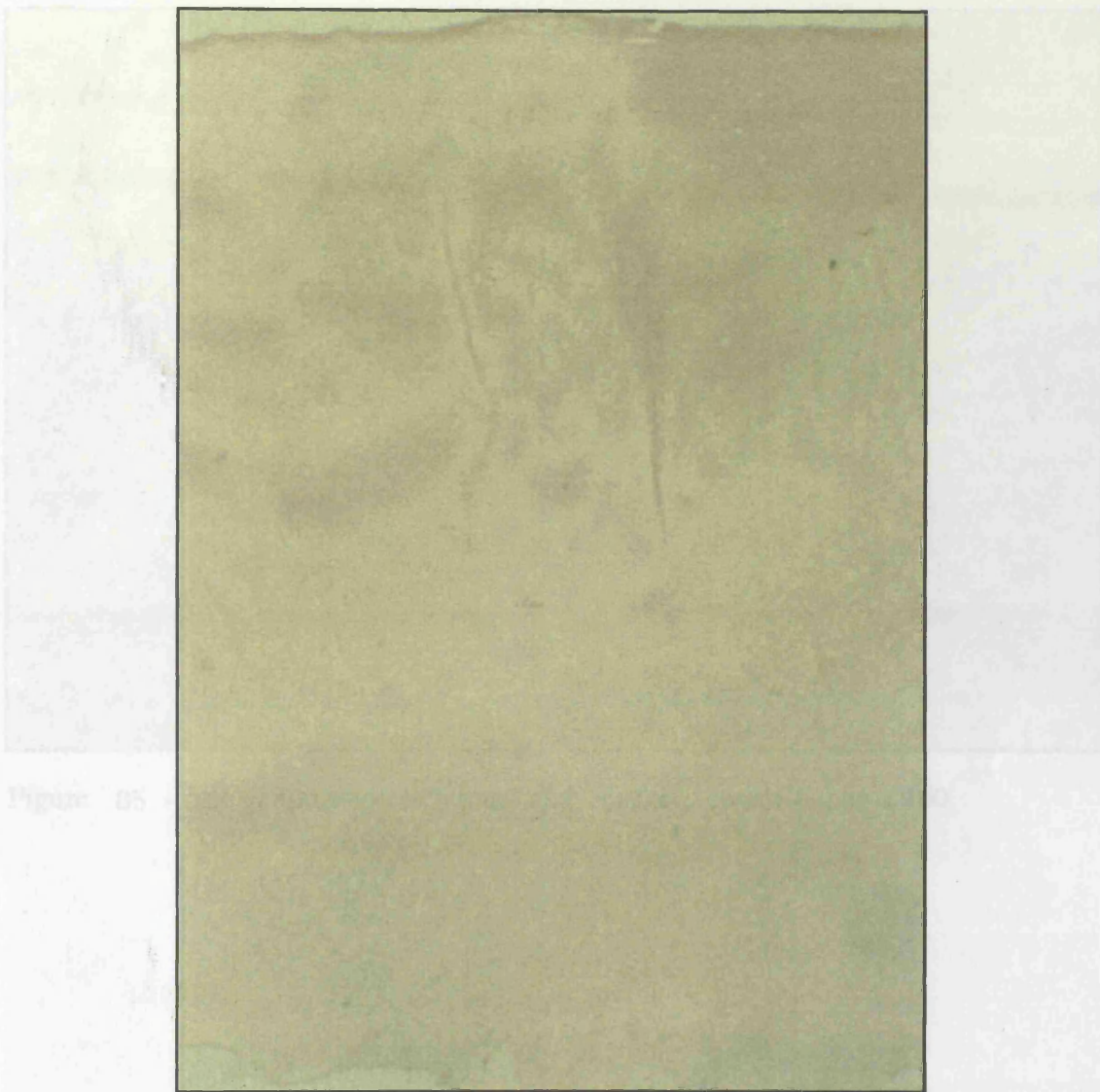


Figure 84 - Normal articular cartilage showing no staining for c-jun. mag x 40.

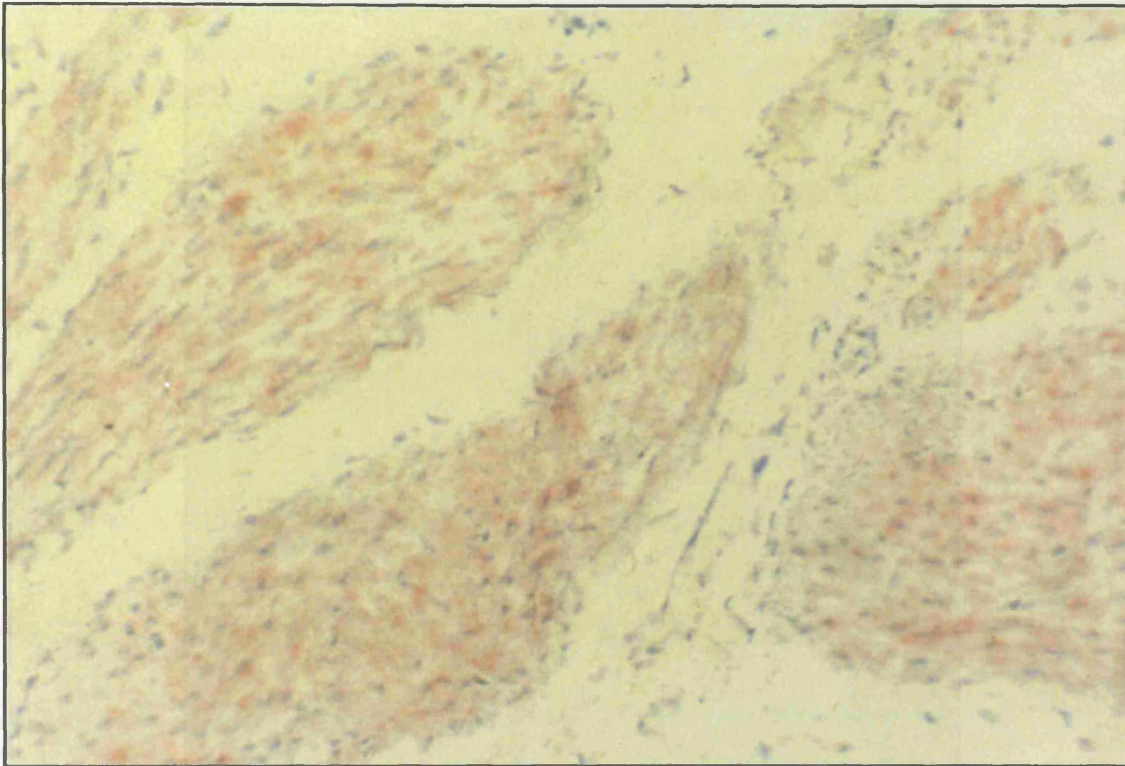


Figure 85 - c-fos expression in human skin (positive control). mag x 100.



Figure 86 - c-fos expression in human skin is limited to the epidermis in the basal region of the deep dermal layer. The epidermis in the superficial and middle layers showed no staining. The granular layer and the rete ridges also had positive staining. mag x 100.

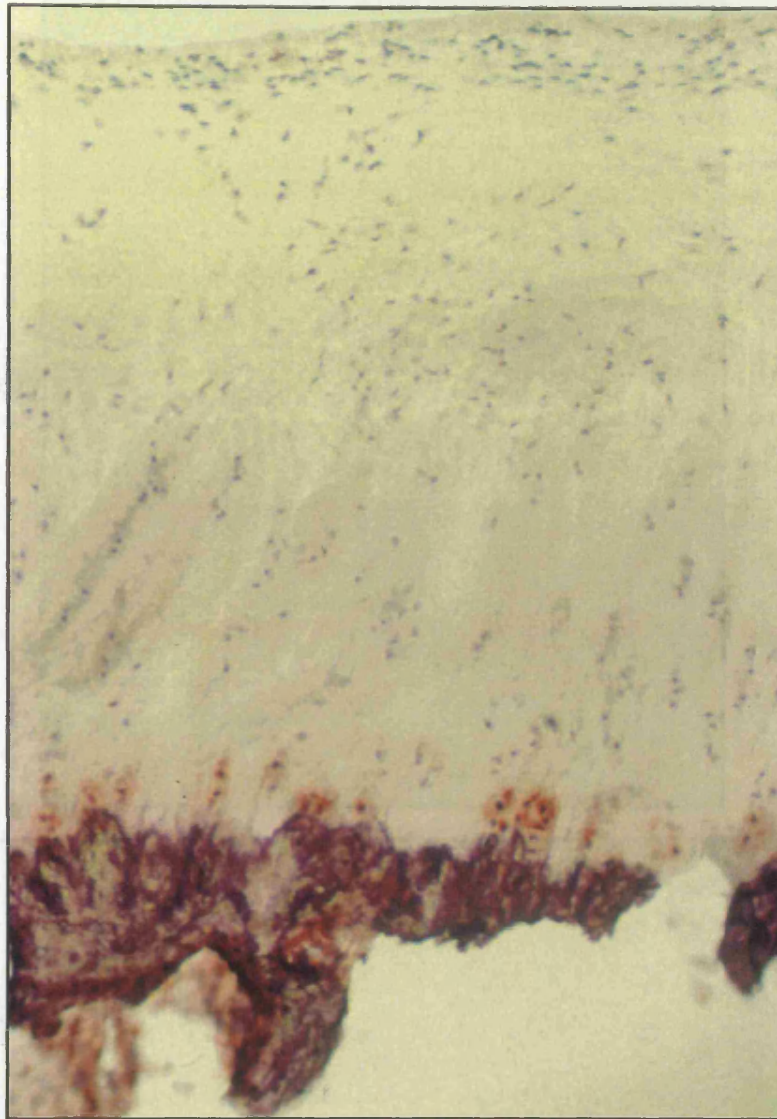


Figure 86 - c-fos expression in osteophyte is limited to chondrocytes at the basal region of the deep cartilage layer. The chondrocytes in the superficial and middle layers showed no staining. The endothelial lining and the osteoid also has positive staining. mag x 100.

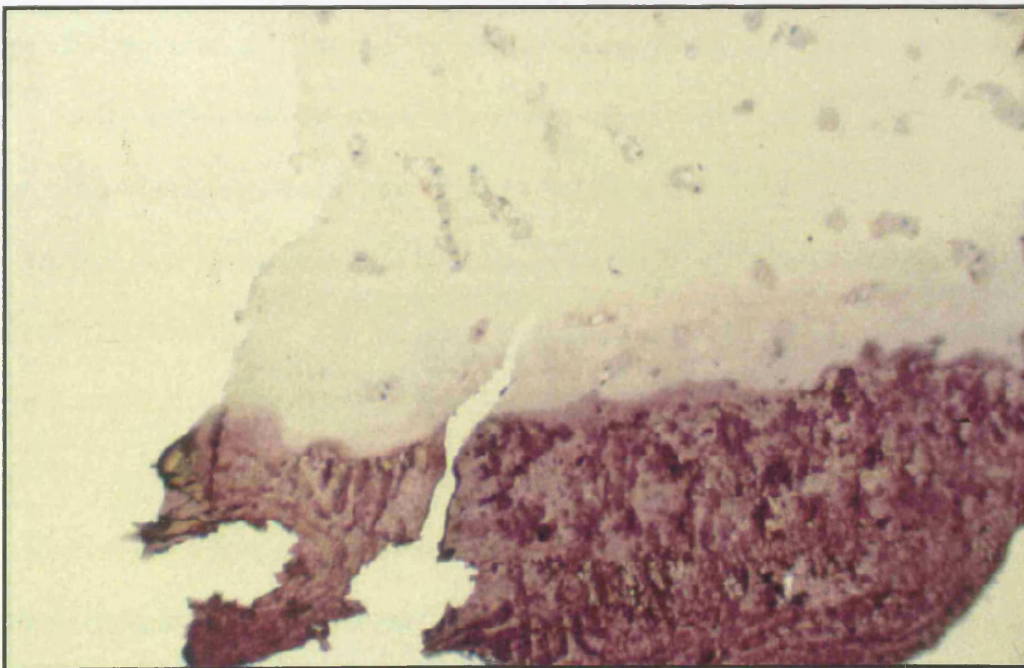
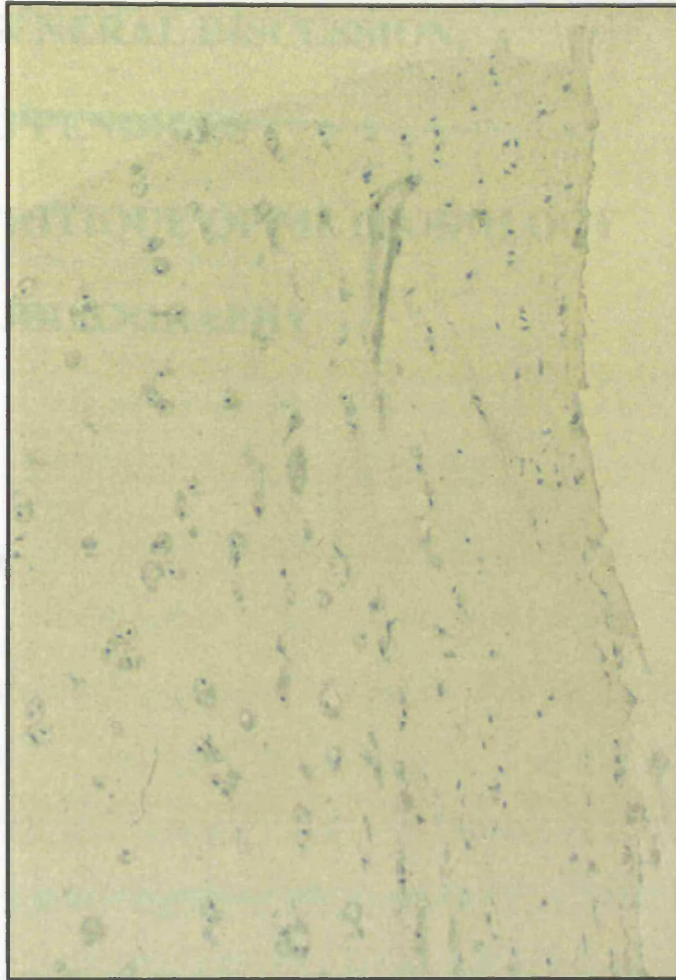


Figure 87a 887b Normal articular cartilage showing no staining for c-fos either in the cartilage mantle or in the subchondral bone. mag x 100.

PART 5 – GENERAL DISCUSSION,

APPENDICES

CRITIQUE OF METHODOLOGY

BIBLIOGRAPHY

General Discussion

In the orthopaedic community, the word osteophyte is synonymous with osteoarthritis. This belief is borne out of the radiological changes observed in advanced joint degenerative diseases and the various classifications of these diseases. It is this concept that has cast a dark cloud over this osteochondral tissue, which is readily expendable during arthroplasties. The reality is that osteophytes are not only pointers to joint degeneration but they act as makers of full thickness articular cartilage defects (FTCD) even when the cartilage injury is occult. This has therefore led to the admission that in actual fact osteophytes may be repair tissues formed in response to FTCD although they are wrongly sited. This later concept is the basis for this research.

Any tissue in the body will respond to injury by an inflammatory response en route to repair. This response in most instances is mediated by blood vessels and not surprisingly therefore this response is limited in articular cartilage, which is an avascular tissue. The limitation in the inflammatory response and subsequently the repair formed in articular cartilage injury is probably compensated for by the formation of osteophytes.

In this study, I have demonstrated a high frequency of co-existence between FTCD and osteophyte formation. The compressive and shear stiffness values of osteophytes, particularly ‘white’ osteophytes compared favourably with that of normal articular cartilage. The comparative histology of osteophytes and normal articular cartilage has also demonstrated that the predominant cartilage type in the cartilage mantle of osteophytes is hyaline-like. The types and the distribution of the native collagen in the cartilage mantle of osteophytes and normal articular cartilage also bear remarkable similarities. The unique architecture of the fibrillary collagen in osteophytes was revealed

by polarising light microscopy. The ultrastructure of osteophytes and osteophyte-articular cartilage junctions further reinforces the similarities between these two intra-articular osteochondral tissues. All these findings probably support the suggestion for the use of osteophytes as osteochondral substitute for FTCD.

The cell culture studies have demonstrated that the chondrocytes in the cartilage mantle of osteophytes are similar to chondrocytes in normal articular cartilage in either two or three-dimensional culture systems. The added advantage of osteophytic chondrocytes over normal articular cartilage chondrocytes is that osteophytic chondrocytes have a faster rate of proliferation. The oncogene studies (particularly c-myc) probably adds credibility to the rapid cellular proliferation which has been observed in osteophytes (from the cell culture study). The fear has always been that the entire cartilage mantle of osteophytes may be programmed to ossify. However, the osteoblastic phenotype studies and the c-fos oncogene expression in the cartilage mantle of osteophytes strongly refute this fear. It is obvious from my work therefore that the cartilage of osteophytes may provide a ready source of chondrocytes for autologous chondrocyte transplantation.

In conclusion, my study has shown that 'white' osteophytes may probably be suitable materials for osteochondral grafting of FTCD and that the cartilage mantle of osteophyte may be a source of chondrocytes for autologous chondrocyte transplantation.

Future Research.

Growth factors are essential for normal cellular function and in relaying information across the cell and nuclear membranes. The sites of expression of these growth factors in peri-articular osteophyte may shed more light to the suitability or otherwise of osteophyte as a graft material.

Experimentally induced FTCD can be treated with autograft of associated osteophyte and the rate of tissue integration and graft survival can be evaluated.

Experiments using three-dimensional culture systems of osteophyte-derived chondrocytes in non-ossifiable carrier medium needs further evaluation.

Appendix 1.

Preparation of epoxy resin.

- 1) Measure one part volume of 'komponent' A (liquid).
- 2) Measure one part volume of 'komponent' B (liquid).
- 3) Measure three-part volume of 'komponent' C (powder).
- 4) Mix 1, 2 and 3 above in a large plastic beaker and pour into plastic moulds (2.5cm in diameter and 5cm tall).

Calibration of epoxy resin stiffness.

- 1) Transfer the plastic mould with the epoxy resin to an electronic material-testing machine (Hounsfield, Redhill) with a 5mm flat tip indenter.
- 2) Increasing load was applied to the resin to a maximum of 500 Newton's (N) over 10 seconds.
- 3) The force-indentation curve was automatically generated using a computerised output.
- 4) The time to a constant force-indentation curve was determined. This marks the time to setting of the resin.

Embedding of specimen.

- 1) Osteophytes, femoral intercondylar notch and visually normal articular cartilage specimens were obtained from patients undergoing total knee replacement.
- 2) Specimens were cut into 10mm squares with about 2mm of underlying subchondral bone measured with a venier calliper.
- 3) Place the specimens on the middle of the surface of freshly prepared epoxy resin with the cartilage surface uppermost.

- 4) The specimens were pushed into the epoxy resin using the flat end of a metal spatula until the cartilage/subchondral bone junction is levelled with the surface of the resin (with the cartilage component of the specimens jutting out).
- 5) While setting, the specimens (cartilage surface) are kept moist with constant instillation of normal saline.

Preparation of specimen for shear testing.

- 1) Plastic moulds were filled half way with epoxy resin and allowed to settle for 10 minutes.
- 2) Specimens were embedded in the centre of the resin with the cartilage surface uppermost.
- 3) Ensure that the cartilage/bone interfaces of the specimens were levelled with the surface of the first layer.
- 4) Resin allowed to set for 80 minutes.
- 5) Circular shaped polytetrafluoroethylene non-clinging films with a 10mm square hole cut in the middle were placed on the specimens.
- 6) Another mixture of epoxy resin was prepared and poured to fill the plastic moulds to cover the protruding cartilage portions of the specimens.
- 7) The entire set up was allowed to set for another 90 minutes.

Appendix 2.

Preparation of osteophyte sections for immunohistochemistry.

Paraffin sections.

- 1) Fix specimens in 10% neutral buffered formalin for three weeks.
- 2) Decalcify specimens in Kristensen's solution for three weeks.
- 3) 7µm thick sections mounted on labelled slides (*PostI* = paraffin section of osteophyte for type I collagen staining, *PostII* = paraffin section of osteophyte for type II collagen staining, *PostX* = paraffin section of osteophyte for type X collagen staining, *PH&E* = paraffin section of osteophyte for haematoxylin and eosin staining).

Frozen section.

- 1) Specimens embedded in Cryo-M-Bed (Bright) on a piece of cork.
- 2) Snap frozen in liquid nitrogen.
- 3) 7µm thick sections obtained using a Bright cryostat with blade designed for skeletal tissue.
- 4) Mounted on silane coated labelled slides (*FostI* = frozen section of osteophyte for type I collagen staining, *FostII* = frozen section of osteophyte for type II collagen staining, *FostIII* = frozen section of osteophyte for type III collagen staining, *FH&E* = frozen section of osteophyte for haematoxylin and eosin staining) and air-dried at room temperature for 30-60 minutes.
- 5) Fix sections in analytical grade acetone for 10 minutes, air-dried and wrap in aluminium foil for storage at -20°C until use.

Immunohistochemistry of paraffin-embedded sections.

- 1) De-wax sections and rinse in phosphate buffered saline (PBS) for 10 minutes.
- 2) Flood with normal rabbit serum at 1 : 5 dilution in PBS for 10 minutes.

- 3) Remove serum (do not rinse) and add primary antibody;
 - a. Goat anti-type I collagen primary antibody at 1 : 20 dilution to *PostI* slides.
 - b. Goat anti-type II collagen primary antibody at 1 : 20 dilution to *PostII* slides.
 - c. Mouse anti-type X collagen primary antibody at 1 : 500 dilution to *PostX* slides.
- 4) Slides incubated with primary antibodies for 1 hour at room temperature and thereafter rinse thoroughly in PBS.
- 5) Rabbit anti-goat FITC conjugated secondary antibody at 1 : 200 dilution added to *PostI* and *PostII* slides and left to incubate for 1 hour at room temperature and thereafter rinsed in PBS, mount in Aquamount before visualization under ultraviolet light.
- 6) To *PostX* slides was added Biotin conjugated anti-mouse secondary antibody at 1 : 200 dilution for 1 hour at room temperature and thereafter rinsed in PBS. Add ABCComplex for 20-30 minutes, rinse and incubate with DAB Sigma fast tablet solution for 10 minutes, counterstain with haematoxylin and dehydrate before mounting in DPX mountant and examine under the light microscope.

Immunohistochemistry of frozen sections.

- 1) Sections brought to room temperature and re-hydrated in PBS for 10 minutes.
- 2) Stain as for paraffin embedded sections;
 - a. Goat anti-type I collagen primary antibody at 1 : 20 dilution to *FostI* slides.
 - b. Goat anti-type II collagen primary antibody at 1 : 20 dilution to *FostII* slides.

c. Goat anti-type III collagen primary antibody at 1 : 20 dilution to *FostIII* slides.

- 3) Slides incubated with primary antibodies for 1 hour at room temperature and thereafter rinse thoroughly in PBS.
- 4) Rabbit anti-goat FITC conjugated secondary antibody at 1 : 200 dilution added to *FostI*, *FostII* and *FostIII* slides and left to incubate for 1 hour at room temperature and thereafter rinsed in PBS, mount in Aquamount before visualization under ultraviolet light.

Appendix 3.

Preparation of osteophyte, osteophyte/normal cartilage composite and normal articular cartilage sections for scanning electron microscopy.

- 1) Fix specimens in 2% cacodylate buffer for 3 weeks at room temperature.
- 2) Thereafter pass specimens through graded alcohol;
 - a) 50% alcohol for 30 minutes,
 - b) 70% alcohol for 30 minutes,
 - c) 90% alcohol for 30 minutes,
 - d) 100% alcohol for 60 minutes,
 - e) Store in 100% acetone until ready for critical point drying.

Critical point drying.

- 1) Transfer specimens into gelatin capsules.
- 2) Transfer gelatin capsules into the boats of the critical point dryer chamber.
- 3) Flush boats with liquid carbon dioxide (CO₂) three times over a period of 10 minutes to remove the acetone.
- 4) Leave specimens for 1 hour.
- 5) Heat chamber with hot water until the temperature in the chamber reaches 35⁰C.
- 6) Reduce the pressure inside the chamber slowly to complete the critical point drying.

Coating of specimens with gold.

- 1) Transfer dried specimens from chamber and glue unto specimen stubs.
- 2) Glued specimens are transferred into the gold coating apparatus.
- 3) Coat specimens with 15 nanometre of gold using the Polaron coating unit.
- 4) Examine specimens using the DS 130 scanning electron microscope.

Appendix 4.

Cell preparation for cell culture.

- 1) Cartilage covering of osteophytes scrapped under aseptic technique.
- 2) Wash specimen in sterile tissue culture medium-modified eagle medium (α -MEM) supplemented with 10% foetal bovine serum, L-glutamine, penicillin and streptomycin.
- 3) Specimens chopped into tiny pieces.
- 4) Transfer specimens into sterile universal containers.
- 5) Add 2% w/v collagenase for 3-4 hours to dissociate the specimens.
- 6) Agitate the sterile universal containers vigorously with the Whirlmixer every 30 minutes.
- 7) At the end of the dissociation period, shake each sample and leave for 30 seconds to allow for sedimentation.
- 8) Remove digested cell suspension and transfer into new sterile universal containers.
- 9) Centrifuge at 2000rpm for 5 minutes.
- 10) Discard supernatant.
- 11) Re-suspend pellets of dissociated cells in α -MEM.

Cell culture technique.

- 1) Count cells using a haemocytometer and plate out in 35mm tissue culture dishes at;
 - a) **Medium density** – 3×10^5 cells/dish and
 - b) **High density** – 8×10^5 cells/dish.
- 2) Passage primary culture (P1) after 3 weeks of incubation (5% carbon dioxide 95% oxygen and at 37°C) and plate cells out at;

- 3) **Medium** (3×10^5 cells/dish) and **High** (8×10^5 cells/dish) densities – **M-P1** and **H-P1**.
- 4) Grow passage 1 (P1) cells for one week and re-passage (P2) and plate out at;
- 5) **Medium** (3×10^5 cells/dish) and **High** (8×10^5 cells/dish) densities – **M-P2** and **H-P2**.
- 6) Grow passage 2 (P2) cells for a further 2 weeks and re-passage (P3) and plate out a) **Medium density cultures at Low density** (1×10^5 cells/dish) **M-P3L** and the **High density** (1×10^7 cells/dish) **H-P3L**.
b) **High density cultures at Medium density** (1×10^5 cells/dish) **M-P3MM** and **High density** (1×10^7 cells/dish) **H-P3MM**.

Passage technique.

- 1) Incubate cultured cells (in the 35mm dishes) in 2mls of trypsin/EDTA solution for 15 minutes at 37°C .
- 2) Transfer loose cells into a sterile universal container.
- 3) Add equal volume of α -MEM to inactivate trypsin.
- 4) Centrifuge cells at 2000rpm for 5 minutes.
- 5) Discard supernatant.
- 6) Re-suspend cells in 2mls of α -MEM.
- 7) Count cells with a haemocytometer and plate out at the densities stated above.

Fixation of cells.

- 1) Culture medium (α -MEM) drained.
- 2) Replace with 2mls of 10% buffered formalin for 48 hours.
- 3) Fixed cells now ready for staining.

Histological staining of fixed cells.

1) At various intervals, P1, P2 and P3 cells are fixed as above and stained with;

a) *Toluidine blue*.

- i) Drain formalin.
- ii) Wash several times with phosphate buffered saline (PBS).
- iii) Add Toluidine blue and leave for 3-5 minutes.
- iv) Remove excess dye.
- v) Wash thoroughly with PBS.
- vi) Examine stained cultures periodically until desirable intensity is obtained.

b) *von Kossa*.

- i) Drain formalin.
- ii) Wash severally with distilled water.
- iii) Add 1% silver nitrate solution.
- iv) Expose to ultraviolet light for 10-20 minutes.
- v) Wash in 10 changes of distilled water.
- vi) Add 2.5% sodium thiosulphite solution and leave to stand for 5 minutes.
- vii) Wash with tap water.
- viii) Counterstain with von Gieson for 5 minutes.
- ix) Wash several times with distilled water.
- x) Mount and examine under light microscope.

Immunocytochemical staining of fixed cells.

(Cell cultures grouped into A, B, C and D)

1) Drain formalin

- 2) Rinse in PBS several times.
- 3) Add 1mg/ml of testicular hyaluronidase prepared in sodium acetate buffer.
- 4) Leave to stand for 1 hour at room temperature.
- 5) Rinse again several times in PBS.
- 6) To group A cell cultures add goat anti-type I collagen primary antibody at 1: 20 dilution.
- 7) To group B cell cultures add mouse anti-type II collagen primary antibody (raised in chicken) at 1: 10 dilution.
- 8) To group C cell cultures add goat anti-type III collagen primary antibody at 1: 20 dilution.
- 9) To group D cell cultures add mouse anti-link protein primary antibody at 1: 20 dilution
- 10) All dilutions were made in 0.1% bovine serum albumin (BSA) and PBSTween (PBSTween is made from 50ml PBS and 25 μ l of Tween i. e. Polyoxyethylenesorbitan monolaurate).
- 11) Allow primary antibody to incubate with the cells for 90 minutes with gentle sustained rhythmic movement of the culture dishes on a rocking machine at room temperature.
- 12) Wash several times with PBSTween.
- 13) Treat groups A and C with rabbit anti-goat horseradish peroxidase (HRP) conjugated secondary antibody in PBSTween at 1: 1000 dilution.
- 14) Treat groups B and D with anti-mouse HRP conjugated secondary antibody in PBSTween at 1:150 dilution.
- 15) Allow secondary antibody to incubate with the cells for 90 minutes with gentle sustained rhythmic movement of the culture dishes on a rocking machine at

room temperature.

16) Wash dishes several times in PBSTween and counterstain with DAB Sigma

fast tablet solution until brownish staining appear.

17) Mount and examine under light microscope.

Appendix 5.

Three-dimensional culture system.

Preparation of type I collagen solution.

- 1) Wash rat-tails in 70% alcohol.
- 2) Remove the skin.
- 3) Break each tail at 2cm intervals and pull tendons out of the broken pieces.
- 4) Wash harvested tendons in PBS and weigh the entire pool.
- 5) Cut tendons into small pieces and digest in 0.5M acetic acid (2mg tendon/ml).
- 6) Leave to stand for 48 hours at 4⁰C with continuous stirring.
- 7) Centrifuge mixture (for 15 minutes at 13,000 rpm) and decant the supernatant containing the extracted collagen.
- 8) Mix supernatant with equal volume of 20% sodium chloride at 4⁰C for 24 hours (to precipitate collagen).
- 9) Centrifuge the solution again (for 15 minutes at 13,000 rpm) and re-suspend the collagen pellets in 0.5M acetic acid.
- 10) Dialyse the solution in small wraps of dialysing tubes (knotted at both ends) against reducing concentrations of acetic acid and finally distilled water at 4⁰C.
- 11) Re-suspend precipitates in 1ml of 0.5M acetic acid (per sample).
- 12) Transfer samples into eppendoff's and freeze-dry overnight.
- 13) Dissolve dried samples in distilled water to obtain approximately 1.5-2mg/ml of type I collagen solution.
- 14) The purity of the collagen was assessed by gel electrophoresis.

Propagation of cultured cells on the surface of type I collagen

'Surface' propagation (ON).

- 1) To 4.2ml of type I collagen solution,
- 2) Add 1ml of 10% DMEM,
- 3) Add 0.1ml HEPES buffer,
- 4) Add 0.6ml sodium bicarbonate solution.
- 5) Measure 1ml aliquot of this cocktail into 35mm culture dishes.
- 6) Incubate at 37°C for 15 minutes – time to setting of the gel.
- 7) Place the gels (set) in the sterile chambers prior to inoculation.
- 8) Harvested cultured cells (Passage 1, Passage 2 and Passage 3 cells) at high densities are inoculated on the gels.
- 9) Add extra media to make 1ml of media covering the gel.
- 10) Incubate propagated gel and feed the cells every second day.

'Deep' propagation (IN).

- 1) Harvested cultured cells (Passage 1, Passage 2 and Passage 3 cells) at high densities are centrifuged at 2000rpm.
- 2) Re-suspend cell pellets in media.
- 3) Prepare the collagen cocktail as above but the volume of the 10 x DMEM is reduced by the same volume as the cell concentration.
- 4) Plate out collagen/cell mixture into 35mm culture dishes.
- 5) Incubate at 37°C for 15 minutes.
- 6) Add 1ml of media and place dishes in the incubator.
- 7) Feed the collagen/cell system every second day.

Alkaline phosphatase assay.

- 1) At each feed for the 'surface' and 'deep' propagated systems, the media that is changed is stored in two eppendoffs – half of the volume in each.
- 2) The 'A' samples were pooled (for each passaged cell stage) and stored at – 80°C until analysed for alkaline phosphatase activity. (The 'B' samples were pooled and freeze-dried and analysed using the Western blotting technique).
- 3) Thaw samples ('A') by placing the eppendoffs in a water bath at 30°C.
- 4) Prepare the reagent solution as per the manufacturer's instruction;
 - i) To 1 tablet of P-nitrophenyl phosphate add,
 - ii) 1 tablet of magnesium compound (manufacturer's) and
 - iii) 25 ml buffer solution.
- 5) Calibrate the Ultrospec 2000, UV/Visible spectrophotometer (Pharmacia, Biotech) with an air chamber.
- 6) A total volume of 3ml is used per sample in the specimen chamber.
- 7) For the control,
 - 3ml of fresh culture media alone,
 - 3ml of buffer solution alone,
 - 2.5ml of buffer solution and 50µl of media were used.
- 8) The mean absorbance change per minute at 405nm is a measure of the alkaline phosphatase activity (measurements made at 0, 1, 2 and 3 minutes after the specimen chamber has been inserted into the machine).
- 9) 3ml of the samples of the pooled media were analysed for alkaline phosphatase as above.

Appendix 6.

Collagen assay of the media from the ‘superficial’ and ‘deep’ propagated culture systems (in type I collagen) using the Western blotting technique.

Electrophoretic separation.

- 1) The freeze-dried samples, both *ON* and *IN* (sample ‘B’ in appendix 5) from each passaged group are weighed.
- 2) Re-suspend the weighed samples in bromophenol blue to make a solution equivalent to 1mg/ml.
- 3) Transfer 60µl of sample to eppendoff’s.
- 4) Arrange eppendoff’s in a metal rack and perforate the lids of the eppendoff’s.
- 5) Lower the metal rack into boiling water ensuring that the tips are immersed in the boiling water for 5 minutes.
- 6) 25µl of each sample was emptied into 6% SDS-PAGE gel wells using a Hamilton’s syringe.
- 7) The syringe was rinsed after each use.
- 8) Allow gel to run for 1 hour at;
15mA per gel,
600V and at
100 Watts.
- 9) Store the gels in sterile dishes containing Towbin’s solution while awaiting transfer of the proteins.

Transfer of protein.

- 1) Sandwich the gel and immobilon P transfer membrane (Millipore) both measuring 7cm x 8cm between two sets of three wet blotting papers also measuring 7cm x 8cm.
- 2) Transfer the set-up into the blotting apparatus.
- 3) Blot the proteins from the gel onto the immobilon P transfer membrane for hour at;
0.8mA per cm² amounting to 44.8mA per gel,
50 volts and
100 Watts.

Staining for collagen type II.

- 1) Transfer the membranes into sterile dishes.
- 2) Wash transfer membrane repeatedly in PBSTween for 15 minutes.
- 3) Flood the membrane with goat anti-type II collagen primary antibody in 0.1% bovine serum albumin (BSA) at 1 : 200 dilution.
- 4) Incubate membrane with primary antibody for 90 minutes at room temperature with gentle sustained rhythmic movement of the culture dishes on a rocking machine.
- 4) Wash membrane in PBSTween repeatedly for 15 minutes.
- 5) Flood membrane with rabbit anti-goat horseradish peroxidase (HRP) conjugated secondary antibody in PBSTween at 1 : 2000 dilution.
- 6) Incubate for 90 minutes with gentle sustained rhythmic movement of the culture dishes on a rocking machine.
- 7) Wash membrane thoroughly with PBSTween.

- 8) Counterstain with DAB Sigma fast tablet solution (dissolve 2 gold and 2 silver tablets in 10ml of water) until brownish bands or columns appear.

Appendix 7.

Osteoblastic phenotype in osteophyte.

Alkaline phosphatase staining – histology.

- 1) Slides of frozen sections obtained (see appendix 2).
- 2) Bring slides to room temperature.
- 3) Re-hydrate in PBS for 10 minutes.
- 4) Prepare staining solution by dissolving;
15mg of sodium alpha-naphthyl phosphate and
20mg of Fast red Tris salt (Diazonium salt) in
20ml of 0.2M Tris buffer at a pH of 10.
- 5) Filter the mixture.
- 6) Flood the re-hydrated slides with the filtered staining solution.
- 7) Allow to stand for 30 minutes.
- 8) Drain excess stain and rinse with PBS.
- 9) Mount on Aquamonut and examine under light microscope.

Alkaline phosphatase staining – immunohistochemistry.

- 1) Bring slides (frozen sections) to room temperature.
- 2) Re-hydrate in PBS for 10 minutes.
- 3) Treat slides with testicular hyaluronidase 1mg/ml in sodium acetate buffer.
- 4) Incubate for 1 hour.
- 5) Rinse with PBS.
- 6) Flood slides with B4-50 primary antibody at 1 : 20 dilution in PBS.
- 7) Incubate for 90 minutes.
- 8) Wash slides repeatedly with PBSTween for 15 minutes.

- 9) Flood slides with rabbit anti-mouse horseradish peroxidase conjugated secondary antibody at 1 : 150 dilution.
- 10) Incubate for 90 minutes.
- 11) Wash repeatedly with PBSTWeen for 15 minutes.
- 12) Counterstain with DAB Sigma fast solution until brown stain appears.

Osteocalcin staining – immunohistochemistry.

- 1) Bring slides (frozen sections) to room temperature.
- 2) Re-hydrate in PBS for 10 minutes.
- 3) Treat slides with testicular hyaluronidase 1mg/ml in sodium acetate buffer.
- 4) Incubate for 1 hour.
- 5) Rinse with PBS.
- 6) Flood slides with rabbit anti-bovine osteocalcin primary antibody at 1 : 20 dilution (Kindly donated by Dr. S Robins of Aberdeen).
- 7) Incubate for 90 minutes.
- 8) Wash slides repeatedly with PBSTWeen for 15 minutes.
- 9) Flood slides with goat anti-rabbit secondary antibody at 1 : 20 dilution.
- 10) Incubate for 90 minutes.
- 11) Wash repeatedly with PBSTWeen for 15 minutes.
- 12) Counterstain with DAB Sigma fast solution until brown stain appears.
- 13) All dilutions were made in 0.1% bovine serum albumin (BSA) and PBSTWeen.

Appendix 8.

Avidin staining technique.

Avidin HRP technique for c-myc and c-jun.

- 1) Wax embedded sections obtained (see appendix 2).
- 2) De-wax through graded alcohol and xylene and water.
- 3) Dip slides in 6% hydrogen peroxide for 10 minutes.
- 4) Wash slides in water and PBS at pH 7.6.
- 5) Flood sections with normal goat serum at 1 : 20 dilution in Tris buffered saline for 10 minutes.
- 6) Drain off excess buffer (do not wash).
- 7) Add primary antibody (100µl/slide) to labelled slides;
 - i) mouse anti-myc primary antibody at 1 : 150 dilution in TBS.
 - ii) Mouse anti-jun primary antibody at 1 : 20 dilution in TBS.
- 8) Incubate slides with primary antibody for 60 minutes.
- 9) Wash in PBS for 20 minutes.
- 10) Add biotinylated goat universal anti-mouse secondary antibody at 1 : 200 dilution in TBS.
- 11) Incubate for 30 minutes.
- 12) Wash in PBS for 20 minutes.
- 13) Add ABC complex horseradish peroxidase solution and leave for 30 minutes.
- 14) Wash in PBS for 30 minutes.
- 15) Add 0.05% DAB solution for 5 minutes.
- 16) Wash in tap water for 5 minutes.
- 17) Counterstain with haematoxylin for 30 seconds (nuclei staining).
- 18) Rinse in running tap water.

- 19) De-hydrate through graded alcohol and xylene.
- 20) Mount in Aquamount and examine under the light microscope.

Avidin conjugated alkaline phosphatase technique for c-fos.

- 1) Slides of frozen sections obtained (see appendix 2).
- 2) Bring to room temperature.
- 3) Wash in water for 2 minutes.
- 4) Flood with TBS buffer for 2 minutes.
- 5) Wipe off excess buffer.
- 6) Flood with normal rabbit serum in TBS at 1 : 20 dilution in for 10 minutes.
- 7) Drain excess serum.
- 8) Add mouse anti-fos primary antibody (100µl/slide) at 1 : 20 dilution in TBS.
- 9) Incubate for 60 minutes.
- 10) Wash in TBS for 20 minutes.
- 11) Add biotinylated rabbit anti-mouse secondary antibody at 1 : 400 dilution in TBS.
- 12) Incubate for 30 minutes.
- 13) Wash in TBS for 20 minutes.
- 14) Add avidin conjugated alkaline phosphatase at 1 : 400 dilution in TBS.
- 15) Incubate for 30 minutes.
- 16) Wash in TBS for 20 minutes.
- 17) Immerse sections in a developer made from;
24mg Levamisole,
50mg Fast Red TR,
50mg Naphthol asB1 phosphate dissolved in
100ml Veronal acetate buffer (VAB) at pH 9.2.

- 18) Leave slides in developer for 60 minutes.
- 19) Wash in water for 5 minutes.
- 20) Counterstain with haematoxylin for 30 seconds.
- 21) Wash in running tap water.
- 22) Mount in Aquamount and examine under the light microscope.

Critique of Methodology.

1. *Compressive stiffness measurement using epoxy resin base.*

Oni and Morrison (1998) had earlier evaluated the mechanical 'quality' of osteophytes using plaster-of-paris as base material. However, in my study epoxy resin was used as the base material after calibrating it because;

1. After the epoxy resin had set (90 minutes after mixing the three components in the specified proportions), the stiffness value was found to be constant and it was not influenced by environmental factors like humidity.
2. The stiffness of plaster-of-paris is variable depending on the humidity since this material has a remarkable ability to imbibe water. Keeping the specimen moist with drops of saline (as was done in this study) will seep into the plaster-of-paris base material and alter its stiffness value. This is one of the factors in favour of epoxy resin.
3. The epoxy resin was far stiffer than the specimen's and this implies that the force/displacement curves and the stiffness values recorded for the specimens (embedded in epoxy resin) were the true values.
4. This study is not expensive and it is readily reproducible.

The set-up for shear testing was unique, simple and straightforward. The application of the specified shear force was directed at the required point.

2. *Comparative histology using various stains.*

The choice of stains was narrowed down to routine cartilage/cartilaginous matrix staining. This was aimed at establishing the presence of hyaline-like cartilage in the osteophytic cartilage mantle.

This study suggested the presence of hyaline-like cartilage as a component of the osteophyte cartilage mantle.

The use of various concentrations of MgCl_2 (Magnesium Chloride) in Alcian blue should have been done to estimate the types and proportions of the proteoglycans in the osteophyte cartilage mantle.

3. *Immunolocalization of the collagen types in the cartilage mantle of osteophytes.*

The collagen content of cartilage is the framework on which the resilience, viscoelasticity and mechanical properties lies. The location and orientation of these collagen fibres are also vital in maintaining the status of the cartilage.

This study was to compliment the mechanical study - to establish the reasons for a less stiff osteophyte cartilage despite their similarities to normal articular cartilage histologically.

The spatial arrangements of these collagen types (I, II, III and X) were found to be similar. This study is reproducible.

However, the same collagen types should have been evaluated in the paraffin embedded and frozen sections. This would have given an appropriate basis for comparison.

4. *The ultrastructure of osteophyte using the scanning electron microscope.*

The preparation of specimens was simple and easily reproducible. The critical freeze-drying was easily achieved.

The in-depth evaluation of the osteophyte/normal articular cartilage junction has thrown more light into the likely aetiopathogenesis of osteophytes.

Various sections of the specimens are visualized sequentially at various magnifications.

5. *The behaviour of osteophytic chondrocytes in monolayer culture.*

This study is reproducible and with the availability of osteophytes (discarded following femoral cuts during total knee replacement), large samples of cells in culture were studied. There was no pre-planned choice of densities for this study but the pilot studies prompted the use of the densities used in this study.

Fixation of the chondrocytes in cultures enabled an evaluation of the type and content of the peri-cellular or cartilage matrix.

The propagation of these chondrocytes in a three-dimensional cultural system (type Collagen) was easily reproducible. The extraction of the type I collagen in our laboratory enabled me to have adequate quality control.

However, the choice of type I collagen three-dimensional culture should hence been substituted because this culture system has a tendency to calcify. The outcome of this study therefore needs more scrutiny.

6. *Osteogenic potentials of osteophytes.*

Osteogenic potentials in osteophyte have been elucidated by a simple histologic and immunolocalization technique for alkaline phosphatase and osteocalcin.

The c-fos evaluation complimented the alkaline phosphatase localization. These two studies were carried out to allay the fears of the proponents of osteophytes as autologous grafts.

Generally the sample sizes did vary between experiments and this should have been addressed.

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